Drinking behavior elicited by central injection of angiotensin II: roles for protein kinase C and Ca\(^{2+}\)/calmodulin-dependent protein kinase II

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**Fleegal, Melissa A., and Colin Sumners.** Drinking behavior elicited by central injection of angiotensin II: roles for protein kinase C and Ca\(^{2+}\)/calmodulin-dependent protein kinase II. Am J Physiol Regul Integr Comp Physiol 285: R632–R640, 2003. First published May 8, 2003; 10.1152/ajpregu.00151.2003.—Prior studies utilizing neurons cultured from the hypothalamus and brain stem of newborn rats have demonstrated that ANG II-induced modulation of neuronal firing involves activation of both protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII). The present studies were performed to determine whether these signaling molecules are also involved in physiological responses elicited by ANG II in the brain in vivo. Central injection of ANG II (10 ng/2 μl) into the lateral cerebroventricle (icv) of Sprague-Dawley rats increased water intake in a time-dependent manner. This ANG II-mediated dipsogenic response was attenuated by central injection of the PKC inhibitors chelerythrine chloride (0.5–50 μM, 2 μl) and Go-6976 (2.3 nM, 2 μl) and by the CaMKII inhibitor KN-93 (10 μM, 2 μl). Conversely, icv injection of chelerythrine chloride (50 μM, 2 μl) and KN-93 (10 μM, 2 μl) had no effect on the dipsogenic response elicited by central injection of carbachol (200 ng/2 μl). Furthermore, injection of ANG II (10 ng/2 μl) icv increases the activity of both PKC-α and CaMKII in rat septum and hypothalamus. These data suggest that signaling molecules involved in ANG II-induced responses in vitro are also relevant in physiological responses elicited by ANG II in the whole animal model.

The above physiological and behavioral effects of ANG II are mediated by rapid receptor-mediated alterations in neuronal activity, resulting in release of neuromodulators and neurotransmitters, which then modulate other neurons. In vitro studies from our laboratory have used neurons cultured from the hypothalamus and brain stem of newborn rats to elucidate the cellular and intracellular actions that underlie the ANG II-mediated changes in neuronal activity. These studies demonstrated that ANG II elicits a positive chronotropic effect via AT\(_1\) receptors (35, 37). Specifically, these experiments have shown that ANG II increases total calcium current (\(I_{\text{Ca}}\)), decreases the voltage-dependent delayed rectifier (\(I_{\text{Kd}}\)-) and A-type (\(I_{\text{A}}\)) K\(^+\) currents, and consequently increases neuronal firing rate (34, 37, 38). The specific intracellular signaling pathways that are involved in these AT\(_1\) receptor-mediated effects have also been investigated, and the results indicate that ANG II modulation of neuronal K\(^+\) and Ca\(^{2+}\) currents involves stimulation of phospholipase C and activation of subsequent Ca\(^{2+}\)-dependent signaling molecules (7, 34, 40). Specifically, these data suggest that the ANG II-stimulated increases in neuronal firing rate and decreases in \(I_{\text{K}}\) involve activation of both protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin dependent protein kinase II (CaMKII) (34, 35, 40), while the ANG II-stimulated increase in \(I_{\text{Ca}}\) appears to only require activation of PKC (34, 38). Moreover, recent experiments indicate the involvement of PKC-α in the ANG II-stimulated decrease in \(I_{\text{K}}\) (33).

While the neuronal culture model has been useful in identifying the intracellular signaling components involved in the rapid cellular actions of ANG II, it is important to establish that these mechanisms operate in vivo. In situ electrophysiological studies have demonstrated that ANG II via the AT\(_1\) receptor alters neuronal K\(^+\) current and firing rate in hypothalamic nuclei, similar to the effects observed in cell culture (9). However, these in vivo studies have not investigated the intracellular signaling molecules involved in the centrally mediated effects of ANG II. The goal of the present study was to ascertain whether the intracellular

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lar signaling molecules identified as important in the ANG II-mediated changes in neuronal activity in vitro are also important in the brain.

