*Cellular and Molecular Neurobiology, Vol. 20, No. 6, 2000*

# AT-1 Receptor and Phospholipase C Are Involved in Angiotensin III Modulation of Hypothalamic Noradrenergic Transmission

**Muriel Rodriguez-Campos,[1] Carina Kadarian,[1] Valeria Rodano,[1] Liliana Bianciotti,[1] Belisario Fernandez,[1] and Marcelo Vatta[1,2]**

*Received July 24, 2000; accepted July 31, 2000*

**SUMMARY**

1. We previously reported that angiotensin III modulates noradrenergic neurotransmission in the hypothalamus of the rat. In the present work we studied the effects of angiotensin III on norepinephrine release and tyrosine hydroxylase activity. We also investigated the receptors and intracellular pathways involved in angiotensin III modulation of noradrenergic transmission.

2. In rat hypothalamic tissue labeled with [$^3$H]norepinephrine 1, 10, and 100 n*M* and 1 $\mu M$ losartan (AT1 receptor antagonist) had no effect on basal neuronal norepinephrine release, whereas 10 and 100 n*M* and 1 $\mu M$ losartan partially diminished norepinephrine secretion evoked by 25 m*M* KCl. The AT2 receptor antagonist PD 123319 showed no effect either on basal or evoked norepinephrine release. The increase in both basal and evoked norepinephrine output induced by 1 $\mu M$ angiotensin III was blocked by 1 $\mu M$ losartan, but not by 1 $\mu M$ PD 123319.

3. The phospholipase C inhibitor 5 $\mu M$ neomicin inhibited the increase in basal and evoked norepinephrine release produced by 1 $\mu M$ angiotensin III.

4. Tyrosine hydroxylase activity was increased by 1 $\mu M$ angiotensin III and this effect was blocked by 1 $\mu M$ LST and 5 $\mu M$ neomicin, but not by PD 123319. On the other hand, 1 $\mu M$ angiotensin III enhanced phosphatidyl inositol hydrolysis that was blocked by 1 $\mu M$ losartan and 5 $\mu M$ neomicin. PD 123319 (1 $\mu M$) did not affect ANG III-induced phosphatidyl inositol hydrolysis enhancement.

5. Our results confirm that angiotensin III acts as a modulator of noradrenergic transmission at the hypothalamic level through the AT1-phospholipase C pathway. This enhancement of hypothalamic noradrenergic activity suggests that angiotensin III may act as a central modulator of several biological processes regulated at this level by catecholamines, such as cardiovascular, endocrine, and autonomic functions as well as water and saline homeostasis.

**KEY WORDS:** angiotensin III; angiotensin receptors; angiotensin antagonists; norepinephrine release; tyrosine hydroxylase activity; renin–angiotensin system.

[1] Cátedras de Fisiología y Fisiopatología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

[2] To whom Correspondence should be addressed at Cátedra de Fisiología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956-7mo Piso, 1113 Buenos Aires, Argentina. e-mail: mvatta@ffyb.uba.ar



0272-4340/00/1200-0747$18.00/0 

## INTRODUCTION

The existence of a renin–angiotensin system (RAS) in the central nervous system (CNS) was reported in the 1970s (Saavedra, 1992; Lenkei *et al.*, 1997). Since then, a complete RAS has been described and all its components demonstrated in CNS (Saavedra, 1992; Lenkei *et al.*, 1997). Brain RAS plays an important role in cardiovascular control, water and salt homeostasis, ingestive behaviors, cerebral blood flow, synthesis and release of hormones and neurohormones, as well as learning and memory processes (Saavedra, 1992; Steckelings *et al.*, 1992; Lenkei *et al.*, 1997; Wright and Harding, 1997; Fitzsimons, 1998).

Angiotensin II (ANG II) heptapeptide metabolite, angiotensin III (ANG III), has been reported as a component of the brain RAS (Wright and Harding, 1997). In 1971, Blair-West *et al.* showed the first biological action of ANG III. They observed that this heptapeptide increased aldosterone secretion in an equipotent manner to ANG II. Several peripheral effects were also reported for ANG III such as arteriolar vasoconstriction and regulation of renal blood flow, renin, corticosterone, and cortisol secretion (Kono *et al.*, 1975; Campbell and Pettinger, 1976; Blair-West *et al.*, 1980; Abdelrahman and Pang, 1992; Radhakrishaman and Sim, 1994). ANG III displays similar central effects to ANG II in controlling cardiovascular function and cerebral blood flow, ingestive behaviors, dipsogenesis, hormonal and neurotransmitter release, and learning and memory processes (Yang *et al.*, 1995; Lenkei *et al.*, 1997; Wright and Harding, 1997).

