Cyclic AMP-independent Involvement of Rap1/B-Raf in the Angiotensin II AT2 Receptor Signaling Pathway in NG108-15 Cells*

Received for publication, March 13, 2002, and in revised form, November 11, 2002
Published, JBC Papers in Press, December 2, 2002, DOI 10.1074/jbc.M202446200

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The angiotensin II (Ang II) type 2 (AT2) receptor is an atypical seven-transmembrane domain receptor. Controversy surrounding this receptor concerns both the nature of the second messengers produced as well as its associated signaling mechanisms. Using the neuronal cell line NG108-15, we have reported previously that activation of the AT2 receptor induced morphological differentiation in a p21Wnt-independent, but p42/p44mapk-dependent mechanism. The activation of p42/p44mapk was delayed, sustained, and had been shown to be essential for neurite elongation. In the present report, we demonstrate that activation of the AT2 receptor rapidly, but transiently, activated the Rap1/B-Raf complex of signaling proteins. In RapN17- and Rap1GAP-transfected cells, the effects induced by Ang II were abolished, demonstrating that activation of these proteins was responsible for the observed p42/p44mapk phosphorylation and for morphological differentiation. To assess whether cAMP was involved in the activation of Rap1/B-Raf and neuronal differentiation induced by Ang II, NG108-15 cells were treated with stimulators or inhibitors of the cAMP pathway. We found that dibutyryl cAMP and forskolin did not stimulate Rap1 or p42/p44mapk activity. Furthermore, adding H-89, an inhibitor of protein kinase A, or Rp-8-Br-cAMP-S, an inactive cAMP analog, failed to impair p42/p44mapk activity and neurite outgrowth induced by Ang II. The present observations clearly indicate that cAMP, a well-known stimulus of neuronal differentiation, did not participate in the AT2 receptor signaling pathways in the NG108-15 cells. Therefore, the AT2 receptor of Ang II activates the signaling modules of Rap1/B-Raf and p42/p44mapk via a cAMP-independent pathway to induce morphological differentiation of NG108-15 cells.

The angiotensin II (Ang II) octapeptide hormone binds two major receptor subtypes, type 1 (AT1) and type 2 (AT2), both of which are expressed in several tissues. One of the most remarkable features of the AT2 receptor is its high level of expression in most fetal tissues (1–3) including the brain (4, 5). Some neuronal cell lines such as NG108-15 (6–8), PC12W (9, 10), and N1E 115 cells (11, 12) also express the AT2 receptor at high levels. The AT1:AT2 receptor ratio increases dramatically after birth (5, 13), suggesting an involvement of the AT2 receptor in fetal development. In the adult, the AT2 receptor expression is limited to some tissues, such as the adrenal gland and specific areas of the brain. Several recent studies have indicated that ligand-independent activation (14) or Ang II stimulation of the AT2 receptor is associated with antiproliferative effects (15, 16), apoptosis (17, 18), and differentiation. Indeed, involvement of the AT2 receptor has been documented in different models of differentiation such as steroidogenesis in gonads or the adrenal gland (19–21), contractility in smooth muscle cells (22, 23), or neurite outgrowth in neuronal cell types (9, 24, 25) (for review, see Refs. 26–29).

The precise nature of the signaling pathways activated by the AT2 receptor is still controversial (for review, see Refs. 26–29). This seven-transmembrane domain receptor is not coupled to any of the classical, well-established, second messengers, such as cAMP or inositol phosphates, and its coupling to a Go protein, reported by several authors (30–34), is not a consensus. However, various mediators, which could individually exert opposite effects, such as CGMP, tyrosine or serine/threonine phosphatases, and the extracellular signal-regulated kinases ERK1/ERK2 (p42/p44mapk) have been associated with activation of the AT2 receptor, depending on cell types and experimental conditions used. More precisely, AT2 receptor activation was shown to decrease p42/p44mapk activities (35, 36), a process often associated with programmed cell death (36–39), or stimulate a sustained increase in p42/p44mapk activity (32), a process more often associated with cellular differentiation (40, 41).

To study the effects of Ang II on differentiation, we have used NG108-15 cells. In their undifferentiated state, neuroblasts or the adrenal gland (19–21), contractility in smooth muscle cells (22, 23), or neurite outgrowth in neuronal cell types (9, 24, 25) (for review, see Refs. 26–29).

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* This work was supported in part by grants from the Canadian Institute for Heath Research (to N. G.-P. and M. D. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Recipient of a studentship from the Fonds de la recherche en santé du Québec.

