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Involvement of MAP Kinase in Angiotensin II-Induced Phosphorylation and Intracellular Targeting of Neuronal AT_1 Receptors

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MAP kinase stimulation is a key signaling event in the AT_1 receptor (AT_1R)-mediated chronic stimulation of tyrosine hydroxylase and norepinephrine transporter in brain neurons by angiotensin II (Ang II). In this study, we investigated the involvement of MAP kinase in AT_1R phosphorylation to further our understanding of these persistent neuromodulatory actions of Ang II. Ang II caused a time-dependent phosphorylation of neuronal AT_1R. This phosphorylation was associated with internalization and translocation of AT_1R into the nucleus. MAP kinase also stimulated phosphorylation of neuronal AT_1R. The conclusion that MAP kinase participates in neuronal AT_1R phosphorylation and its targeting into the nucleus is supported further by the following. (1) MAP kinase-mediated phosphorylation of AT_1R was blocked by the AT_1R antagonist losartan; (2) AT_1R co-immunoprecipitated with MAP kinase; (3) MAP kinase-kinase inhibitor PD98059 attenuated Ang II-induced phosphorylation of AT_1R; and (4) PD98059 blocked Ang II-induced nuclear translocation of AT_1Rs. In summary, these observations demonstrate that Ang II-induced phosphorylation of AT_1R is mediated by its activation of MAP kinase. A possible role of AT_1R translocation into the nucleus on persistent neuromodulatory actions of Ang II has been discussed.

Key words: angiotensin II; AT_1 receptor; phosphorylation; nuclear receptor; nuclear localization signal (NLS); MAP kinase; hypothalamic–brainstem neurons

Angiotensin II (Ang II) exerts its neuromodulatory actions on the brain by regulating the activities of neuronal enzymes involved in the turnover of catecholamines (Steckelings et al., 1992; Raizada et al., 1994). It binds to the AT_1 receptor (AT_1R) subtype and stimulates tyrosine hydroxylase (TH), dopamine β-hydroxylase (DβH), and norepinephrine transporter (NET) (Lu et al., 1996a; Yu et al., 1996). In fact, whereas acute stimulation by Ang II involves post-transcriptional events, chronic exposure of neurons with Ang II results in a persistent stimulation of these neuromodulatory activities and involves transcription of TH, DβH, and NET genes (Lu et al., 1996a; Yu et al., 1996). These observations are intriguing and suggest that the neuronal AT_1R, a member of G-protein-coupled receptors (GPCRs), may be unique in that persistent stimulation of neuromodulation by Ang II is independent from its desensitization induced by Ang II. In view of this uniqueness of neuronal AT_1R, we set out to investigate the signal transduction mechanism involved in Ang II regulation of neuromodulation. These studies have revealed a distinct signaling pathway for this GPCR involving Ras-Raf-1 and MAP kinase (Yang et al., 1996a), suggesting that AT_1R interaction with the heterotrimetric Gq would result in the dissociation of $G_{\beta\gamma}$ from Gα. $G_{\beta\gamma}$, then, directly or through the recruitment of one or more cytoplasmic or membrane-associated factors, causes the activation of Ras, which leads to the activation of MAP kinase (Van Corven et al., 1993; Burgering and Bos, 1995; Inglese et al., 1995; Shaw, 1995). The role of the pleckstrin homology (PH) domain in this communication between $G_{\beta\gamma}$ and Ras has already been proposed for other GPCRs (Inglese et al., 1995; Shaw, 1995). Thus, it would seem that $G_{\beta\gamma}$ and not Gα plays a role in the AT_1R-mediated signaling process involving MAP kinase.

Phosphorylation of GPCRs by GPCR kinases (GRKs) is an important step for termination of the signaling pathway stimulated by the interaction of agonist to its receptor (Lefkowitz, 1993; Permont et al., 1995). Because the AT_1R signaling mechanism leading to the cellular actions in the neurons seems to be unique, we decided to investigate the phosphorylation of this receptor by Ang II and the potential role of this phosphorylation on its persistent stimulatory actions of Ang II on the neurons. Our observations demonstrate that Ang II stimulates phosphorylation of AT_1R and that this may be mediated by MAP kinase. Phosphorylation is associated with AT_1R internalization and its targeting into the nucleus.

MATERIALS AND METHODS

One-day-old Wistar Kyoto rats were obtained from our breeding colony, which originated from Harlan Sprague Dawley (Indianapolis, IN). DMEM, plasma-derived horse serum (PDHS), and trypsin (150 U/mg) were from Central Biomedia (Irwin, MD). [γ-^{32}P]ATP (3000 Ci/mmol), [^{32}P]-orthophosphate (1 mCi = 37 MBq), and chemiluminescence assay reagents were from DuPont NEN (Boston, MA). Nitrocellulose membranes were from Micron Separations (Westboro, MA). Ang II and myelin basic protein (MBP) were purchased from Sigma (St. Louis, MO). Losartan potassium (Dup 753) was a gift from DuPont/Merck (Wilmington, DE). PD123319 was from RBI (Natick, MA). PD98059 was from Calbiochem (La Jolla, CA). [^{125}I]-Sar^1-Ile^8-Ang II (specific activity = 2200 Ci/mmol) was purchased from Dr. Robert Speth, Washington State

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University (Pullman, WA). Polyclonal anti-rabbit-AT_1R antibody (306, catalog number SC579) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). It was prepared by using a peptide corresponding to amino acids$^{306-359}$ of the AT_1R. The antibody was specific for AT_1R and was mouse, rat, and human reactive. No cross-reaction of the AT_1R antibody with the AT_2R was observed. Monoclonal antibody to AT_1R was prepared by using a synthetic peptide corresponding to amino acids$^{8-17}$ of the AT_1R and characterized as described elsewhere (Barker et al., 1993). Anti-MAP kinase (C-14), a polyclonal antibody that specifically recognizes ERK-2 and to a much lesser extent ERK-1, and protein A/G PLUS-agarose were purchased from Santa Cruz Biotechnology. An anti-rat MAP kinase polyclonal antibody (ERK I–III) that recognized the p42 MAP kinase and p44 MAP kinase and activated mouse GST-p42 MAP kinase were from Upstate Biotechnology (Lake Placid, NY). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) and were the highest quality available.

Hypothalamus–brainstem neuronal cells in primary culture

Hypothalamus–brainstem areas of 1-d-old Wistar Kyoto rat brains were dissected, and brain cells were dissociated by trypsin. The hypothalamic block contained the paraventricular nucleus and the supraoptic, anterior, lateral, posterior, dorsomedial, and ventromedial nuclei. The brainstem block contained medulla oblongata and pons. Dissociated brain cells were plated in poly-L-lysine-precoated tissue culture dishes (2×10^7 cells/100 mm diameter dish or 3×10^6 cells/35 mm diameter dish) in DMEM containing 10% PDHS, and neuronal culture was established essentially as described previously (Raizada et al., 1984, 1993). The cultures were allowed to grow for 10–15 d before their use in experiments. Immunohistochemical analysis has indicated repeatedly that these cultures contain 85–90% neuronal cells and 10–15% astroglial cells (Raizada et al., 1984, 1993).

Immunoprecipitation

Neuronal cultures, established in 100-mm-diameter culture dishes, were treated with Ang II. The cell lysates were prepared by adding 1 ml lysis buffer [25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, and 0.8 μg/ml leupeptin] and scraping the cells off the culture dish. Cell lysates were centrifuged at $6000 \times g$ for 10 min at 4°C, and the protein content of resulting supernatants was determined using a Bio-Rad (Richmond, CA) Bradford protein assay kit. Lysates containing 400 μg of protein were subjected to an immunoprecipitation protocol as follows. Lysates were incubated with 1 μg of rabbit anti-AT_1R or anti-MAP kinase antibody overnight at 4°C. Immunoprecipitates were collected on protein A/G PLUS-agarose, washed three times with lysis buffer, and used in additional experiments (Yang et al., 1996a).