One of the most striking actions of ANG II in the brain is the AT<sub>1</sub> receptor-mediated stimulation of water intake, an effect that is one component of this peptide's regulatory actions on fluid balance (2, 5, 10, 21, 24, 28). Intracerebroventricular injection of ANG II elicits a rapid and acute drinking response that occurs within ~1 min, similar to the time course necessary for ANG II-mediated changes in membrane ionic currents and neuronal activity (~1–3 min). Furthermore, this effect involves activation of neuronal AT<sub>1</sub> receptors in the subfornical organ (SFO), paraventricular nucleus (PVN), and median preoptic nucleus (MnPO) (10, 12, 13, 19, 21, 22, 27, 30). In the experiments described here, we assess the roles of PKC and CaMKII in the drinking response produced by injection of ANG II into the lateral cerebroventricle (icv) and third ventricle (i3v) of Sprague-Dawley (SD) rats. The results indicate that 1) central injection of ANG II activates PKC-α and CaMKII in the septal and hypothalamic regions of the SD rat brain; and 2) the dipogenic response produced by icv or i3v injection of ANG II involves activation of both PKC-α and CaMKII.

MATERIALS AND METHODS

Chemicals and Supplies

ANG II, KN-93, carbamylcholine chloride (carbachol), and anti-rabbit peroxidase-conjugated secondary antibody were all purchased from Sigma (St. Louis, MO), Chelerythrine chloride and Go-6976 were obtained from BioMol (Plymouth Meeting, PA). Rabbit anti-PKC-α, rabbit anti-PKC-β1, and rabbit anti-phosphoCaMKII-α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-paxillin was purchased from BD Transduction Laboratories (San Jose, CA), and anti-mouse peroxidase-conjugated secondary antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Stainless steel guide cannulas and microinjectors were special ordered from Plastics One (Roanoke, VA). Protein assay dye, 10% Tris-HCl Criterion gels, and nitrocellulose were purchased from BioRad Laboratories (Hercules, CA). Western Lightning Chemiluminescence Reagent Plus was purchased from Perkin Elmer Life Sciences (Boston, MA).

Animals

Adult male rats were purchased from Charles River Laboratories (Wilmington, MA). Rats were housed individually in light-controlled facilities. Animals were fed ad libitum and had free access to water. All procedures were approved by the University of Florida Institutional Animal Use and Care Committee.

Surgical Procedure

Adult male rats (200–250 g) were anesthetized with ketamine (30 mg/kg body wt im) and xylazine (6 mg/kg body wt im). Once fully anesthetized, rats were placed in a Kopf stereotaxic frame, a midline incision was made on the top of the head, and the skull was cleaned. For icv injections, a stainless steel guide cannula (22 gauge) was placed into the right lateral cerebroventricle using the following stereotaxic coordinates: bregma –1.30 mm, lateral 1.50 mm, from skull –4.50 mm (26). Since many of the ANG II-responsive nuclei surround the third ventricle, i3v injections were made using a stainless steel guide cannula (26 gauge) placed into the third cerebroventricle according to the following stereotaxic coordinates: bregma –2.56 mm, lateral 0.00 mm, from skull –10.00 mm (26). Postsurgery, rats were injected with bana mine (1.1 mg/kg body wt im) and allowed to recover for 1 wk.