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†††† The abbreviations used are: Ang II, angiotensin II; AT1 and AT2, type 1 and 2 Ang II receptors, respectively; 8-Br-cAMP-S, 8-bromo-adenosine cyclic 3’,5’-phosphorothioate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CMV, cytomegalovirus; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GST, glutathione S-transferase; mapk, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NGF, nerve growth factor; PKA, protein kinase A.
pathway for the activation of the p42/p44 MAPK cascade must be considered. We have demonstrated recently that nitric oxide and cGMP are involved in the AT2 receptor effects on neurite outgrowth. However, this pathway appeared to be a parallel, complementary, rather than an intermediary step of the AT2 signaling cascade directed to p42/p44 MAPK activation (33).

In PC12 cells stimulated with NGF, initiation of neurite outgrowth was accompanied by a sustained increase in p42/p44 MAPK activities (44, 45), mediated by Rap1, a small guanine nucleotide-binding protein of the Ras family. Complete, a neuron-specific member of the Ras family of kinases (46–48). The active GTP-bound form of Rap1 is known to bind, in vitro, to p21regulated effectors of the Raf family of kinases, in particular B-Raf and the Raf guanine nucleotide exchange factors (RafGEx), RafGDS (49, 50). Moreover, NGF stimulated Rap1 activity via the exchange factor Crk/C3G and was also shown to activate this pathway through a cAMP-dependent mechanism, involving (51) or not involving protein kinase A (52). Similar mechanisms could also be considered for NG108-15 cells because chronic treatment with cAMP analogs is a well known differentiating factor for these cells (42, 43). The aim of the present study was to investigate whether the Rap1/B-Raf pathway could be involved in the Ang II-induced sustained increase in p42/p44 MAPK activity participating in the morphological differentiation of the NG108-15 cells and to verify whether cAMP differentiation of the NG108-15 cells and to verify whether cAMP could be involved in the signaling pathway of the AT2 receptor.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), HAT supplement (hypoxanthine, aminopterin, thymidine), gentamycin, and LipofectAMINE were from Invitrogen. [3H]ATP was from NEN (DuPont, Boston, MA). A stock solution of [3H]cAMP (50 Ci/ml) was from Amersham Biosciences (Arlington Heights, IL). [3H]adenine (50 Ci/ml) and cAMP-specific phosphodiesterase were from Amersham Biosciences (Arlington Heights, IL). Monoclonal anti-Rap1, anti-Rap2, and anti-Rap3 antibodies were from StressGen Biotechnologies (Victoria, BC, Canada). Anti-rabbit antibodies were from Amersham Biosciences. Complete, Raf-1, and recombinant MEK1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham Biosciences. Anti-phosphotyrosine (1:500), anti-Rap1/Krev (1:200), anti-B-Raf (1:500), anti-Phospho-ERK1/2 (1:1,000), anti-p42/p44 MAPK (1:1,000), anti-Raf-1, and anti-p21Ras antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). According to the experiments, cells were cultured in DMEM containing 10% FBS, HAT supplement, and 50 mg/liter gentamycin. All other chemicals were of grade A purity.

Cell Culture—NG108-15 cells (provided by Drs. M. Emerit and M. Hamon; INSERM, Unité 238, Paris, France) were cultured (passages 12–21) in DMEM with 10% FBS, HAT supplement, and 50 mg/liter gentamycin at 37 °C in 75-cm² Nuncel Delta flasks in a humidified atmosphere of 95% air and 5% CO2 (43). Subcultures were performed at subconfluence (70%–80%) in 24-well Petri dishes and incubated for 3 days (treated every day beginning 24 h after plating, for a 3-day treatment). Cells were treated without (control cells) or with 100 nM Ang II in the absence or presence of the inhibitors to be tested: H-89 (10 μM for short time experiments or 0.5 μM for 3-day treatment) or Rp-8-Br-cAMP-S (50 μM for all treatments).

Cell Transfection—NG108-15 cells were transfected with pCMV-Rap1N17, pCDN3.1/Rap1GAP, or with pCDN3.1. Plasmid DNA (3 μg/ml) was mixed with 40 μg/ml LipofectAMINE and incubated at room temperature for 30 min. For transfection, NG108-15 cells were grown to subconfluence (70–80%) in 24-well Petri dishes and incubated for 3 h at 37 °C with the DNA-lipid complex. Transfection medium was then replaced with fresh medium and incubated for 24 h post-transfection. At the end of this period random samples were harvested for determination of p42/p44 MAPK activity as described elsewhere (33). Morphological studies were done on stably transfected cells, grown in a Geneticin (G-418, 200 μg/ml)-containing medium.