Immunoblotting

Immunoprecipitates were suspended in 20 μl of Laemmli's sample buffer in a boiling water bath for 3 min and then centrifuged. The resulting supernatants (10 μl) were electrophoresed in 10% SDS-PAGE, and proteins were transferred onto nitrocellulose membrane. The membrane was blocked by 5% nonfat dry milk in TBST (20 mM Tris HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) for 1 hr followed by incubation for 1 hr at room temperature with rabbit anti-MAP kinase antibody or rabbit anti-AT_1R antibody. Protein-bound antibody was detected by incubation of the membrane with horseradish peroxidase-labeled secondary antibody and enhanced by chemiluminescence assay reagents. The bands recognized by the primary antibody were visualized by exposure to film (Yang et al., 1996a).

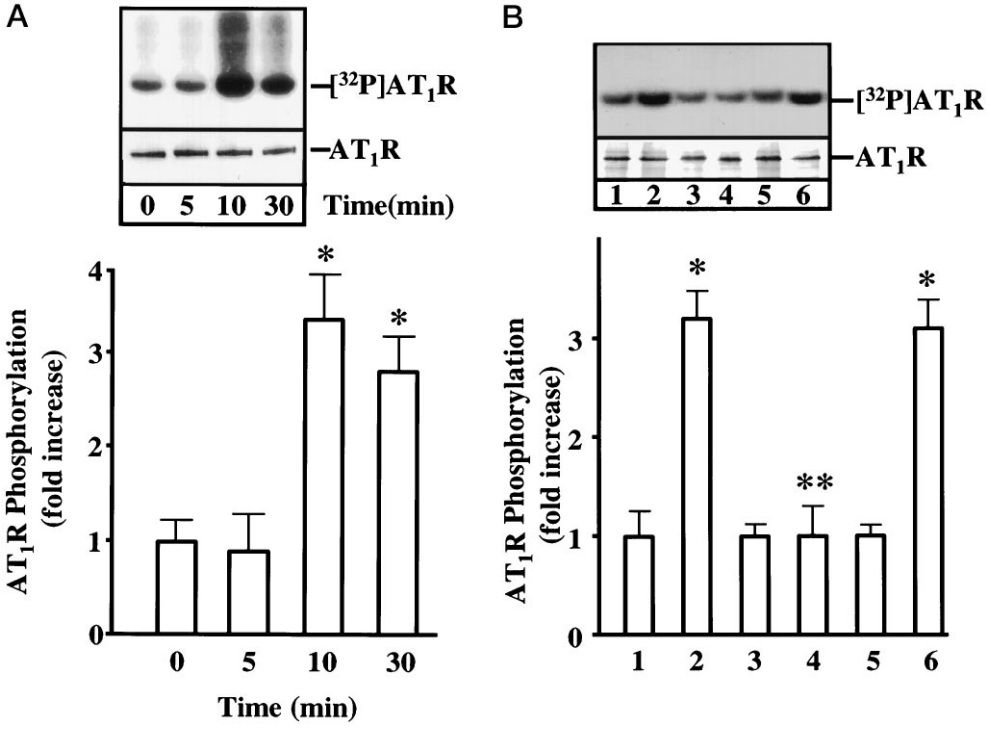

Figure 1. Ang II stimulation of AT_1R phosphorylation. *A*, Time course. Neuronal cultures were prelabeled with [^{32}P]-orthophosphate for 4 hr, and cells were lysed in lysis buffer as described in Materials and Methods and incubated with 100 nM Ang II for the indicated time periods. Incorporation of TCA-precipitable [^{32}P] was measured, and $\sim 4.3 \times 10^6$ dpm were used to immunoprecipitate AT_1Rs. [^{125}I]-Sar^1-Ile^8-Ang II (30 fmol/mg) binding activities were subjected to SDS-PAGE and autoradiography, as described in Materials and Methods. The same membrane was probed with AT_1R antibody (*AT_1R*) after the development of the autoradiogram to normalize for [^{32}P] incorporated into AT_1R donated as [^{32}P]-AT_1R. *Top*, Representative autoradiogram; *bottom*, quantitation of radioactive band representing AT_1R from three separate experiments. Data from three separate experiments were averaged (± SE) and presented as fold increase by using zero time control as baseline. *Single asterisk* shows significant ($p < 0.05$) difference from 0 time control. *B*, Receptor specificity. Cultures were incubated without (*1, 3, 5*) or with (*2, 4, 6*) 100 nM Ang II for 10 min in the absence (*1, 2*) or presence (*3, 4*) of 10 μM losartan or 10 μM PD123319 (*5, 6*). Data were analyzed essentially as described for *A*. *Top*, Representative autoradiogram; *bottom*, quantitation of [^{32}P]-AT_1R after normalization of densities with immunoreactive AT_1R. Data are presented as fold increase by using control as baseline. *Single asterisks* show significant difference ($p < 0.05$) from 1, whereas *double asterisk* shows significant difference ($p < 0.05$) from 2.

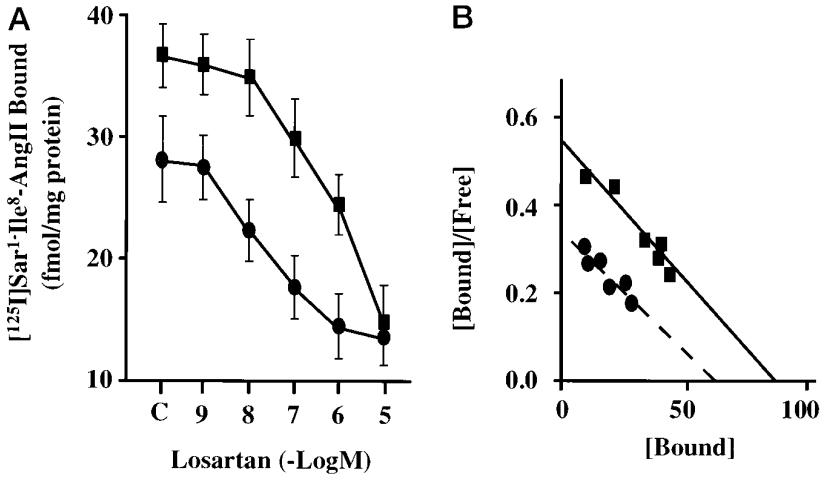

Figure 2. Effect of Ang II on [^{125}I]-Sar^1-Ile^8-Ang II binding to cell surface AT_1Rs in neurons. Neuronal cultures were incubated with 100 nM Ang II for 15 min at 37°C. This was followed by measurement of cell surface binding of [^{125}I]-Sar^1-Ile^8-Ang II to AT_1Rs, essentially as described in Materials and Methods. *A*, Competition–inhibition of untreated (■) and Ang II-treated (●) neurons, with indicated concentrations of losartan. *B*, Scatchard analysis of the data from Figure 3*A*.

[^{125}I]-Sar^1-Ile^8-Ang II binding assay

Binding in AT_1R immunoprecipitates. Cell-free lysates were subjected to immunoprecipitation by rabbit-anti-AT_1R antibody as described above. Immunoprecipitates containing AT_1R were collected on protein A/G PLUS-agarose and washed three times with lysis buffer and once with binding buffer [PBS, pH 7.2, containing 1.0% bovine serum albumin (BSA)]. Binding of [^{125}I]-Sar^1-Ile^8-Ang II to these immune complexes was carried out as described previously (Abramowski and Staufenbiel, 1995). In brief, immune complexes containing ~30 fmol of [^{125}I]-Sar^1-Ile^8-Ang II binding activity suspended in 0.5 ml binding buffer were incubated with 1 nM [^{125}I]-Sar^1-Ile^8-Ang II in the presence of 10 μM PD123319 for 1 hr at room temperature to determine total binding. PD123319, an AT_2R antagonist, was used in all binding assays to block the binding of [^{125}I]-Sar^1-Ile^8-Ang II to AT_2Rs. In addition, increasing concentrations of losartan (1 nM-10 μM) were used for the competition–inhibition experiments. All reactions were run in triplicate. The binding reaction was terminated by filtration and collection of [^{125}I]-Sar^1-Ile^8-Ang II bound to receptors on Whatman GF/B filters presoaked with 0.3% polyethyleneimine. Filters were washed three times with ice-cold PBS, pH 7.2, to remove unbound radioligand, and bound radioactivity was counted by a Beckman DP5500 gamma counter. Binding was expressed as femtomoles of [^{125}I]-Sar^1-Ile^8-Ang II bound per milligrams of cellular protein used to immunoprecipitate the receptor. Specific binding was calculated by subtracting the [^{125}I]-Sar^1-Ile^8-Ang II bound to complex in the presence of losartan from that bound in its absence. Scatchard analysis was carried out from the competition–inhibition experiments for the calculation of K_d and B_{max} using the EBDA-ligand Program (Elsevier-Biosoft).