Injection Protocols

To confirm correct placement of the guide cannula, rats were injected icv or i3v with ANG II (10 ng/2 μl) and monitored for a drinking response for 30 min postinjection. Additionally, if the protocol allowed, rat brains were dissected to further verify that the cannula was correctly placed. The dose of ANG II employed here is at the lower end of the range used in similar studies and consistently yielded a rapid and vigorous drinking response. These routes of administration allow ANG II and all pharmacological inhibitors to reach AT<sub>1</sub> receptors within the SFO and PVN. Rats with a positive drinking response (>6 ml of water in 30-min time period) were entered into the study. For the remainder of the study, rats with icv or i3v cannulas randomly received either injections of 0.9% saline (2 μl), ANG II (10 ng/2 μl), inhibitor (2 μl), or an inhibitor (2 μl) pretreatment (15 min) followed by ANG II (10 ng/2 μl) until each rat had received all four treatments. A similar protocol was used for another group of icv-cannulated rats, except carbachol (200 ng/2 μl) was substituted for ANG II as the dipsogen. At least 2 days separated each injection. All injections were given between 7:30 AM and 12:00 PM to control for differences in water intake throughout the day. After completion of one inhibitor sequence, all rats were injected with ANG II (10 ng/2 μl) to verify that a positive drinking response still existed and to ensure the inhibitors had no long-term effects on the ANG II-induced drinking response. Rats were either killed or used in further protocols. A total of 109 male SD rats were used to complete all these studies.

Analysis of PKC and CaMKII Activities

Changes in the activity of PKC-α and PKC-β1 were assessed within the septum-hypothalamus and brain stem (medulla) regions after icv injection of ANG II. Activation of PKC was determined by measuring the translocation of PKC protein from the cytosolic to membrane fractions within cell extracts. This was achieved by Western blot analysis of PKC-α and PKC-β1 proteins in both the cytosolic and membrane fractions of the cellular extracts, an approach widely used by others (16, 17). Simultaneous changes in the activity of CaMKII after icv injection of ANG II were assessed by Western blot analysis of phosphorylated CaMKII-α, the active form of this enzyme within the cytosolic fraction. Detailed methodologies for these procedures were as follows.

Extraction of cytosolic and membrane proteins. Rats demonstrating a positive drinking response were injected with either ANG II (10 ng/2 μl) or 0.9% saline (2 μl) and monitored for water intake. Five minutes postinjection, rats were killed, and their brains were removed. Brains were placed in ice-cold PBS, and the septum and hypothalamus regions (according to bregma: rostral to caudal 0.0 to –5.5 mm; dorsal to ventral 2.5 to 10.2 mm) (26) and brain stem (according to bregma: rostral to caudal –8.0 to −15.0 mm; dorsal to ventral 7.0 to 11.0 mm) (26) were dissected and frozen on dry ice. Frozen brains were stored at −80°C until they could be processed.
The frozen septal-hypothalamic regions and brain stems were weighed and homogenized on ice in 5 vol (5 × weight in g) of extraction buffer (pH 7.5) containing (in mM) 20 Tris·HCl, 150 NaCl, 2 EDTA, 1 EGTA, 1 PMSF, and 1 NaVO₄. To remove cellular debris, samples were centrifuged at 1,000 g for 30 min at 4°C followed by centrifugation of the supernatant at 45,000 g for 20 min at 4°C. The supernatant was saved as the membrane fraction and stored at –80°C. The pellet was resuspended in extraction buffer supplemented with 0.5% Triton X-100 and were shaken for 60 min at 4°C. The resuspended pellet was centrifuged at 45,000 g for 20 min at 4°C, and the supernatant was saved as the cytosolic fraction and stored at –80°C. Protein concentrations for both cytosolic and membrane fractions were determined using a Bradford-based protein assay.

Western Blot analysis of PKC, phosphoCaMKII, and paxillin proteins. Laemmli sample buffer (1.5×) was added to the membrane and cytosolic fractions (10 μg of protein for septal-hypothalamic region and 5 μg of protein for the brain stem) and boiled for 5 min at 100°C. SDS-PAGE and Western blot analyses were performed as described previously (3, 15). Briefly, proteins were separated using 10% Tris-HCl gels and then transferred to nitrocellulose. Western blot analysis was performed according to protocol from Santa Cruz Biotechnology. Anti-PKC-α (1:10,000), anti-PKC-β (1:10,000), and anti-phosphoCaMKII-α (1:500) primary antibodies and anti-rabbit secondary antibody (1:16,000) were used to detect PKC-α (80 kDa), PKC-β (80 kDa), and phosphorylated CaMKII-α (50 kDa). To ensure equal loading of samples, anti-paxillin primary antibody (1:10,000) and anti-mouse secondary antibody (1:10,000) were used to detect paxillin (68 kDa), a protein not affected by ANG II treatment. Bands were visualized using Western Lightning Chemiluminescence Reagent Plus and were quantified using a GS-710 Densitometer and the Quantity One protein analysis package (BioRad Laboratories). Data are presented as arbitrary units.