B-Raf and Raf-1 Kinase Activity Measurements—NG108-15 cells were plated at a density of 2 × 10^5 cells in 100-mm Petri dishes and used for experiments at 80% confluence. Cells were incubated from 0 to 8 days in medium with 100 nM Ang II, then washed with Hanks’ balanced solution and lysed in 1 ml of ice-cold buffer (70 mM β-glycerophosphate buffer (pH 7.2) containing 0.5% Triton X-100, 100 μM NaVO₃, 2 mM MgCl₂, 1 mM EGTA, 5 μg/ml leupeptin, 20 μg/ml aprotinin, 1 mM dithiothreitol for B-Raf or 10 mM Tris (pH 7.4) containing 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% bovine serum albumin, 20 μM aprotinin, 1 mM phenylmethlysulfonyl fluoride, and 2 mM NaVO₃ for Raf-1). Briefly, total B-Raf and Raf-1 were, respectively, immunoprecipitated from 2-ml cell extracts after Ang II treatment. The precipitate was washed several times. Immune complex kinase assays were performed as described, using recombinant MEK1 as substrate and [γ-32P]ATP as a phosphate donor for 30 min at 30 °C, and the reaction was stopped by adding Laemmli buffer (50–56%). The assay products were separated by electrophoresis on 10% SDS-polyacrylamide gels, revealed with Kodak Biomax MS films and analyzed with Image Quant software (Molecular Dynamics).

Rap1 Activity Measurements—The activated form of Rap1 was pulled down with glutathione S-transferase (GST)-RaiGDS fusion protein from cell lysates as described elsewhere (56–58). Wild-type NG108-15 cells or cells stably transfected with pCDN3.1. RapN17, or Rap1GAP were cultured in 35- or 100-mm Petri dishes and, for wild-type cells, incubated from 0 to 60 min with 100 nM Ang II or 100 nM CGP42112 (an AT2 receptor agonist). Cells were washed with Hanks’ balanced solution and lysed in 1 ml of ice-cold lysis buffer containing 2.5% CHAPS, 50 mM Tris-HCl (pH 7.6), 140 mM NaCl, 50 mM NaVO₃, and Complete® mixture of inhibitors. Lysates were centrifuged to eliminate insoluble material, and 150 μg of total protein was incubated with 15 μl of a 50% slurry of glutathione-Sepharose beads coupled with 5 μg of GST-RaiGDS for 1 h at 4 °C. Beads were washed four times with lysis buffer, and activated Rap1 protein was eluted with Laemmli buffer and resolved by Western blotting (15% SDS-PAGE) transferred on polyvinylidene difluoride membrane and revealed with a Rap1 polyclonal antibody, 1:2,000.

Determination of Cells with Neurites—NG108-15 cells were plated at a density of 5 × 10^4 cells in 35-mm Petri dishes and incubated for 3 days in FBS-containing medium without (control) or in the presence of 100 nM Ang II + 0.5 μM Rp-8-Br-cAMP-S or Ang II + 0.5 μM Rp-8-Br-cAMP-S (each introduced daily with the inhibitors applied 30 min prior to Ang II) and were then examined under a phase contrast microscope. Cells with at least one neurite longer than a cell body were counted as positive for neurite outgrowth.

Western Blot Analysis—Cells cultured in 35-mm Petri dishes were loaded on 10 or 15% SDS-polyacrylamide gels. Kaleidoscope prestained standards were loaded to evaluate molecular weights of separated proteins. Gels were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 1% gelatin and probed with primary antibodies at the indicated dilutions, followed by the appropriate secondary antibody conjugated to horseradish peroxidase. Detection was performed by chemiluminescence with ECL (Amersham Pharmacia). Filters were exposed on Kodak X-AR film.

Measurement of cAMP Accumulation—The ability of cells to activate cAMP production and accumulation were measured by two different methods. The capacity of cells to stimulate adenylyl cyclase activity was determined by measuring the conversion of [3H]ATP to [3H]cAMP, as described previously (59). In short, cultured cells were incubated at 37 °C in the DMEM (with 10% FBS) containing 1.5 μCi/ml [3H]adenine. After 1 h, the cultures were washed and incubated with 100 nM Ang II, 10 nM CGP42112, or 20 μM forskolin in a Hanks’ balanced solution buffer containing 1 μM isobutylmethylxanthine (an inhibitor of phosphodiesterase activity). After 15 min at 37 °C, cells were collected, solubilized, and chromatographed on Dowex and alumina columns.

A standard amount of cAMP produced in cells was assayed using an enzyme immunoassay kit, as proposed by the manufacturer (Amersham Biosciences). Briefly, NG108-15 cells were cultured in DMEM + 10% FBS in 35-mm Petri dishes. Cells were stimulated for various times with 100 nM Ang II, and for the last 15 min of stimulation, a final concentration of 1 μM isobutylmethylxanthine was added. At the end of the experimental period, the cAMP contained in control or stimulated cells was assayed using an enzyme immunoassay kit, as proposed by the manufacturer (Amersham Biosciences). Results are presented as fmol of cAMP produced/μg of proteins. 
The protein content of each sample was determined using Bio-Rad DC method.