Binding in intact neurons. Cell surface AT_1R levels were measured with the use of intact neuronal cells attached to culture dishes. Neuronal cultures were established in 35-mm-diameter culture dishes, and binding of [^{125}I]-Sar^1-Ile^8-Ang II to AT_1R was determined as follows. After treatment with Ang II, growth medium was aspirated from culture dishes, and cultures were rinsed with PBS, pH 7.2, with 2–5 min incubation between rinses. This allowed for the dissociation of any unlabeled Ang II that bound to cell surface AT_1Rs during preincubation. Triplicate cultures were incubated with 1 ml of reaction mixture containing 1.0 nM [^{125}I]-Sar^1-Ile^8-Ang II, 1.0% BSA, and 10 μM PD123319 for the determination of total binding. In addition, triplicate cultures that also contained increasing concentrations of losartan (1 nM-10 μM) were used for the competition–inhibition experiments. Binding was performed at 4°C for 60 min; the dishes were then washed three times with ice-cold PBS, pH 7.4. Cells were dissolved in 0.1N NaOH (0.5 ml/dish), and data for the quantitation of cell surface AT_1R were analyzed essentially as described previously (Yang et al., 1996b).

Fractionation of neurons into nuclear fraction

Neuronal cultures, established in 100-mm-diameter tissue culture dishes, were rinsed twice with PBS, pH 7.4, and cells were collected by scraping the monolayer with the help of a Teflon scraper. Cells were fractionated into total cell lysate, nuclear, and cell extract without nuclear fraction, essentially as described elsewhere with minor modifications (Abmayr and Workman, 1992). Briefly, the cell pellet was incubated in a solution containing 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.0, 0.5 mM dithiothreitol (DTT), and 0.2 mM PMSF for 10 min at 4°C followed by homogenization with 15 gentle strokes using a type B pestle in a Dounce homogenizer. A >90% lysis of neurons was accomplished by this method, as evidenced by a microscopic examination. Certain amounts of homogenates were saved and used for whole-cell lysate fraction. The remaining homogenates were centrifuged at 3300 × *g* for 15 min, and nuclear fraction was collected. This nuclear fraction was used directly for confocal microscopy to localize AT_1R immunoreactivity. For other biochemical determinations, the nuclear pellet was lysed by nuclear lysis buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, and 30 mM KCl). The nuclear extract was collected by centrifugation at 25,000 × *g* for 30 min at 4°C. Both nuclear and cell extract without nuclear fraction were dialyzed overnight against a dialysis buffer (20% glycerol, 20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM EDTA). Nuclear specific protein, lamin B, and cytosolic enzyme aldolase were used to determine the relative purity of these fractions (Clegg, 1984; Gerace, 1986). Nuclear fractions contained 100% of immunoreactive lamin B, whereas 4–8% of the total aldolase was found associated with this fraction. Aldolase activity was found predominately (96%) in the fraction of cell extract without nuclei, which contained no detectable lamin B. Distribution of these proteins in these compartments is consistent with the presence of traditional markers and shows that this fractionation yielded relatively pure nuclear preparation from neurons. Equal amounts of proteins in both fractions were used to immunoprecipitate AT_1Rs as described above.

Measurement of MAP kinase activity by in-gel assay

MAP kinase activity was measured essentially as described elsewhere (Yang et al., 1996a). Briefly, neuronal cells grown in 100-mm-diameter culture dishes were rinsed three times with ice-cold PBS, pH 7.4, and lysed by incubation with 0.5 ml of lysis buffer (25 mM Tris-HCl, 25 mM NaCl, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin, and 0.8 μg/ml leupeptin) for 10 min at 4°C. Cell-free lysates containing equal amounts of protein from triplicate culture dishes were pooled and incubated with 1 μg of rabbit anti-AT_1R antibody at 4°C overnight. Immunoprecipitates were collected on protein A/G PLUS-agarose, centrifugated, and electrophoresed on 10% SDS-PAGE containing 0.5 mg/ml MBP. The gel was then washed twice in 20% 2-propanol, 50 mM Tris-HCl, pH 8.0, and twice in 50 mM Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol. Each wash lasted for 1 hr at room temperature with gentle shaking. MAP kinase activity was measured by incubating the gel with 40 mM HEPES, pH 8.0, 2 mM DTT, 10 mM $MgCl_2$, 0.5 mM EGTA, 40 μM ATP, and 10 μCi [γ-^{32}P]ATP (3000 Ci/mM) for 30 min at room temperature, followed by autoradiography as described previously (Yang et al., 1996a).

Phosphorylation of AT_1R by exogenous MAP kinase

Phosphorylation of AT_1Rs after their immunoprecipitation from neuronal cell lysate by anti-rabbit-AT_1R antibody was carried out with the use of exogenous MAP kinase by a protocol based on that described by Paxton et al. (1994). Briefly, neuronal cell lysates were prepared as