At least three subtypes of receptors were characterized in the CNS, AT1 (A and B), AT2, and AT4 (Saavedra, 1992; Lenkei *et al.*, 1997; Wright and Harding, 1997). The AT1 receptor is mainly localized in the hypothalamus and brain stem, whereas AT2 is localized in the midbrain and pituitary (Saavedra, 1992; Lenkei *et al.*, 1997; Wright and Harding, 1997).

The intracellular signaling mechanism upon AT1 receptor stimulation involves the activation of phospholipase C (PLC) activity, which increases inositol 1,4,5-triphosphate and 1,2-diacylglycerol formation, resulting in an increase of intracellular $Ca^{2+}$ and protein kinase C activity, respectively (Saavedra, 1992; Wright and Harding, 1997). It is well known that the AT1 receptor mediates all or most of the known central actions of ANGs, while the physiological function and mechanisms of signaling are of AT2 unclear (Lenkei *et al.*, 1997; Wright and Harding, 1997).

Increasing evidence supports the hypothesis that ANG III is the effective peptide of the ANGs family in the CNS. Zini *et al.* (1996) reported that ANG III is the main brain RAS effector in the control of vasopressin release and in the basal firing level of vasopressinergic neurons. Furthermore, the inhibition of the conversion of ANG III from ANG II reduces drinking and blood pressure response, suggesting that this conversion is an obligatory step for the brain ANG system (Wright and Harding, 1997).

The hypothalamus is an important integrative center involved in the control of cardiovascular, endocrine, and autonomic functions and hydrosaline homeostasis (Dampney 1994; Oparil *et al.*, 1995). In addition, this central region presents noradrenergic neurons and binding sites for ANGs (Saavedra, 1992; Dampney, 1994; Oparil *et al.*, 1995; Lenkei *et al.*, 1997; Wright and Harding, 1997). Hypothalamic

norepinephrine (NE) participates in the control and modulation of cardiocirculatory activity and water and salt regulation (Dampney, 1994; Oparil *et al.*, 1995). Both central ANGs and NE have been implicated in clinical and experimental hypertension, suggesting that ANGs and catecholamines may be involved in the development and/or maintenance of hypertensive disease (Saavedra, 1992; Steckelings *et al.*, 1992; Dampney, 1994; Oparil *et al.*, 1995; Lenkei *et al.*, 1997; Wright and Harding, 1997; Fitzsimons, 1998).

We previously reported that ANG III diminishes neuronal uptake and endogenous content and increases basal and evoked neuronal release of NE in the CNS and adrenal medulla (Vatta *et al.*, 1991; Papouchado *et al.*, 1994, 1995). In addition, the hypothalamus contains catecholaminergic neurons and AT1 receptors, suggesting anatomic support for the ANG (ANG III in this case) regulation of the NE system (Saavedra, 1992; Dampney, 1994; Oparil *et al.*, 1995; Gelband *et al.*, 1997; Lenkei *et al.*, 1997; Wright and Harding, 1997). For these reasons we decided to study the effects of ANG III on diverse steps of NE metabolism, as well as the receptors and intracellular pathway involved. Our findings show that, in the rat hypothalamus, ANG III increases both basal and evoked neuronal NE release and tyrosine hydroxylase (TH) activity through the AT1 receptor subtype and the activation of PLC.

## METHODS

### Animals and Tissue Preparation

Male Sprague-Dawley rats (from the Facultad de Farmacia y Bioquimica, UBA) weighing 250–300 g were used. Animals were lodged in steel cages and kept in a controlled room at 22–24°C with a 12-hr light/dark cycle. Rats were allowed free access to tap water and food. All efforts were made to reduce the number of animals used.

Animals were decapitated, and brains were quickly removed and hypothalami immediately dissected, cooled, and weighted. Hypothalami were lightly minced and then transferred into a glass tube with a nylon mesh fitted at the bottom to allow free interchange with the medium. All experiments were carried out *in vitro*.