Data Analysis

All results are expressed as means ± SE. Statistical significance was determined using either a repeated-measures one-way ANOVA, one-way ANOVA, or the Mann-Whitney rank sum test. If the ANOVA yielded statistical significance (P < 0.05), pairwise comparisons were performed using the Bonferroni t-test. Differences were significant at P < 0.05; n refers to the number of animals.

RESULTS

Role of PKC in the ANG II-Induced Drinking Response

Intracerebroventricular injection of ANG II (10 ng/2 μl) via the icv route stimulated a significant increase in water intake within 5 min (Fig. 1). Furthermore, this dipsogenic response was sustained over the next 10 min and then tapered off (Fig. 1). The total volume of water consumed 30 min after ANG II (10 ng/2 μl) injection was significantly greater than the total volume ingested 30 min after 0.9% saline (2 μl) injection (2,600% above control; Fig. 1). Likewise, i3v injection of ANG II (10 ng/2 μl) produced a significant increase in water intake (1,800%) compared with saline-treated (0.9%, 2 μl) control rats.

Having established the drinking responses to icv- and i3v-injected ANG II, we determined whether inhibition of PKC modified these responses. The data shown in Fig. 2A clearly show that injection of the general PKC inhibitor chelerythrine chloride (0.5–50 μM, 2 μl) into the lateral cerebroventricle (icv) reduced the ANG II (10 ng/2 μl icv)-stimulated drinking response in a dose-dependent manner (21, 42, and 61% respectively). Similarly, i3v injection with chelerythrine chloride (50 μM, 2 μl) significantly attenuated the drinking response (~33%) produced by ANG II (10 ng/2 μl) injected via the same route (Fig. 2B). Compared with saline-treated (0.9%, 2 μl) controls, central injection of chelerythrine chloride (0.5–50 μM, 2 μl) alone had no effect on water intake over the 30-min time period (Fig. 2, A and B). Furthermore, after icv or i3v injection of chelerythrine chloride (0.5–50 μM, 2 μl), it was observed that rats were still active and alert (i.e., rats roamed the cage, groomed themselves, and would approach the water bottle randomly).

Because in vitro studies demonstrated that PKC-α is important in ANG II-mediated effects on neuronal IKᵥ (20), we determined if the ANG II-mediated drinking response also involved activation of this PKC isoform. Figure 2C shows that Go-6976 (2.3 nM, 2 μl), a PKC inhibitor specific for PKC-α at this dose (20), attenuated the ANG II-induced drinking response (32%). Go-6976 (2.3 nM, 2 μl) alone did not alter water intake compared with rats injected with 0.9% saline (2 μl; Fig. 2C) nor were there any gross behavior effects (i.e., rats were active and alert). These data suggest that the PKC-α isoyme is involved in the ANG II-mediated drinking response.

Previous studies have demonstrated that central injection of the muscarinic acetylcholine receptor agonist carbachol elicits a drinking response, and this effect involves activation of an inhibitory G protein (31). To verify that results of inhibition of PKC were specific to the ANG II-induced drinking response, the effects of chelerythrine chloride on the dipsogenic response of carbachol were also investigated. Compared with sa-
Fig. 2. ANG II-induced drinking response is attenuated by protein kinase C (PKC) inhibition. 

A: rats were injected icv with the general PKC inhibitor chelerythrine chloride (Ch, 0.5–50 μM, 2 μl) followed by an icv injection of ANG II (10 ng/2 μl), and drinking was recorded over the next 30 min. Graph shows mean ± SE water intake for the above treatment conditions. Also shown are mean ± SE water intakes after icv injection of 0.9% saline (2 μl), ANG II (10 ng/2 μl), or Ch (0.5–50 μM, 2 μl) alone. Sample sizes are as follows: saline n = 19, ANG II n = 21, Ch(50) n = 8, Ch(50)/ANG II n = 8, Ch(5)/ANG II n = 12, Ch(0.5)/ANG II n = 13, Ch(0.5)/ANG II n = 5, Ch(0.5)/ANG II n = 6. *P < 0.05 from control. †P < 0.05 from ANG II. 