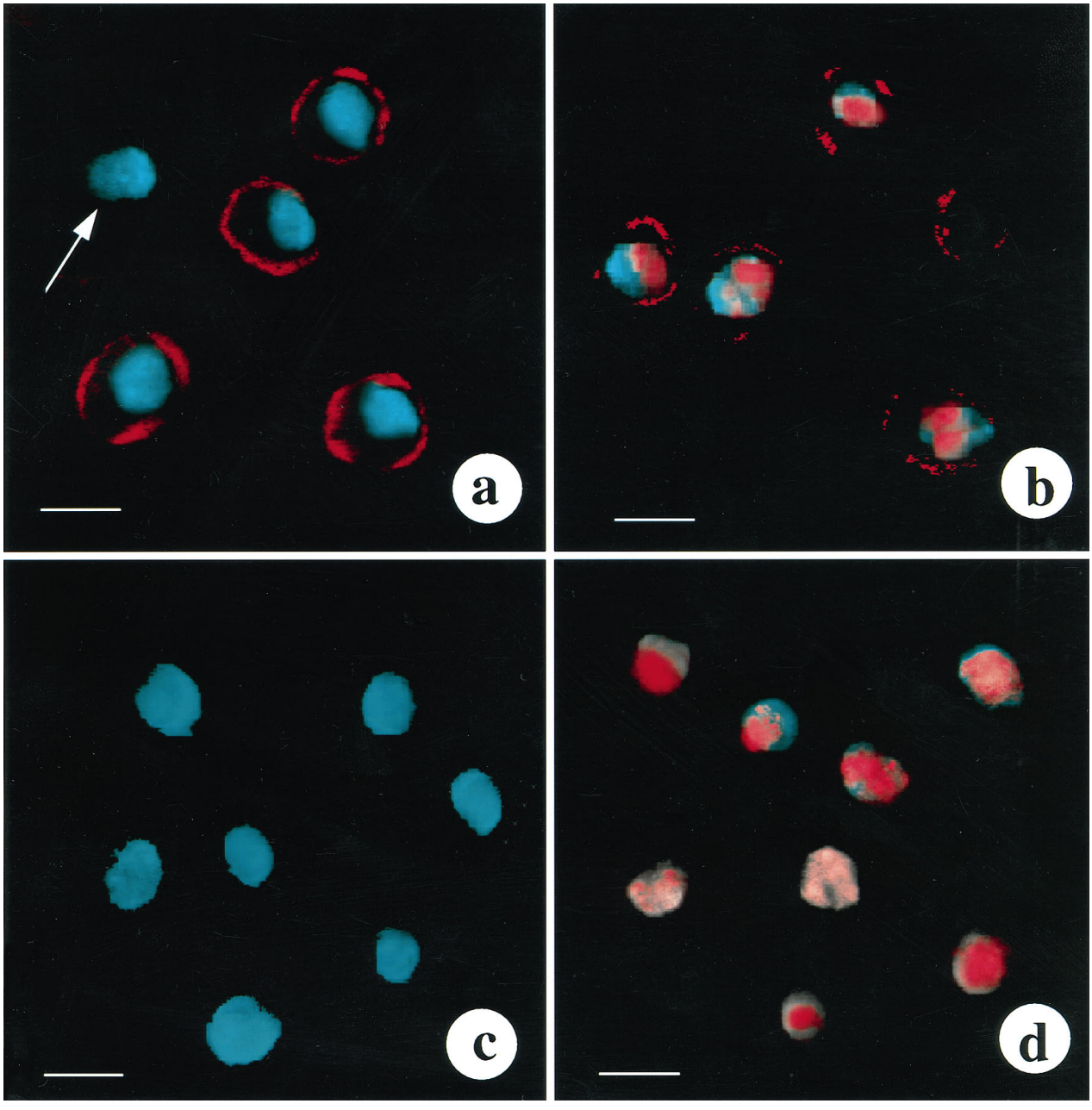

Figure 3. Effect of Ang II on localization of AT_1Rs in neurons after Ang II treatment. Neuronal cultures were incubated without (*a, c*) or with (*b, d*) 100 nM Ang II for 30 min at 37°C. Samples *a* and *b* were used to conduct confocal microscopy after immunofluorescent staining, essentially as described in Materials and Methods. Samples *c* and *d* were subjected to a nuclear isolation protocol, essentially as described elsewhere (Abmayr and Workman, 1992). This was followed by plating the nuclei on the slide, fixation, permeabilization, immunofluorescence with the use of monoclonal anti-AT_1R antibody, and confocal microscopy as described in Materials and Methods. *Arrow* represents an AT_1R negative neuron. Scale bars, 4 μm.

described above. Lysates containing 400 μg protein were incubated with 1 μg rabbit anti-AT_1R antibody overnight at 4°C, and AT_1R immunoprecipitates were collected on protein A/G PLUS-agarose and rinsed three times with lysis buffer and once with kinase assay buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.015% Triton X-100). Immunoprecipitates were suspended in 10 μl kinase assay buffer. For measurement of phosphorylation, 10 μl of AT_1R immunoprecipitate (~30 fmol [^{125}I]-Sar^1-Ile^8-Ang II binding activity) was incubated without or with 0.3 U of MAP kinase, 0.1 mg/ml BSA, and 0.2% β-mercaptoethanol in a final volume of 20 μl. The reaction was started by the addition of 10 μl of ATP mixture (0.3 mM ATP, 30 mM $MgCl_2$, and 200 μCi [γ-^{32}P]-ATP in 1 ml kinase assay buffer) and run for 0–30 min at 30°C. After the reaction was stopped by the addition of phosphoric acid, samples were blotted onto Whatman GF/B filter paper, and then the paper was washed four times with ice-cold 0.5% phosphoric acid and finally once with acetone, essentially as described elsewhere (Paxton et al., 1994). The paper was allowed to dry, and radioactivity was quantitated by liquid scintillation counting. Reaction mixtures that contained 3 μg of MBP instead of AT_1R immunoprecipitate were used as the standard for phosphorylation assays. In certain experiments, the kinase reaction was stopped by the addition of 5× Laemmli's sample buffer instead of phosphoric acid; samples were heated and centrifuged, and the supernatant was electrophoresed in 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and subjected to autoradiography. The same membrane was later probed with AT_1R antibody. [^{32}P]-labeled AT_1R bands were quantitated using a UVP Imagestore 5000 system and quantitated with the use of the SW5000 Gel Analysis program. Data were normalized for equal loading by analysis of the densities of unlabeled AT_1R immunoreactivity.

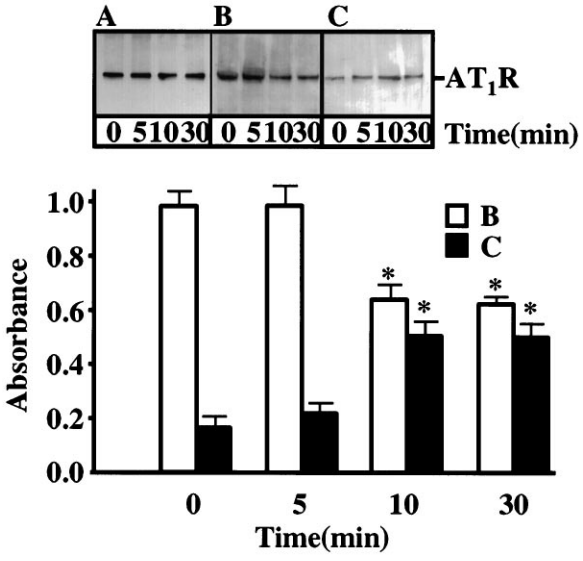

Figure 4. Ang II-induced redistribution of AT_1Rs in neurons. Neuronal cultures, established in 100-mm-diameter culture dishes, were treated with 100 nM Ang II for the indicated time periods. Whole cells (*A*), nuclear fraction (*C*), and rest of the cell fraction (*B*) were collected (Abmayr and Workman, 1992). AT_1Rs from these fractions were immunoprecipitated and quantitated essentially as described in Materials and Methods. *Top*, A representative immunoblot; *bottom*, quantitation of bands representing AT_1R from three separate experiments. Data are presented as absorbance of and have been normalized with zero time absorbance for rest of cell fraction. *Asterisk* indicates significantly different ($p < 0.05$) from zero time.

Labeling of neuronal cells with [^{32}P]-orthophosphate and analysis of phosphorylated AT_1R

Neuronal cultures were established for 15 d in 100-mm-diameter culture dishes. Growth medium was removed, and cultures were incubated with phosphate-free DMEM containing dialyzed PDHS for 4 hr at 37°C followed by prelabeling the cells with 1 mCi/ml ^{32}P-orthophosphate for 4 hr at 37°C. Ang II was added, and incubation was continued for various time periods. Cultures were immediately rinsed three to four times with ice-cold PBS, and lysates were prepared in lysis buffer as described above. Cell lysates were centrifuged at 6000 *g* for 10 min. Proteins from 100 μl of each cell lysate were precipitated by 10 μl of 100% trichloroacetic acid (TCA), and incorporation of [^{32}P] was measured by liquid scintillation counter. Samples containing the same amounts of [^{32}P] radioactivity were used to immunoprecipitate AT_1Rs, essentially as described above. Agarose beads containing AT_1R were collected at 3000 *g* for 10 min, washed three times in lysis buffer, resuspended in 20 μl of Laemmli's sample buffer, and heated to 100°C for 3 min. Supernatant was electrophoresed in 10% SDS-PAGE, and proteins were transferred to PVDF membrane (Bio-Rad). The membrane was dried and subjected to autoradiography at −80°C for 2–3 d. After autoradiography to detect [^{32}P]-labeled AT_1Rs ([^{32}P]-AT_1R), the same membrane was probed for total AT_1Rs by immunoblot analysis. Densities of bands in [^{32}P]-AT_1R and AT_1R were quantitated with the use of UVP Imagestore System and SW5000 Gel Analysis program. Data were normalized for equal loading with the use of total AT_1R.