### Neuronal [$^3$H] Norepinephrine Release

The [$^3$H]NE release determination was performed as described by Vatta *et al.* (1999a). Briefly, hypothalami were incubated at 37°C for 30 min in 2 ml of modified Krebs bicarbonate solution (KBS), pH 7.4 in the presence of pargyline, tropolone and hydrocortisone (Sigma Chemical Co., St. Louis, MO) at 100 m*M* each drug to inhibit monoamine oxidase activity, catecholamine-*O*-methyltransferase activity, and extraneuronal NE uptake, respectively. NE stores were labeled with 2.5 $\mu$Ci/ml L-[7,8-$^3$H]NE (1.18 TBq/mmol of specific activity; Amersham Pharmacia Biotech, UK) for a 30-min incubation period. Labeling was followed by eight consecutive washes (5 min each) with KBS. In the last wash, 30 $\mu$*M* cocaine (Catedra de

Toxicologia, Facultad de Farmacia y Bioquimica, UBA) was added in order to inhibit neuronal NE reuptake. Hypothalami were then incubated four times for 5 min each period (one basal and three experimental periods). In the experimental periods the different drugs were added, ANG III and Neomicin (Nmc) (Sigma), Losartan (LST) (supplied by DuPont-Merk Pharmaceutical Co., Wilmington, DE), and PD 123319 (supplied by Parke-Davis Pharmaceutical, An Arbor, MI). The incubation media of the four periods were saved for the determination of [$^3$H] activity. Results are expressed as the ratio of the radioactivity released between each experimental period (5, 10, or 15 min) and the basal period (E/B).

## Tyrosine Hydroxylase Activity

TH activity was measured as described by Hendry and Iversen (1971) and Zigmond and Chalazonitis (1979). Hypothalami were preincubated for 15 min at 37°C with KBS and then incubated for 60 min in the presence of the different drugs. After the incubation period tissues were washed and homogenized in 500 $\mu$l of cold water and TH activity was determined in a 100-$\mu$l aliquot. A sample of the homogenate was saved for protein assay (Lowry *et al.*, 1951). Samples and blanks were incubated for 20 min at 37°C with HEPES buffer (pH 6.0) containing 10 $\mu M$ L-[3,5-$^3$H]TH (1.70 TBq/mmol of specific activity; Amersham), tetrahydrobiopterin (0.7 m*M*; ICN Biomedicals, Inc., Costa Mesa, CA), and $\beta$-mercaptoethanol (0.2 *M*, ICN) in 200-$\mu$l final volume. The reaction was stopped by the addition of 1 ml 0.4 N $HClO_4$ containing 10 $\mu$g of unlabeled L-DOPA (ICN) as a carrier and solution was adjusted to pH 8.6 with Tris-EDTA buffer (0.05 M). The [$^3$H]DOPA was separated from [$^3$H]tyrosine by chromatography in aluminum oxide columns and eluted with 1.5 ml of 0.5 *M* acetic acid. Tritium activity was determined by conventional scintillation methods. TH activity results are expressed as the percentage of the control group ± SEM (Vatta *et al.*, 1999b).

## Inositol Phosphate Assay

Hypothalami were incubated in 500 $\mu$l of KBS/10 n*M* LiCl as described by Vatta *et al.* (1999b). Thirty minutes before the end of the incubation period the different drugs were added. Phosphoinositide separation was performed according to Berridge *et al*. (1982). Briefly, tissues were washed for 5 min with cold KBS/LiCl and homogenized with 500 $\mu$l of KBS/LiCl and 2 ml of chloroform:methanol (1:2 v/v). To separate the phases, 620 $\mu$l of chloroform and 1 ml of water were added to the homogenates followed by centrifugation at 20000 × *g* for 15 min. The upper phase was applied to an anion exchange column (Bio-Rad AG1-X8 resin, 100–200 mesh, formiate form). The columns were washed with 10 ml of 5 m*M* myoinositol and the fraction mainly containing $IP_3$ and its isomers was eluted with 1 *M* amonium formiate/0.1 *M* formic acid. This fraction represents phospholipase C activity (Taylor *et al.*, 1986). The [$^3$H] activity in this fraction was measured by conventional liquid scintillation counting methods. Results are expressed as the percentage of the control value ± SEM (Vatta *et al.*, 1999b).

### Statistical Analysis

All values are presented as the mean ± SEM. Differences among groups were statistically assessed using the ANOVA test followed by the *t* test modified by Bonferroni. In all cases, *p* values of 0.05 or less were considered statistically significant.