**RESULTS**

**Ang II Stimulates B-Raf Kinase Activity**—NG108-15 cells were treated with 100 nM Ang II for periods ranging from 5 to 120 min. Total B-Raf was immunoprecipitated from cell lysates using an anti-B-Raf antiserum, and the kinase activity, using MEK1 as a substrate, was determined as described under “Experimental Procedures.” Quantification of MEK1 phospho-

**Fig. 1. Effect of Ang II on B-Raf kinase activity in NG108-15 cells.**

A, NG108-15 cells (8 × 10⁶ cells in 100-mm Petri dishes) were stimulated for the indicated time periods in culture medium (DMEM containing 10% FBS). B-Raf kinase activity induced by 100 nM Ang II was analyzed as described under “Experimental Procedures” by immune complex kinase assay using anti-B-Raf antiserum, recombinant MEK1 as a substrate, and [γ-32P]ATP as the phosphate donor. The reaction product was resolved by SDS-PAGE and detected by autoradiography. Numbers on the left indicate the molecular mass of proteins in kDa. B, densitometric analysis of the mean ± S.E. of three different experiments. C, total extracts of NG108-15 cells (NG) were submitted to SDS-PAGE followed by Western blot analysis to verify the presence of Rap1 and B-Raf proteins as described under “Experimental Procedures.” A 2-day-old rat brain homogenate (B) was used as a positive control. Western blots are representative of three individual experiments. The lower band that appeared on the Western blot analysis of p21rap1 has been described by the manufacturer (Santa Cruz Biotechnology) to be a nonspecific cross-reaction of this antibody.

The protein content of each sample was determined using Bio-Rad DC method.

**Data Analysis**—The data are presented as mean ± S.E. of the number of experiments indicated in the text. Homogeneity of variance was assessed by Bartlett’s test, and p values were obtained from Dunnett’s tables.

**Ang II Stimulates Rap1 Activity**—In vitro, GTP-bound Rap1, the active form of this G protein, associates tightly with Rap1-GDS (50). Because B-Raf kinase activity usually arose from an upstream stimulation of Rap1, we used this property to measure the amount of activated Rap1, as described by Franke et al. (58). Cell lysates were incubated with GST-RalGDS fusion protein precoupled to glutathione beads. The activated Rap1 that bound to RapGDS was separated by SDS-PAGE and analyzed by Western blot analysis using anti-Rap1 antibodies. The densitometric analysis of the mean ± S.E. of four different experiments is shown.

**Fig. 2. Activation of Rap1 by the AT2 receptor of Ang II in NG108-15 cells.** NG108-15 cells (8 × 10⁶ cells in 100-mm Petri dishes) were stimulated in culture medium (DMEM containing 10% FBS). A and B, time course effect of 100 nM Ang II and C, dose-dependent effect of CGP42112 (after 1 min of incubation) on Rap1 activity. Rap1 activity was measured as described under “Experimental Procedures.” Cell lysates were incubated with GST-RalGDS fusion protein precoupled to glutathione beads. The activated Rap1 that bound to RapGDS was separated by SDS-PAGE and analyzed by Western blot analysis using anti-Rap1 antibodies. The densitometric analysis of the mean ± S.E. of four different experiments is shown.

rulation, expressed as a ratio of MEK1 detected by autoradiography (Fig. 1A) over the total MEK1 determined by Western blotting (not shown), showed that Ang II induced a rapid and transient increase in B-Raf kinase activity. The effect was observed within 5 min of stimulation, reached a peak at 10 min, and then decreased to control levels by 20 min (Fig. 1B). Rap1 kinase activity was also measured but did not show any significant differences over control level (data not shown). Western blot analyses of cell homogenates indicated that Rap1 (left panel) and B-Raf (right panel) proteins are indeed present at high levels in the NG108-15 cells (Fig. 1C). Because Rap1 is found ubiquitously and B-Raf has been demonstrated to be expressed predominantly in neuronal tissues, the expression of both proteins was compared using newborn rat brain homogenate as a positive control. Interestingly, we observed that, like in the rat brain, NG108-15 cells expressed the p95B-Raf, but the 68-kDa isoform was undetectable.