Immunofluorescence localization of AT_1R

Neuronal cultures established in 35-mm-diameter dishes were rinsed with PBS, pH 7.4, and fixed in −10°C methanol for 5 min. After preincubation with fetal bovine serum for 30 min at 37°C to suppress nonspecific binding of the antibody, cells were incubated with a mouse monoclonal anti-AT_1R antibody at 1 μg/ml concentration in PBS containing 0.5% BSA. After the cells were rinsed five times with PBS at room temperature, they were incubated for an additional 60 min at 37°C with rhodamine-conjugated anti-mouse IgG. Cells were counterstained with DAPI to identify nuclear DNA and nuclei as described elsewhere (Lubke et al., 1994). Appropriate controls in which either primary antibody was replaced by growth medium without AT_1R antibody or without secondary antibody were also run in parallel to determine nonspecific staining. The cells were processed for fluorescent microscopy as described previously (Lu et al., 1996a,b). DAPI-stained nuclei and rhodamine staining representing AT_1R were examined with the use of a confocal microscope. Data were collected by using a 40×/numerical Olympus IMT-2 inverted light microscope in which focal position, excitation lamp shutter, excitation and remission barrier filters, and digital camera shutter were under the control of a stand-alone computer (Hiraoka et al., 1991; Swedlow et al., 1993). Two-dimensional images were processed as described previously (Lu et al., 1996a,b).

Data Analysis

Each experiment was conducted in triplicate culture dishes. Cells in these dishes were derived from multiple brains of 1-d-old rats. Each experiment was repeated three times, unless indicated otherwise. Images from autoradiograms were captured in UVP Imagestore 5000 system, and radioactive bands were quantitated essentially as described elsewhere (Yang et al., 1996a,b). Data from at least three autoradiograms were quantitated and corrected for equal loading by quantitating total AT_1R immunoreactivity or other standard protein. They are presented as mean ± SE. Statistical analysis was performed by using ANOVA and Dunnett's tests.

RESULTS

Effects of Ang II on AT_1R phosphorylation

[^{32}P]-orthophosphate-prelabeled neuronal cultures were incubated with 100 nM Ang II to determine whether occupancy by Ang II of AT_1Rs stimulates its phosphorylation. Figure 1*A* shows that AT_1R antibody immunoprecipitated a radiolabeled band of ~49 kDa. Ang II caused a time-dependent increase in the radioactivity represented by this band, and an approximately sixfold stimulation was observed within 10 min. The molecular weight (MW) of this band corresponded to the reported size for the AT_1R by this antibody (Paxton et al., 1993). Figure 1*B* shows that Ang II-induced phosphorylation of the receptor was mediated by Ang II interaction with the AT_1R, because it was blocked by losartan, an AT_1R subtype-specific antagonist, and not by PD123319, an AT_2R subtype antagonist.

The effect of Ang II on AT_1R internalization was determined next, because studies have shown that phosphorylation of the receptor is key in the agonist-induced internalization of many GPCRs (Lohse, 1993; Freedman et al., 1995). Neuronal cultures were treated with 100 nM Ang II for 15 min, followed by measurement of [^{125}I]-Sar^1-Ile^8-Ang II binding to cell surface AT_1R. Figure 2*A* shows that Ang II caused a significant decrease in the binding of [^{125}I]-Sar^1-Ile^8-Ang II to cell surface AT_1Rs compared with untreated cultures. The decrease was the result of a decrease in B_{max} (60 ± 9 fmol/mg protein in Ang II-treated vs 86 ± 10 fmol/mg protein in control neurons) rather than changes in the K_d values (6.0 ± 0.7 nM in Ang II-treated vs 5.2 ± 0.8 nM in control neurons) (Fig. 2*B*). This indicated that phosphorylation by Ang II is associated with internalization of AT_1Rs. Immunofluorescence combined with confocal microscopy was used to determine the fate of AT_1R after neurons were treated with Ang II. Figure 3*a* shows that AT_1Rs were diffusely and uniformly distributed on the plasma membrane of neuronal cell soma. Incubation with 100 nM Ang II for 30 min resulted in an AT_1R fluorescence accumulation into the nuclear region, as evidenced by their colocalization with DAPI, a nuclear-specific stain (Fig. 3*b*). Conformation of the nuclear localization of AT_1R immunoreactivity was further achieved by immunofluorescence staining in isolated nuclei with the use of a monoclonal anti-AT_1R antibody. Figure 3*d* shows that nuclei isolated from 100 nM Ang II-treated neurons have significant AT_1R staining. Nuclei from untreated neurons lacked this staining (Fig. 3*c*). AT_1R staining on the plasma membrane and in the nucleus was specific, because controls without primary anti-

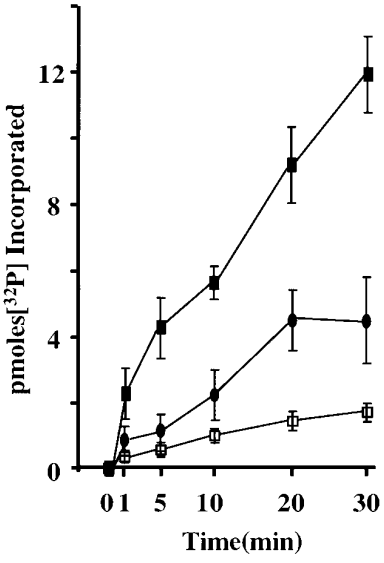

Figure 5. *In vitro* phosphorylation of AT_1Rs by MAP kinase. AT_1R was isolated by immunoprecipitation of neuronal cell lysates with the polyclonal AT_1R antibody as described in Materials and Methods. Immunoprecipitate containing ~30 fmol/mg protein [^{125}I]-Sar^1-Ile^8-Ang II binding activity was incubated without (□-□) or with (●) 0.3 U of MAP kinase at 30°C for indicated time periods to determine the incorporation of [^{32}P] into AT_1R, essentially as described in Materials and Methods. MBP (3 μg) was used as assay control (■). Data are presented as picomoles of radioactivity incorporated as a function of time and are mean ± SE of three experiments.

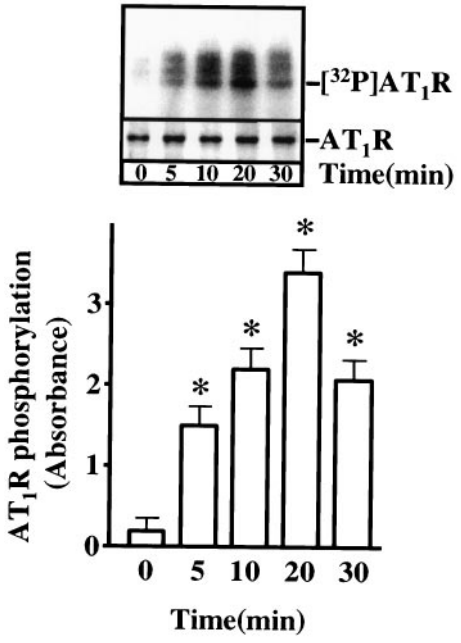

Figure 6. Immunoblot analysis of *in vitro* phosphorylated AT_1Rs by MAP kinase. AT_1Rs were subjected to MAP kinase-mediated phosphorylation essentially as described in legend to Figure 5. Phosphorylated receptor preparation was electrophoresed in 10% SDS-PAGE, and protein was transferred to membrane and subjected to autoradiography. *Top*, A representative autoradiogram; *bottom*, quantitation of [^{32}P]-labeled band corresponding to AT_1R after normalization with AT_1R immunoreactivity for equal loading. Data are mean ± SE ($n = 3$). *Asterisks* indicate significantly different ($p < 0.05$) from zero time control.

body or secondary antibody showed no staining. Specificity of nuclear staining of the AT_1R was further confirmed with the use of the polyclonal anti-AT_1R antibody. This antibody provided essentially the same result as that seen with the monoclonal antibody, although the latter antibody gave sharper staining with minimum background.

Nuclear fractions of control and Ang II-treated neuronal cultures were isolated, and immunoblotting was carried out to confirm the Ang II-induced nuclear translocation of AT_1Rs depicted by the confocal microscopic data. Figure 4*A* shows that the intensity of the immunoreactive band corresponding to AT_1R of the whole cells did not change after treatment with 100 nM Ang II. Its intensity, however, was increased in the nuclear fraction of neurons treated with Ang II as a function of time (Fig. 4*C*). This was associated with a decrease in immunoreactivity in the extract from the rest of the cell (Fig. 4*B*). As a consequence, the nuclear fraction showed a threefold increase in AT_1R immunoreactivity in 30 min.