## RESULTS

### ANG III Effects on Neuronal NE Release in the Presence of AT1 or AT2 Antagonists or a PLC Inhibitor

In order to assess the subtype of ANG receptor involved in ANG III-induced release, experiments we carried out in the presence or in the absence of LST or PD 123319 (AT1 and AT2 receptor antagonists, respectively). We have shown that 1 $\mu M$ ANG III is an effective concentration to increase neuronal NE release in the hypothalamus (Vatta *et al.*, 1991). In order to determine if LST and/or PD 123319 had any effect on basal or evoked NE secretion, a concentration–response study was performed. Results showed that 1, 10, and 100 n*M* and 1 $\mu M$ LST or PD 123319 had no effect on basal neuronal NE secretion (Figs. 1 and 3, upper panel), while 10 and 100 n*M* and 1 $\mu M$ LST partially diminished NE release evoked by 25 m*M* KCl at 10 and 15 min (Fig. 2, upper panel). LST (10 and 100 n*M* and 1 $\mu M$) showed a tendency to diminish evoked NE secretion at 5 min, but it was not statistically significant. In addition, 1 n*M* LST and all concentrations of PD 123319 did not elicit any effect on evoked NE secretion (Figs. 2 and 4, upper panel). One $\mu M$ LST inhibited the increase in basal and evoked (by 25 m*M* KCl) neuronal NE release induced by 1 $\mu M$ ANG III (Figs. 1 and 2, lower panels). On the other hand, 1 $\mu M$ PD 123319 did not alter basal or evoked NE release induced by 1 $\mu M$ ANG III (Figs. 3 and 4, lower panel)

As AT1 stimulation induces phosphoinositol breakdown, we assessed the effect of ANG III on basal and evoked NE secretion in the presence of the PLC inhibitor, neomicin (Nmc). Results showed that 5 $\mu M$ Nmc did not modify either basal or evoked (25 m*M* KCl) NE release (Fig. 5). When hypothalami were incubated with 1 $\mu M$ ANG III in the presence of 5 $\mu M$ Nmc, the increase of NE induced by the peptide in basal and evoked experiments was inhibited (Fig. 4).

### ANG III Effects on TH Activity in the Presence of AT1 or AT2 Receptor Antagonist or a PLC Activity Inhibitor

With the aim of determining the effect of ANG III on TH activity and the involvement of AT1 and AT2 receptors and PLC in this process, we investigated the effect of ANG III on hypothalamic TH activity in the presence of LST or PD 123319 and Nmc. Figure 6 shows that 1 $\mu M$ ANG III significantly increased TH activity, whereas 1 $\mu M$ LST, 1 $\mu M$ PD 123319, or 5 $\mu M$ Nmc did not modify it. When hypothalami were incubated with 1 $\mu M$ ANG III in the presence of LST or