B: rats were injected in the 3rd cerebroventricle (icv) with the general PKC inhibitor Ch (50 μM, 2 μl), and drinking was recorded over the next 30 min. Bar graph shows mean ± SE water intake for the above treatment condition. Also shown are mean ± SE water intakes after icv injection of 0.9% saline (2 μl), ANG II (10 ng/2 μl), or Ch (50 μM, 2 μl) alone; n = 9 for all treatment conditions. *P < 0.05 from control. †P < 0.05 from ANG II. 

C: rats were injected icv with the selective PKC-α inhibitor Go-6976 (2.3 nM, 2 μl). Fifteen minutes later rats were injected icv with ANG II (10 ng/2 μl), and drinking was recorded over the next 30 min. Graph shows mean ± SE water intake for the above treatment condition. Also shown are mean ± SE water intakes after icv injection of 0.9% saline (2 μl), ANG II (10 ng/2 μl), or Go-6976 (2.3 nM, 2 μl) alone; n = 8 for rats treated with Go-6976, and n = 9 for all other treatment conditions. *P < 0.05 from control. †P < 0.05 from ANG II. 

D: rats were injected icv with the general PKC inhibitor Ch (50 μM, 2 μl). Fifteen minutes later rats were injected with carbacol (Carb, 200 ng/2 μl icv), and drinking was recorded over the next 60 min. Bar graph shows mean ± SE water intake for the above treatment condition. Also shown are mean ± SE water intakes after icv injection of 0.9% saline (2 μl), Carb (200 ng/2 μl icv), or Ch (50 μM, 2 μl) alone; n = 15 for all treatment conditions. *P < 0.05 from control. 

line-treated (0.9%; 2 μl) control rats, icv injection of carbacol (200 ng/2 μl) increased water intake by ~400% over a 60-min time period (Fig. 2D). However, this dipsogenic effect of carbacol was not affected by prior icv injection of the rats with the PKC inhibitor chelerythrine chloride (50 μM, 2 μl; Fig. 2D).

**ANG II Increases PKC-α Activity in the Rat Septum and Hypothalamus**

To further substantiate a role for PKC-α in the ANG II-induced drinking response, we determined whether the PKC-α isozyme was activated in the septum and hypothalamus and brain stem after icv injection of ANG II. In addition, we studied the effects of ANG II on PKC-β1 activity for the sake of comparison. Injection of ANG II (10 ng/2 μl, 5 min) icv stimulated a translocation of PKC-α from the cytosol to the membrane in a hypothalamic block containing the SFO, PVN, and MnPO (Fig. 3A). This translocation was indicated by an ~77% increase in the levels of PKC-α protein in the membrane fraction of ANG II-treated rats compared with saline (0.9%; 2 μl) controls, and 2) a 65% decrease in the ratio of cytosolic vs. membrane PKC-α protein levels in the ANG II-treated rats (Fig. 3A). Conversely, injection of ANG II (10 ng/2 μl, 5 min) icv had no significant effect on the activity of PKC-α in the brain stem (Fig. 3B), as indicated by a lack of significant translocation from the cytosol to membrane in ANG II-treated rats. Furthermore, icv injection of ANG II had no significant effect on the activation of PKC-β1 in
the septum-hypothalamus (mean ± SE ratio of cytosolic vs. membrane PKC-βI protein levels: saline 72.1 ± 42.4; ANG II 130.4 ± 97.8) and brain stem (mean ± SE ratio of cytosolic vs. membrane PKC-βI protein levels: saline 12.4 ± 1.91; ANG II 21.7 ± 7.92). This indicates that the action of ANG II is specific for PKC-α. As a gel loading control, levels of paxillin were assessed in both the cytosol and membrane fractions of the septum-hypothalamus and brain stem. In all conditions, paxillin protein levels were similar (Fig. 3).