Role of MAP Kinase in AT_1R phosphorylation

We studied the identity of protein kinase, which may be involved in Ang II-stimulated phosphorylation of AT_1R. The role of MAP kinase was investigated in view of our previous observation that Ang II stimulates this kinase in neuronal cells (Lu et al., 1996b; Yang et al., 1996a) and that MAP kinase may be involved in the translocation of proteins across the nuclear membrane. In addition, the role of this kinase on phosphorylation of estrogen and epidermal growth factor receptors has been reported (Morrison et al., 1993; Kato et al., 1995). AT_1R was immunoprecipitated with the polyclonal AT_1R antibody, and immunoprecipitates were incubated with exogenous MAP kinase in the presence of [^{32}P]-ATP. Figure 5 shows that a time-dependent phosphorylation of AT_1R was seen in the immunoprecipitates, even in the absence of exogenous MAP kinase. Phosphorylation was at low levels and was the first indication that endogenous MAP kinase could be co-immunoprecipitated with AT_1R. Incubation with exogenous MAP kinase significantly increased the incorporation of [^{32}P] into the immunoprecipitate in a time-dependent manner. The phosphorylation reached a plateau in 20 min with 0.3 U of MAP kinase, at which time it was fourfold higher than control samples without exogenous MAP kinase. MBP phosphorylation was used as a standard for MAP kinase substrate in these experiments. SDS-PAGE of *in vitro* phosphorylated AT_1R immunoprecipitate by MAP kinase showed a [^{32}P]-labeled band that corresponded to the molecular size of AT_1R (~49 kDa) (Fig. 6). Density of this band increased as a function of time, which paralleled the *in vitro* phosphorylation time course (Fig. 5). Immunoblot with AT_1R antibody showed a single band of ~49 kDa, consistent with the reported size of the AT_1R (Fig. 6).

A series of co-immunoprecipitation experiments using polyclonal anti-AT_1R antibody were carried out in Ang II-stimulated neuronal cells to further confirm an interaction between the AT_1R and MAP kinase. Figure 7*A* shows that MAP kinase immunoreactivity co-precipitated with AT_1R in Ang II-treated neurons. Maximal association was observed in 5–10 min. In-gel kinase assay was carried out in immunoprecipitated AT_1R preparation isolated from Ang II-treated neurons. Figure 7*B* shows a significant MAP kinase activity in these immunoprecipitates: an approximately threefold increase was observed in 10 min, and only one band was observed. This is consistent with our previous observation that Ang II stimulates only one isoform of MAP kinase in neurons (Yang et al., 1996a). Ang II receptor subtype specificity

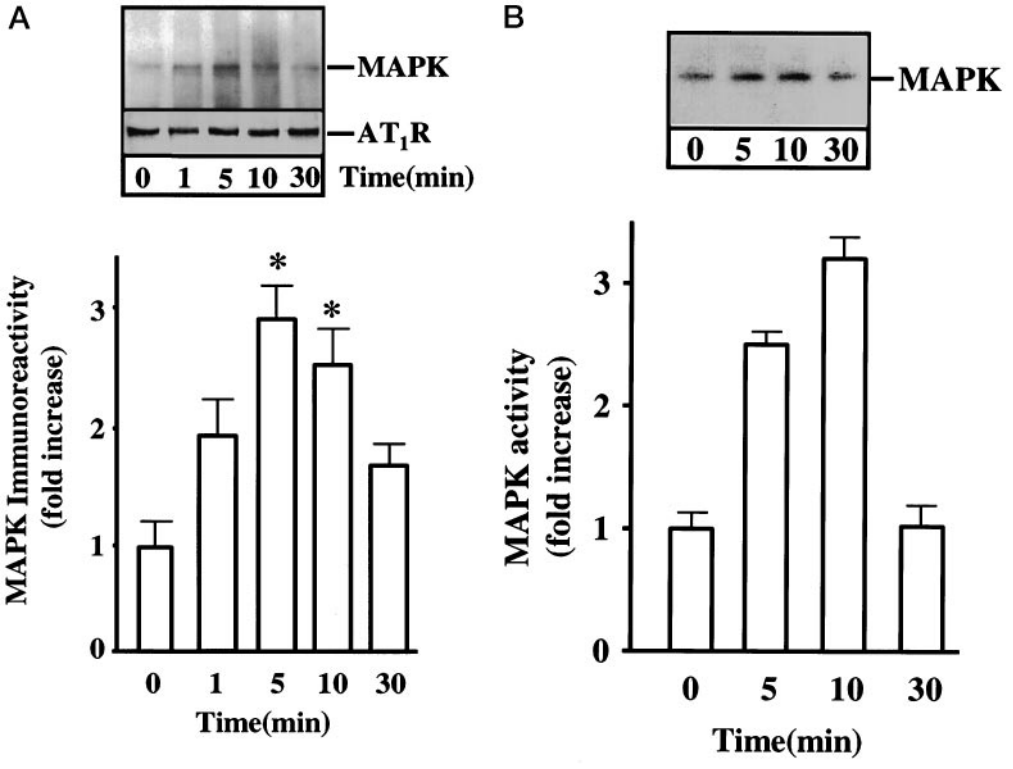

Figure 7. Co-immunoprecipitation of AT_1R with MAP kinase in Ang II-treated neurons. *A*, Co-immunoprecipitation of MAP kinase by AT_1R antibody. Neuronal cultures were incubated with 100 nM Ang II for indicated time periods. Cell lysates were prepared and subjected to immunoprecipitation with the polyclonal AT_1R antibody as described in Materials and Methods. Immunoprecipitates were electrophoresed on SDS-PAGE and immunoblotted with MAP kinase antibody as described in Materials and Methods. Membranes were also immunoblotted with AT_1R antibody to normalize for equal loading. *Top*, A representative immunoblot depicting a band corresponding to MAP kinase (P42); *bottom*, quantitation of radioactive bands corresponding to MAP kinase. Data are mean ± SE ($n = 3$). *Asterisks* shows significantly different ($p < 0.05$) from zero time. *B*, MAP kinase activity in co-immunoprecipitates. Immunoprecipitates, prepared essentially as described in *A*, were used to run in-gel kinase assay to measure MAP kinase activity as described elsewhere (Yang et. al., 1996a).

was determined in Ang II-induced interaction of AT_1R with MAP kinase. Co-precipitation of MAP kinase with AT_1R was blocked by losartan, an AT_1R antagonist, and not by PD123319, an AT_2R antagonist (Fig. 8). Further evidence for co-immunoprecipitation of these two proteins was obtained by the observation that immunoprecipitation of Ang II-treated neurons with MAP kinase antibody contained a protein band of ~49 MW corresponding to AT_1R (data not shown).

Next, neuronal cultures were pretreated with 10 μM PD98059 for 30 min, a relatively selective inhibitor of MAP kinase–kinase under these conditions (Alessi et al., 1995), to determine its effect on Ang II-induced phosphorylation of AT_1R. Figure 9 shows that PD98059 completely attenuated Ang II-induced AT_1R phosphorylation. PD98059 by itself had no effect. Figure 10*A* shows that preincubation of neurons with 10 μM PD98059 also blocked Ang II-induced translocation of AT_1R immunoreactivity into the nucleus. In addition, confocal microscopy of nuclei isolated from PD98059-treated neurons showed no AT_1R immunoreactivity after Ang II treatment (Fig. 10*B*). Collectively, these observations suggest that Ang II stimulation of MAP kinase may be involved in AT_1R phosphorylation and its translocation to the nucleus.

Finally, the effect of phosphorylation by MAP kinase on binding of [^{125}I]-Sar^1-Ile^8-Ang II to AT_1Rs was measured to determine whether phosphorylated receptors retain AT_1 binding activity. Figure 11*A* shows that MAP kinase-phosphorylated AT_1R had very little [^{125}I]-Sar^1-Ile^8-Ang II binding activity compared with nonphosphorylated receptor. This lack of binding was the result of a significant decrease in the B_{max} for phosphorylated receptors (79 ± 8 fmol/mg protein) compared with control (17 ± 5 fmol/mg protein) (Fig. 11*B*).