**Ang II Stimulates Rap1 Activity**—In vitro, GTP-bound Rap1, the active form of this G protein, associates tightly with Rap1-GDS (50). Because B-Raf kinase activity usually arose from an upstream stimulation of Rap1, we used this property to measure the amount of activated Rap1, as described by Franke et al. (58). Cell lysates were incubated with GST-RalGDS fusion protein precoupled to glutathione beads. The activated Rap1 that bound to RapGDS was then separated by SDS-PAGE and
analyzed by Western blot using anti-Rap1 antibodies. As shown in Fig. 2A, Ang II stimulation significantly increased the total amount of Rap1-GTP associated with GST-RalGDS within 90 s, persisted no longer than 5 min, and then decreased under the basal level. The maximum response was observed after 1 min of stimulation with Ang II (1.35 ± 0.08-fold increase, n = 9) (Fig. 2B, n = 4). As shown in Fig. 2C, increasing concentrations of the AT2 receptor agonist CGP42112 also increased Rap1 activity after a 1-min incubation, indicating that activation of Rap1 occurred specifically through the AT2 receptor.

Rap1 Inhibition Prevents AT2-induced p42/p44 MAPK Activation—As described previously, activation of Rap1 and B-Raf was shown to lead to sustained p42/p44 MAPK activity (46–48). To investigate further the involvement of Rap1 in the AT2 receptor signaling, we transfected NG108-15 cells with Rap1N7 or Rap1GAP constructs. Rap1N7 is a dominant negative mutant form of Rap1, and Rap1GAP increases the GTPase activity of Rap1, thus favoring the GDP-bound form (inactive form) of the protein (60, 61). As shown in Fig. 3A, in transfected NG108-15 cells (pcDNA3.1/Rap1GAP or pCMVRapN17), the active form of Rap1 (the GTP-bound form) was decreased substantially, compared with the native or pcDNA3.1-transfected NG108-15 cells. In pcDNA3.1-transfected cells used as control, Ang II induced a delayed phosphorylation of p42/p44 MAPK (as determined with an antibody directed against the phosphorylated form of p42/p44 MAPK). This effect is observed within 30 min of Ang II application (2.2 ± 0.4-fold increase over control), sustained for 60 min (1.8 ± 0.3-fold increase over control), and then decreased to basal level at 120 min (Fig. 3, B and E). However, in Rap1N7- or Rap1GAP-transfected cells, Ang II failed to activate p42/p44 MAPK (Fig. 3, C–E). These results demonstrated that Rap1 activation is necessary for Ang II to activate p42/p44 MAPK.

Rap1 Activation Is Involved in AT2-induced Morphological Differentiation—We have shown previously that a sustained activation of the p42/p44 MAPK pathway was an essential event to promote neurite outgrowth in NG108-15 cells treated with Ang II (41). Control pcDNA3.1-transfected cells had similar rounded morphology, with only very short neurites, in some cells, as in nontransfected NG108-15 cells (Fig. 4, A and C). After a 3-day treatment with 100 nM Ang II, cells possessed many neurites as native cells (Fig. 4, B and D). Similar treatment conducted in cells transfected with pCMVRapN17 (Fig. 4, E and F) or pcDNA3.1/Rap1GAP (Fig. 4, G and H) was unable to induce neurite outgrowth and elongation. Quantification of these results demonstrated that a 3-day treatment with Ang II had no effect on neurite outgrowth in cells expressing the dominant negative form of Rap1 (Rap1N7) or the specific inhibitor for Rap1 (Rap1GAP) (Fig. 5). Indeed, in control and pcDNA3.1-transfected NG108-15 cells, Ang II induced a substantial increase in the number of cells with neurites (from 6.3 ± 2.3% to 29.9 ± 3.5% in native NG108-15 cells and from 6.1 ± 2.1% to 27.1 ± 7.6% in pcDNA3.1-transfected cells). This effect was abolished in cells expressing Rap1N7 or Rap1GAP. These observations indicated that Rap1 activation is required for the AT2-induced neurite outgrowth.

Cyclic AMP and PKA Are Not Involved in AT2-induced p42/p44 MAPK Activation—Several studies have documented the requirement of PKA for such a Rap1-dependent activation of the p42/p44 MAPK. To determine whether PKA could be involved in the observed Ang II-induced p42/p44 MAPK phosphorylation, we stimulated NG108-15 cells with Ang II in the presence or in the absence of 10 μM H-89, an inhibitor of PKA. As shown in Fig. 6, A and B, pretreatment of the cells with H-89 did not alter the activation of p42/p44 MAPK induced by Ang II. Because it has been shown that cAMP is able to activate p42/p44 MAPK in a PKA-independent mechanism (52), we verified such possibi-
ties using cells stimulated with forskolin (an activator of adenylyl cyclase). In NG108-15 cells, 10 μM forskolin decreased the basal level of phosphorylated p42/p44 mapk, an effect reversed when cells were preincubated with Rp-8-Br-cAMP-S, an inactive analog of cAMP (Fig. 6C). This inhibition of p42/p44 mapk was observed within 5–10 min after forskolin application and persisted for at least 2 h. The same results were also observed with a cell-permeable cAMP analog, dibutyryl cAMP (1 mM) (data not shown). These results indicate that cAMP was not involved in the signaling pathway of the AT2 receptor which leads to a sustained, but delayed, p42/p44 mapk activation.