**Role of CaMKII in the ANG II-Induced Drinking Response**

Studies in neuronal cultures have also indicated that along with PKC, CaMKII is involved in the ANG II modulation of neuronal $I_{\text{Kv}}$ and firing (33–35, 37, 40). In the next set of experiments, we determined whether CaMKII was important for the ANG II-induced drinking response. Injection of the CaMKII inhibitor KN-93 (10 μM, 2 μl) either icv or i3v significantly attenuated the drinking response produced by ANG II (10 ng/2 μl) administered via the same route (38 and 33%, respectively; Fig. 4, A and B). Importantly, central injection of KN-93 (10 μM, 2 μl) alone had no effect on water intake or behavior compared with central injection of 0.9% saline (2 μl; Fig. 4, A and B). In addition, this effect of KN-93 (10 μM, 2 μl) was specific to the ANG II-induced drinking response because it did not alter the increase in water intake elicited by icv injection of carbachol (200 ng/2 μl; Fig. 4C).

Because our studies demonstrated that both PKC-α and CaMKII play a role in the ANG II-stimulated drinking response, the next set of experiments was performed to determine if inhibition of both PKC and CaMKII would abolish the ANG II drinking response completely. Coinjection (2 μl) of both chelerythrine chloride (50 μM) and KN-93 (10 μM) significantly reduced the ANG II (10 ng/2 μl)-stimulated drinking response (23%), but the effect of each inhibitor was not additive (Fig. 5).

**ANG II Increases CaMKII Activity in the Rat Septum and Hypothalamus**

To substantiate the above findings, the next set of experiments investigated the activation of CAMKII in the hypothalamus and brain stem after central injection of saline and ANG II. ANG II (10 ng/2 μl, 5 min) injected icv increased the activity of CaMKII-α, as determined by the levels of phosphorylated CaMKII-α, in the septum-hypothalamus (Fig. 6) by ~92%. Phosphorylated CaMKII-α protein was not detectable in the brain stem of 0.9% saline- or ANG II-treated rats (data not shown). The levels of paxillin protein were similar under each treatment condition, indicating that there was equal loading of each sample onto the gel (Fig. 6).
Role of Phosphatidylinositol 3-Kinase and c-Jun NH2-Terminal Kinase in the ANG II-Induced Drinking Response

In vitro studies from our laboratory have also demonstrated that ANG II elicits cellular and physiological responses that are delayed (>30 min) when compared with the ANG II-stimulated changes in neuronal activity (<5 min) (33). Additionally, studies have demonstrated that these ANG II-mediated longer-term effects involve a variety of signaling molecules, which include phosphatidylinositol 3-kinase (PI3-K) and c-Jun NH2-terminal kinase (JNK) (14, 33, 39). Temporally, because activation of PI3-K and JNK occurs after the changes in neuronal activity and the induction of the drinking response (14, 33, 39), it is not expected that inhibition of these molecules would alter the ANG II-stimulated dipsogenic response. Indeed, as Fig. 7 demonstrates, icv injection of either the selective PI3-K inhibitor LY-294002 (10 μM, 2 μl; Fig. 7A) or the pharmacological JNK inhibitor II (100 nM, 2 μl; Fig. 7B) did not significantly reduce the increase in water intake elicited by ANG II (10 ng/2 μl) injected via the same route.

DISCUSSION

The data presented here demonstrate that the drinking response induced by central injection of ANG II involves activation of the signaling molecules PKC-α and CaMKII. As indicated in Fig. 1, icv injection of ANG II stimulates a rapid and vigorous drinking response (~5 min) that is sustained for ~10–15 min and then begins to taper off with random bursts occurring for the remainder of the 30 min. A similar response profile is observed with icv-injected ANG II. The beginning of the ANG II-induced drinking time course is
Fig. 6. Central injection of ANG II activates CaMKII-α in the rat septum and hypothalamus. A: representative blots showing phosphorylated CaMKII-α (pCaMKII-α) and paxillin protein levels in the cytosolic fractions of septal-hypothalamic extracts from 3 saline-treated (0.9%, 2 μl icv) and 4 ANG II-treated (10 ng/2 μl icv) rats. B: bar graph shows mean ± SE pCaMKII-α protein levels for each treatment condition; n = 9 for saline controls and n = 12 for ANG II-treated rats. *P < 0.05 compared with saline-treated rats.