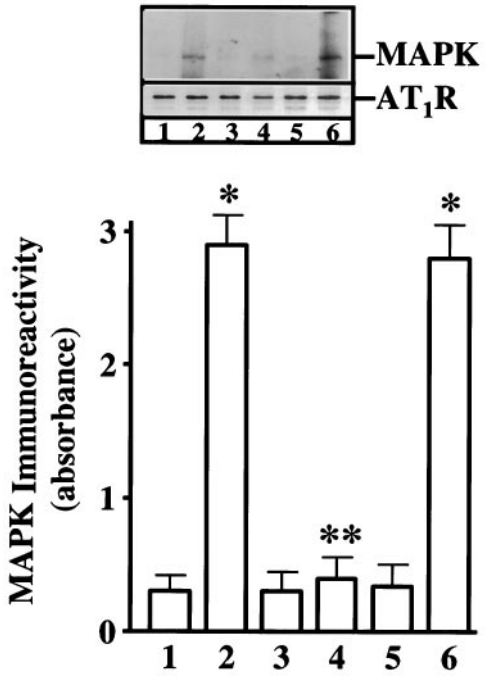

Figure 8. Effect of Ang II receptor antagonists on co-immunoprecipitation of AT_1R with MAP kinase. Neuronal cultures were incubated without (*1, 3, 5*) or with (*2, 4, 6*) 100 nM Ang II for 10 min in the presence of 10 μM losartan (*3, 4*) or 10 μM PD123319 (*5, 6*). Cell lysates were immunoprecipitated with AT_1R antibody, and the immunoprecipitate was subjected to SDS-PAGE and immunoblotted with MAP kinase antibody as described in Materials and Methods. *Top*, A representative immunoblot; *bottom*, quantitation of bands corresponding to MAP kinase. Data are mean ± SE ($n = 3$). *Single asterisks* indicate significantly different ($p < 0.05$) from control (2 and vs 1). *Double asterisk* indicates significantly different from Ang II-treated cells (4 vs 2).

DISCUSSION

Observations presented in this study demonstrate that Ang II stimulates phosphorylation of AT_1R, which is associated with its

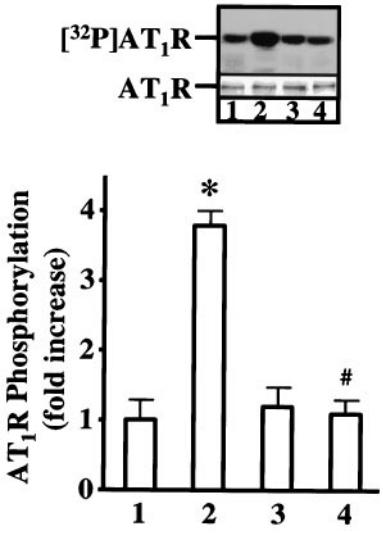

Figure 9. Effect of PD98059 on Ang II-induced phosphorylation of AT_1Rs. Neuronal cultures were prelabeled with [^{32}P]-orthophosphate for 4 hr and incubated without (*1, 3*) or with (*2, 4*) 100 nM Ang II for 10 min in the absence (*1, 2*) or presence (*3, 4*) of 10 μM PD98059. *Top*, Representative autoradiogram; *bottom*, data (mean ± SE; $n = 3$) were normalized for equal loading by immunoblotting of the membranes with AT_1R antibody, essentially as described for Figure 1. *Asterisk* indicates significantly different from 1 ($p < 0.05$); # indicates significantly different from 2 ($p < 0.05$).

translocation into the nucleus. Evidence is also presented to establish that MAP kinase is involved in this phosphorylation and nuclear targeting of AT_1R.

Ang II stimulates phosphorylation of neuronal AT_1R. The site(s) of this phosphorylation could be localized in the intracellular domain, consistent with the presence of threonine, serine, and tyrosine residues in this region of the receptor (Catt et al., 1993; Inagami et al., 1993). Phosphorylation of neuronal AT_1R was associated with its translocation into the nucleus. The presence of nuclear AT_1R in neurons is consistent with earlier reports in which nuclear Ang II receptors have been demonstrated in hepatocytes (Booz et al., 1992; Tang et al., 1992). The precise mechanism involved in AT_1R phosphorylation remains to be worked out fully; however, our data strongly support the notion that MAP kinase plays a crucial role. Ang II stimulates MAP kinase in a Ras-Raf-1-dependent process (Yang et al., 1996a). Stimulation of MAP kinase leads to the propagation of downward signals, which ultimately results in the regulation of neuromodulatory actions of Ang II (Lu et al., 1996b; Yang et al., 1996a). The data presented here suggest another important role of MAP kinase activation by Ang II. They show that MAP kinase is involved in the phosphorylation of AT_1Rs. Evidence for this includes the following: (1) exogenous MAP kinase phosphorylates AT_1R; (2) MAP kinase co-immunoprecipitates with AT_1R; (3) endogenous MAP kinase, co-immunoprecipitated with the receptor, also phosphorylates AT_1R; (4) the AT_1R antagonist losartan blocks both co-immunoprecipitation and colocalization of AT_1R with MAP kinase; (5) MAP kinase-kinase inhibition by PD98059 attenuates Ang II-induced AT_1R phosphorylation; and (6) PD98059 also blocks Ang II-induced nuclear translocation of AT_1Rs. Collectively, these observations provide strong evidence for a direct role of MAP kinase in AT_1R phosphorylation. Additional support for this view is evident in the presence of a MAP kinase recognition sequence (amino acids$^{232–233}$) in the AT_{1b}R subtype, which together with the AT_{1a} subtype are shown to be present in our neuronal cells (Raizada et al., 1993). It is pertinent, however, to add a note of caution in our conclusion. Alternative possibilities of the involvement of other GRKs, such as βARK1, also should not be ruled out at the present time in Ang II-induced AT_1R phosphorylation. For example, it may be possible that Ang II stimulates MAP kinase, which activates other GRKs that in turn phosphorylate the AT_1R. Such a hypothesis is supported by recent data demonstrating AT_1R phosphorylation by βARK1 (Oppermann et al., 1996). This is supported further by the lack of MAP kinase recognition sequence in the AT_{1a}R subtype. Thus, whether MAP kinase indirectly (AT_{1a} and AT_{1b} subtypes) or directly (AT_{1b}) phosphorylates the receptor remains to be elucidated.

Three questions arise from these observations concerning the role of MAP kinase. First, is MAP kinase involved in the translocation of AT_1R to the nucleus? It is tempting to suggest that MAP kinase, in addition to phosphorylating AT_1R, also may phosphorylate relevant protein(s) in the nuclear pore complex so that the phosphorylated AT_1R and other signaling molecules, including MAP kinase itself, are transported across the nuclear membrane. The following evidence supports this notion. (1) MAP kinase is localized predominately around the nuclear membrane in the neurons and is translocated into the nucleus after stimulation with Ang II (Lu et al., 1996b), and (2) MAP kinase plays a role in the translocation of other neuronal signaling proteins such as Stat3 in neurons. Our studies have indicated that Ang II-mediated translocation of Stat3 requires activation of MAP kinase, because its depletion causes accumulation of Stat3 in the cytoplasmic compartment (unpublished observation). (3) p62 and gp210, two proteins of nuclear pore complex that are involved in nuclear translocation of cytoplasmic proteins, contain MAP kinase phosphorylation motifs (Wozniak et al., 1989). This would suggest that MAP kinase phosphorylation of these proteins may be significant in the nuclear translocation of AT_1R.