To support further our observations that cAMP is not involved in the activation of p42/p44 mapk in our model, Rap1 activity was measured in NG108-15 cells incubated in the presence of forskolin at concentrations as high as 20 μM. This cAMP-elevating agent failed to increase significantly the association of Rap1 with GST-RalGDS (1.1 ± 0.1-fold increase over control) (Fig. 6, D and E), at a time (5 min) where p42/p44 mapk was strongly inhibited with any concentrations of forskolin tested (from 10 nM to 20 μM) (not shown). In comparison, Ang II induced a significant increase in Rap1 activity of 1.35 ± 0.08-fold over control. These results suggest that in NG108-15 cells, cAMP was not sufficient to increase Rap1 activity, reinforcing its absence of effect in the AT2 receptor signaling mechanisms.

Cyclic AMP and PKA Are Not Involved in AT2-induced Neurite Elongation—To document further the absence of any involvement of PKA and/or cAMP in the AT2-induced neurite elongation, cells were left untreated (Figs. 7A and 8A) or treated for 3 days with 100 nM Ang II (Figs. 7B and 8B), in the absence or in the presence of 0.5 μM H-89 (Fig. 7, C and D) or 50 μM Rp-8-Br-cAMP-S (Fig. 8, C and D) (to block all cAMP-dependent mechanisms). Both inhibitors were added, daily, 30 min before the application of Ang II. Phase contrast micrographs indicated that cells stimulated with Ang II alone or in the presence of H-89 (Fig. 7) or Rp-8-Br-cAMP-S (Fig. 8) exhibit similar neurite elongations. Quantification of these results indicated that cells stimulated with Ang II alone or in the presence of H-89 exhibited numerous well-developed neurites (29.0 ± 3.9% and 30.6 ± 2.3%, respectively), compared with control cells (8.6 ± 1.7%) (Fig. 9A). Similar results were found for cells stimulated in the presence of Rp-8-Br-cAMP-S (Fig. 9B). These observations confirmed that inhibition of PKA did not impair the ability of Ang II to induce neurite outgrowth in NG108-15 cells.

The AT2 Receptor Is Not Coupled to cAMP Production—As shown in Fig. 10A, incubation of cells with 100 nM Ang II or 10 nM CGP42121 (an AT2 receptor agonist at that concentration), for period ranging from 0 to 120 min, had no effect on cAMP accumulation. In contrast, 20 μM forskolin induced a 110-fold increase in cAMP accumulation, indicating that adenylyl cyclase is functional in the NG108-15 cells. Because cAMP could also be produced much later after hormonal stimulation, we also measured the intracellular content of cAMP in NG108-15 cells after longer period of Ang II treatment. As shown in Fig. 10B, the same kinds of result were obtained using an enzyme immunoassay to determine the cAMP content in cells. Together, these results clearly established that the AT2 receptor signaling did not involve cAMP as a second messenger for Rap1 and p42/p44 mapk activation as well as for neurite elongation.

DISCUSSION

In the present study, we have demonstrated that the binding of Ang II to the AT2 receptor, in NG108-15 cells, increased Rap1/B-Raf activities, two events accompanied by a concomitant decrease in p21WAF1 and a sustained increase in p42/p44 mapk activities. However, in contrast to many G protein-coupled receptors that activate the Rap1/B-Raf cascade, the action of
Ang II is independent of cAMP or PKA, again confirming the atypical nature of this seven-transmembrane domain receptor. Application of Ang II, on NG108-15 cells, led to a transient stimulation of B-Raf kinase activity, as measured by an in vitro kinase assay using MEK1 as a substrate, but it did not affect the basal level of Raf-1 activity. One of the better described activators of B-Raf is the small G protein Rap1 (62). Using RapGDS as affinity probe, we found that Ang II treatment of NG108-15 cells significantly increased the active, GTP-bound form, of Rap1 within 1 min and B-Raf within 5 min, whereas Raf-1 activity was not modified.