Fig. 7. Inhibition of either phosphatidylinositol 3-kinase (PI3-K) or c-Jun NH2-terminal kinase (JNK) does not alter the ANG II-induced drinking response. A: rats (n = 14) were injected icv with the selective PI3-K inhibitor LY-294002 (LY, 10 μM). Fifteen minutes later rats received an icv injection of ANG II (10 ng/2 μl), and water intake was recorded over the next 30 min. Bar graph shows mean ± SE water intake for the above treatment condition. Also shown are mean ± SE water intakes for 0.9% saline-treated (2 μl icv), ANG II-treated (10 ng/2 μl icv), and LY-treated (10 μM, 2 μl icv; n = 14) rats. *P < 0.05 from control. B: rats were injected icv with the pharmacological agent JNK inhibitor II (JI, 100 nM) followed by an icv injection of ANG II (10 ng/2 μl), and water intake was recorded over the next 30 min. Bar graph shows mean ± SE water intake for the above treatment condition. Also shown are mean ± SE water intakes for 0.9% saline-treated (2 μl icv), ANG II-treated (10 ng/2 μl icv), and JI-treated (100 nM, 2 μl icv) rats; n = 8 for all treatment conditions. *P < 0.05 from control.

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similar to the time course for ANG II modulation of K+ and Ca2+ currents (~1–3 min). Because many physiological responses elicited by the brain are due to changes in neuronal activity, it is logical that the same mechanisms involved in ANG II modulation of neuronal activity in vitro are involved in ANG II-mediated responses in vivo. Indeed, central injection of the general PKC antagonist chelerythrine chloride significantly reduces the ANG II-induced drinking response, indicating a role for PKC. Further investigation into the PKC isozyme involved indicated that PKC-α is involved in the ANG II-mediated drinking response, similar to the ANG II modulation of IKv and neuronal activity (34, 35, 40). Furthermore, these data suggest that the ANG II drinking response involves activation of PKC-α in the septum and hypothalamus but not the brain stem. However, these data indicate that other PKC isozymes may be involved in the ANG II-stimulated dipsogenic effect because Go-6976 does not completely inhibit the drinking response elicited by ANG II. Additionally, similar to its role in the ANG II modulation of IKv and neuronal activity (34, 35, 40), CaMKII plays a role in the ANG II-mediated drinking response because central injection of the CaMKII antagonist KN-93 reduces the ANG II-induced dipsogenic effect. These results also suggest that activation of CaMKII-α in the septum and hypothalamus is partially responsible for the ANG II-induced drinking response.