Second, is phosphorylation of AT_1R necessary for its nuclear targeting? Our observations showing that the MAP kinase kinase inhibitor PD98059 inhibits both Ang II-induced AT_1R phosphorylation and its nuclear translocation strongly support the role of phosphorylation. Third, how does AT_1R, a plasma membrane protein, transport across the nuclear membrane? It has been suggested that the presence of a nuclear localization signal (NLS) in many peptide hormones and their membrane receptors plays an important role in the translocation into the nucleus (Scearce et al., 1993; Jans, 1994; Radulescu, 1995). For example, it has been suggested (Radulescu, 1995) that the presence of NLS in the α subunit of insulin receptor is the key in the nuclear translocation of this receptor and in the effects of insulin on gene regulation. Thus, we hypothesized that the AT_1R may contain NLS consensus sequence, which could guide this protein across the nuclear membrane. It was intriguing to find two consensus sequences in this receptor that hold the potential for NLS (Dingwall et al., 1988; Kang et al., 1994). They are amino acids 304–310 on the C-terminal tail (LGKKFKK) and 221–225 on the third intracellular loop (ALKKA). This would confirm that, like the insulin receptor, AT_1R is translocated into the nucleus and that the mechanism of this translocation involves these NLSs. Mutagenesis studies involving this NLS region of the AT_1R will be needed to confirm this point of view.

Finally, the role of AT_1R phosphorylation and its translocation into the nucleus on persistent stimulatory actions of Ang II on neuromodulation needs to be examined. Our data show that Ang

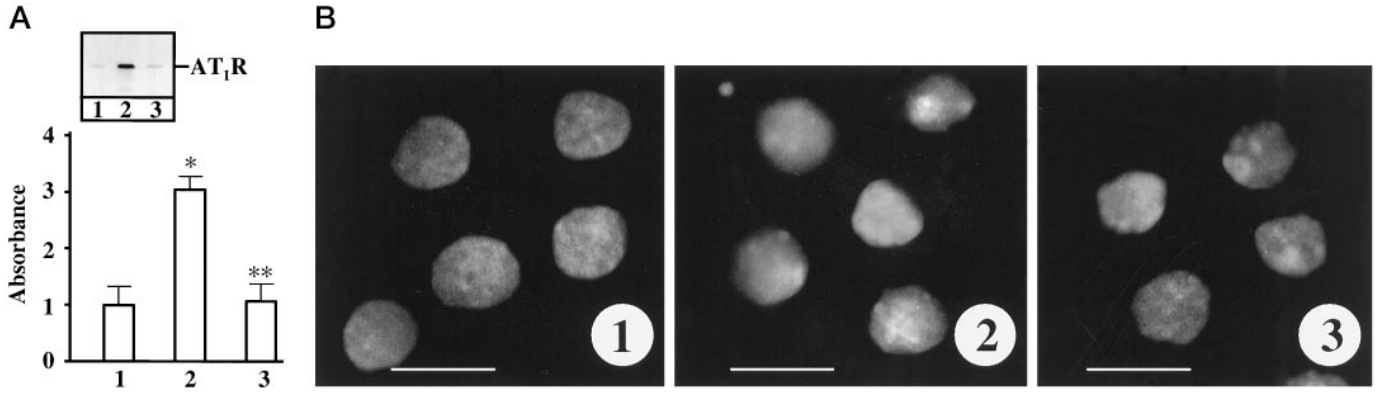

Figure 10. Effect of PD98059 on Ang II-induced AT_1R translocation into the nuclear fraction. *A*, Immunoblotting of AT_1R in nuclear fraction. Neuronal cultures were incubated without (*1*) or with (*2, 3*) 100 nM Ang II for 30 min at 37°C in the absence (*1, 2*) or presence (*3*) of 10 μM PD98059. Neurons were collected, and the nuclear fraction was isolated and subjected to immunoprecipitation with the AT_1R polyclonal antibody. Immunoprecipitates were analyzed on SDS-PAGE as described in Materials and Methods. *Top*, Representative immunoblot; *bottom*, data are presented as absorbance of AT_1R band density and are mean ± SE of three experiments. *Single asterisk* indicates significantly different from 1 ($p < 0.05$); *double asterisk* indicates significantly different from 2 ($p < 0.05$). *B*, Confocal microscopic images of AT_1R immunoreactivity in nuclei. Neurons were treated without (*1*) or with (*2, 3*) 100 nM Ang II in the absence (*2*) or presence (*3*) of 10 μM PD98059 for 30 min at 37°C. Nuclei were isolated, fixed on slides, and subjected to confocal microscopic analysis with the use of monoclonal AT_1R antibody, essentially as described in Materials and Methods.

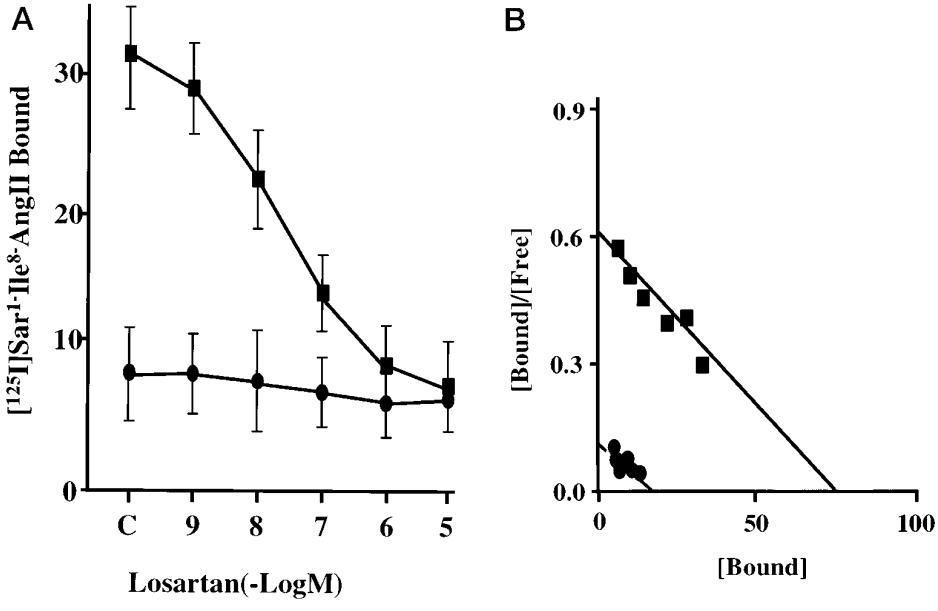

Figure 11. Effect of MAP kinase-mediated phosphorylation on binding of $[^{125}I]$-Sar^1-Ile^8-Ang II to AT_1R. Neuronal cell lysates were used to immunoprecipitate AT_1R. Immunoprecipitates containing ~30 fmol ^{125}I-Sar^1-Ile^8-Ang II binding activity were used to incubate without (■) or with (●) 0.3 U of MAP kinase, essentially as described in legends to Figures 5 and 6 and in Materials and Methods. This was followed by determination of the ability of phosphorylated AT_1R to bind $[^{125}I]$-Sar^1-Ile^8-Ang II. *A*, Competition–inhibition of $[^{125}I]$-Sar^1-Ile^8-Ang II binding in control (■) and MAP kinase-phosphorylated (●) receptors with indicated concentrations of losartan as described in Materials and Methods. Each point represented triplicate samples and mean ± SE of three experiments. *B*, Scatchard analysis of data from Figure 11*A*.

II stimulates phosphorylation of AT_1Rs, which is associated with their internalization. Also, phosphorylated AT_1R lacks Ang II binding activity. This would suggest that, like other GPCRs, Ang II induces events associated with AT_1R desensitization (Boulay et al., 1994; Kai et al., 1994). Norepinephrine transporter and TH activities, however, are chronically stimulated by Ang II for 4–24 hr despite this desensitization (Lu et al., 1996a; Yu et al., 1996). On the basis of our data, it is tempting to suggest that a selective targeting of certain populations of AT_1Rs to the nucleus where it may act as a transcription regulator may hold the key to explaining the chronic neuromodulatory response of Ang II. Additional experiments will be needed to clarify the relationship between Ang II-mediated internalization-induced desensitization and nuclear translocation of AT_1Rs with chronic actions of Ang II in the neurons. Nonetheless, these observations are relevant, because they demonstrate three unique features of this GPCR. (1) Phosphorylation of the receptor is MAP kinase-mediated; (2) chronic stimulation of cellular response is not related to desensitization of the receptor; and (3) the presence of NLS in the AT_1R sequence may be the basis of its translocation into the nucleus.

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