Members of the Raf family of proteins include Raf-1, A-Raf, and B-Raf. Neurons are known to express the ubiquitous isoform Raf-1 and the specific neuronal isoform B-Raf (63). In neurons stimulated with NGF, B-Raf is the major isoform activated, with only 5% of the total activity attributed to Raf-1 (64, 65). The findings that both proteins, Rap1 and B-Raf, are activated after Ang II treatment are thus compatible with the observation that activation of p42/p44 MAPK is stimulated in a p21ras-independent mechanism by the AT2 receptor (41). Moreover, our results demonstrated that Rap1 activation is respon-
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Fig. 9. Quantification of neurite outgrowth induced by Ang II. NG108-15 cells were grown as described under “Experimental Procedures” and treated with 100 nM Ang II in the absence (control, C) or in the presence of 0.5 μM H-89 (A) or 50 μM Rp-8-Br-cAMP-S, an inactive analog of cAMP (B). Cells with at least one neurite longer than a cell body were counted as positive for neurite outgrowth. Results are expressed as percentage of cells with neurites over the total amount of cells in the micrographs. Results are the mean ± S.E. of three different experiments, each performed in triplicate. *, p < 0.01, difference compared with control cells.

Fig. 10. Effect of Ang II and CGP42112 on adenylyl cyclase activity and cAMP accumulation. NG108-15 cells were grown as described under “Experimental Procedures” and treated without (control, C) or with 100 nM Ang II, 10 nM CGP42112, or 20 μM forskolin (FSK) for the indicated time periods. The effects of Ang II and CGP42112 on adenylyl cyclase activity (A) and of Ang II on the intracellular cAMP level (B) were quantified as described under “Experimental Procedures.” Results are the mean of two different experiments, each performed in triplicate.

Fig. 11. Activation kinetics of Rap1, B-Raf, and p42/p44\textsuperscript{mapk} after Ang II activation in NG108-15 cells. The kinetics data for Rap1 and B-Raf are from Figs. 1 and 2. The kinetic data for p42/p44\textsuperscript{mapk} activities are from Figs. 3 and 6 combined with data published previously (33, 41). Results are shown as protein activity (-fold increase over basal level) over the duration of the stimulation with 100 nM Ang II.

Fig. 12. Schematic representation of the AT\textsubscript{2} receptor signaling mechanisms in the NG108-15 cells. The Ang II AT\textsubscript{2} receptor induction of neurite outgrowth and elongation involves at least two distinct, independent, but complementary pathways. First, after binding of Ang II, the activated AT\textsubscript{2} receptor rapidly inactivates p21\textsuperscript{ras} and activates the Rap1/B-Raf pathway, leading to a delayed p42/p44\textsuperscript{mapk} phosphorylation. Another pathway involving the nitric oxide/soluble guanylyl cyclase/cGMP cascade of signaling is also necessary to observe neurite outgrowth. Stimulation of these two parallel pathways could modulate gene expression and the phosphorylation state of different microtubule-associated proteins (MAPs) to control microtubules stability/dynamic responsible for neurite elongation. NOS, nitric-oxide synthase; NO, nitric oxide; sGC, soluble guanylyl cyclase; unP-MAPs, unphosphorylated MAPs; P-MAPs, phosphorylated MAPs.
substantially decreased), Ang II-induced p42/p44MAPK activation is abolished. In addition, these transfected cells exposed to Ang II have the same morphology as the native, untreated NG108-15 cells. As we have shown previously that a sustained activation of the p42/p44MAPK pathway was an essential event to promote neurite outgrowth in NG108-15 cells treated with Ang II (41), these results indicate that Rap1 activation is necessary for Ang II to induce p42/p44MAPK activation and that Rap1 is required for the Ang II-induced neurite outgrowth.

How Rap1 could be activate in our model remain unknown. In several models, cAMP and cAMP-activated proteins appeared to be of great importance, and many studies have reported the requirement of PKA for NGF activation of Rap1 as well as for p42/p44MAPK (47). Indeed, several studies indicate that treatment of cells with cAMP or analogs induced neurite outgrowth, both in PC12 (44) and in NG108-15 cells (24, 42, 43). Activation of Rap1 by cAMP favored its association with Raf-1, consequently inhibiting the transient p21ras-dependent activation of p42/p44MAPK (66). In parallel, the action of Rap1 on B-Raf accounted for the sustained stimulatory effect on p42/p44MAPK activity (51, 55, 66). In PC12 cells stimulated with NGF, cAMP is required for a sustained increase of p42/p44MAPK activity (51), even if in these cells, the NGF TrkA receptor is coupled directly to Rap1 through the adaptor protein CrkII (47).