Observations made during these studies also indicate that antagonism of PKC and CaMKII inhibit the drinking response due to icv injection of ANG II in the first 10 min, but not the random bursts. Thus it may be hypothesized that one of these kinases (i.e., PKC) is important for the initiation of the drinking response elicited by ANG II, while another kinase (i.e., CaMKII) may be involved in maintaining the drinking response for the first 10 min. Therefore, it is plausible to conclude that inhibition of both signaling molecules would inhibit the dipsogenic response completely. However, coadministration of PKC and CaMKII antagonists only partially inhibits the ANG II-induced drinking re-
response. These results were intriguing because they differ from those found in vitro, which demonstrated that inhibition of both PKC and CaMKII completely abolishes the ANG II-stimulated decrease in \( I_{Kv} \) and stimulation of neuronal activity (40). There are several conceivable explanations that may explain the differences between the in vivo and in vitro systems. One possibility may be that PKC and CaMKII are both equally involved in the same aspects of the ANG II-mediated drinking response; thus inhibition of both enzymes will not completely inhibit the ANG II-mediated effect. A second thought may be that these two enzymes are sequentially activated (i.e., PKC is upstream of CaMKII or vice versa), and thus coinhibition is not additive because they are both part of the same signaling network. The data also could suggest that while both enzymes are involved in the ANG II-induced drinking response, PKC and CaMKII are not the only two kinases important in this ANG II-mediated effect. For this reason, experiments were performed to investigate other signaling molecules that may be involved in the ANG II-induced drinking response. These data show that inhibition of PI3-K and JNK reduces the drinking response, but this effect is not significant. This could suggest that PI3-K and JNK play a very minor role in the ANG II-stimulated dipsogenic response. However, this also suggests that there are other signaling molecules in addition to PKC and CaMKII that are more significantly involved in this ANG II-mediated response, and these still are yet to be determined. Another explanation for this difference between the cell culture system and the in vivo model may be the administration of the drug. In the cell culture system, the individual drugs are diluted separately and then individually added to the dish. On the other hand, the drugs for the in vivo experiments were made as an inhibitor cocktail for icv injection. The two chemical compounds may interact with each other, thus reducing their relative potencies. Finally, this difference between results in the in vitro and in vivo system may be due to the fact that the whole animal brain is much more complicated than the simplified cell culture system. Inhibition of one signaling molecule in the brain may elicit one effect, while inhibition of two signaling molecules at the same time may elicit a totally different response. This difference supports the necessity to verify that ANG II-mediated actions discovered through the use of in vitro systems also occur in the whole animal model.

The results presented here also demonstrate that activation of PKC and CaMKII is specific to the dipsogenic response elicited by ANG II because inhibition of PKC and CaMKII has no effect on the carbachol-induced drinking response. Moreover, these results also verify that the reduction in the ANG II-mediated dipsogenic response is due to the inhibition of PKC and CaMKII by the chelerythrine chloride and KN-93, respectively, and not by secondary effects of the inhibitor on the alertness and activity of the rat. Additionally, these results suggest that different intracellular signaling molecules are involved in the ANG II-mediated and carbachol-induced drinking responses. This is consistent with the previously published study that demonstrated that the dipsogenic response elicited by carbachol involves an inhibitory G protein (31). Furthermore, these results support studies presented in the literature that demonstrated that the ANG II-mediated dipsogenic response does not require activation of cholinergic pathways because the acetylcholine antagonist atropine has no effect on the ANG II-induced drinking response (10).

Another important observation made during these studies was the activation of PKC and CaMKII in the septum-hypothalamus regions but not in the brain stem. This is of interest because nuclei of the brain stem such as the nucleus tractus solitarius are known to be important in ANG II-mediated physiological responses. There are several different interpretations that could be made. First, it could suggest that the septum and hypothalamus are more important in the ANG II-induced dipsogenic response than the brain stem. However, a more plausible interpretation is that different intracellular signaling molecules are activated by ANG II in cells in the septum and hypothalamus than in the brain stem. This means that PKC and CaMKII are the signaling molecules predominantly activated in the septal-hypothalamic regions while different signaling molecules are activated by this peptide in the brain stem.

In summary, these initial experiments are important because they demonstrate for the first time that intracellular signaling molecules involved in ANG II-mediated cellular actions are also involved in physiological responses due to this peptide. While the in vitro cell culture system is beneficial because it allows investigators to study individual neurons in a controlled environment, it has its limitations. Due to these limitations, it is important to verify that the knowledge regarding AT\(_1\) receptor-mediated cellular signaling discovered in vitro is relevant in ANG II-mediated physiological responses in the whole animal model. These data demonstrate that the findings from in vitro cell culture systems on AT\(_1\) receptor-mediated activation of intracellular signaling pathways can be extrapolated to the physiological responses regulated by ANG II. Specifically, these results provide the first evidence that PKC-\(\alpha\) and CaMKII in the brain are involved in the ANG II-mediated drinking response.

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DISCLOSURES

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