However, in the NG108-15 cells stimulated with Ang II, the present results indicate that cAMP (or analogs) and PKA are not involved in the increased activities of Rap1 and B-Raf. We report that the PKA inhibitor H-89 or an inactive cAMP analog, Rp-8-Br-cAMP, neither impaired the ability of Ang II to induce p42/p44MAPK activation nor did either affect morphological differentiation. Furthermore, forskolin-induced cAMP production in NG108-15 cells inhibited p42/p44MAPK phosphorylation but did not modify Rap1 activity. These results corroborate our previous observations that coinubation of Ang II with dibutyryl cAMP inhibits differentiation induced by each stimulus alone (24). Furthermore, Ang II did not stimulate cAMP production or accumulation, demonstrating that the cAMP/PKA pathway is not involved in the AT2 receptor signaling mechanisms leading to p42/p44MAPK activation and neuronal differentiation of NG108-15 cells. Those results corroborate the observations of Sanchez et al. (67) which indicate that in the human neuroblastoma cell line SH-SY5Y, cAMP-induced neurite outgrowth was independent of p42/p44MAPK activation.

Fig. 11 shows, in the same time scale, the sequential activation of Rap1/B-Raf and p42/p44MAPK. The delay between B-Raf and p42/p44MAPK could be explained by the presence of an intermediate type of reaction, such as activation of MEK. In NGF-treated cells, York et al. (56) have shown a sustained Rap1 activation mediated by TrkA internalization and phosphoinositide 3-kinase activation. In this system, the authors proposed that phosphoinositide 3-kinase favors clathrin-dependent TrkA internalization into the endocytic compartment where Rap1 is localized. However, such a mechanism is incompatible with the known behavior of the AT2 receptor. Indeed, in contrast to many G protein-coupled receptors, including the AT1 receptor, the AT2 receptor does not internalize (68, 69). In addition, the differences in activation kinetics could be also the result of variations in the molecular composition and the stability of protein complexes recruited by the different receptors (65).

Our recent observations on the AT2 receptor signaling mechanisms in NG108-15 cells are summarized in Fig. 12. After binding of Ang II, the activated AT2 receptor rapidly inactivates p21ras (5–120 min) (41) and enhances the level of the GTP-bound form of Rap1 (1–5 min). Activated Rap1 then enhances the activity of B-Raf (5–15 min) which in turn stimulates p42/p44MAPK phosphorylation (30–60 min). Finally, the return of p42/p44MAPK phosphorylation to basal level, occurring much later (seen after 120 min of Ang II treatment), may be under a phosphotyrosine phosphatase activity such as SHP-1, shown to be activated after the AT2 receptor stimulation (39).

Ang II also stimulates the nitric oxide/soluble guanylyl cyclase/cGMP cascade of signaling (33). Our studies have shown that this cascade, together with MAPK activation, is involved in neurite outgrowth, through a mechanism independent of Ras activation. All of these pathways may have as final targets regulation of gene expression and modulation of the phosphorylation states of different microtubule-associated proteins such as MAP2, tau, and MAP1b, as we have shown previously in NG108-15 cells (24) as well as in granule cells from rat cerebellum (25).

Three important conclusions can be drawn from the present work. First, Ang II, in NG108-15 cells, induced a rapid activation of Rap1 and B-Raf, two proteins that account for the sustained activation of p42/p44MAPK induced by the AT2 receptor. Second, AT2 signaling mechanisms leading to Rap1/B-Raf-dependent p42/p44MAPK activation and neurite elongation are clearly cAMP-independent. Third, the capability of cAMP to induce neurite outgrowth and neuronal differentiation in the NG108-15 cells occurs independently of the sustained increase in p42/p44MAPK activity. Altogether, these observations suggest that when present in the cell type studied, such as neurons, the Rap1/B-Raf signaling cascade could play a pivotal role in transduction (63). Thus, the presence of Rap1/B-Raf may explain the controversial results surrounding the AT2 receptor action (in particular apoptosis versus differentiation). In addition, as many G protein-coupled receptors, the AT2 receptor may probably recruit some not yet identified partners, which altogether, contribute to the complex regulation of morphological neuronal differentiation.

Lessons from knockout mice or from neurological disorders reinforce the idea that the AT2 receptor may be important for neuronal development. Indeed, perturbations in exploratory behavior and locomotor activity were observed (70, 71) as well as an anxiety-like behavior (72). In addition, a decrease in the expression of the AT2 receptor is observed in areas of the adult brain implicated in the development of neurological disorders such as Alzheimer’s disease, Huntington’s disease, or Parkinson’s disease (caudate nucleus, putamen and substantia nigra, temporal cortex) (73). Finally, recent observations indicate that AGR2R2 mutations are correlated with mental retardation (74). Together, such observations indicate inappropriate neuronal differentiation and plasticity (in adult) when AT2 is absent or genetically modified.

Acknowledgments—We are grateful to Dr. J. L. Bos (Utrecht University, Utrecht, The Netherlands) for the GST-RalGDS fusion protein and Dr. P. J. Stork (Oregon Health Sciences University, Portland, OR) for the pCMVRapN17 and pcDNA3.1/Rap1GAP. We also thank Lucie Chounard for experimental assistance.

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