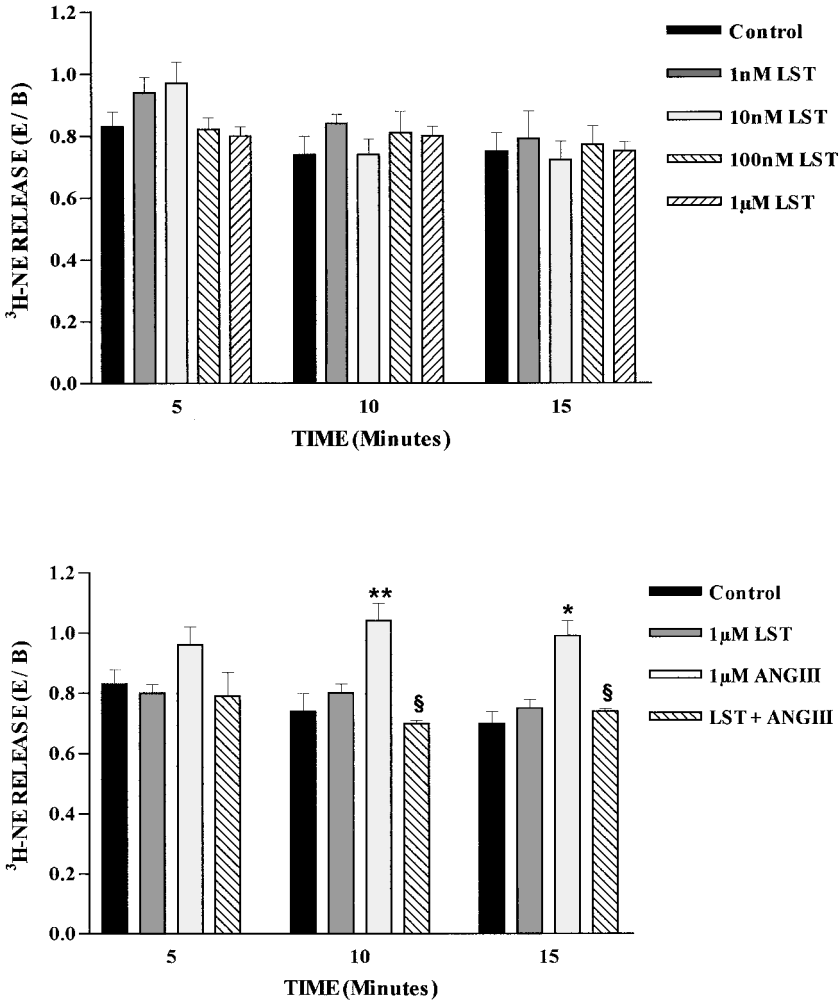


**Fig. 1.** Basal neuronal [$^3$H]norepinephrine ([$^3$H]NE) release in the presence of angiotensin III (ANG III) and/or the AT1 receptor antagonist losartan (LST). E/B represents the ratio between $^3$H release in each experimental period (5, 10, or 15 min) and the basal period*,**$p < 0.001$, 0.005, respectively, versus control group; $^{\S}p < 0.001$ versus 1 $\mu M$ ANG III group. Values are mean ± SEM. Number of experiments is five to seven. Upper panel: Effect of different concentrations of LST on basal [$^3$H]NE release. Lower panel: Effect of LST on basal [$^3$H]NE release induced by ANG III.

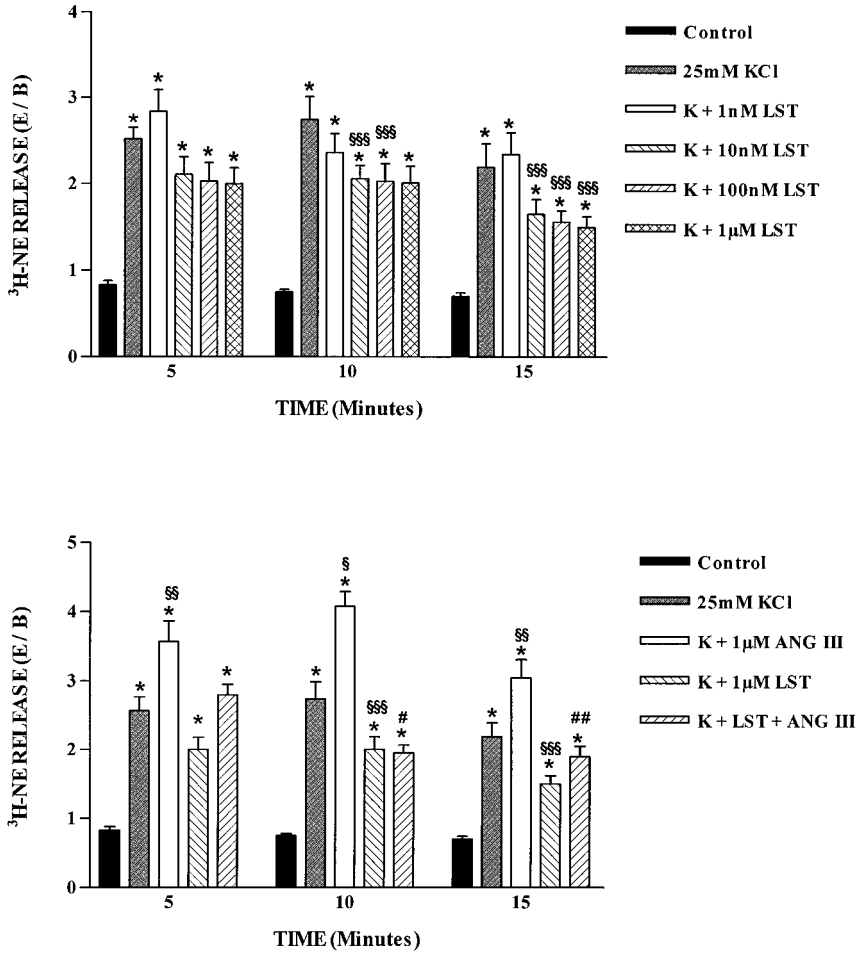


**Fig. 2.** Neuronal [$^3$H]norepinephrine ([$^3$H]NE) release evoked by 25 m*M* KCl in the presence of angiotensin III (ANG III) and/or the AT1 reeptor antagonist losartan (LST). E/B represents the ratio between tritium release in each experimental period (5, 10, or 15 min) and the basal period. *$p < 0.001$ versus control group; $^{§,§§,§§§}p < 0.001$, 0.02, 0.05, respectively, versus 25 m*M* KCl group; $^{\#,\#\#}p < 0.001$, 0.01, respectively versus 1 $\mu M$ ANG III group. Values are mean ± SEM. Number of experiments is five to nine. Upper panel: Effect of different concentrations of LST on evoked neuronal [$^3$H]NE release. Lower panel: Effect of LST on evoked neuronal [$^3$H]NE release induced by ANG III.

Nmc, ANG III-induced TH activity was inhibited (Fig. 6). In addition, AT2 blockade by 1 $\mu M$ PD 123319 did not modify ANG III-induced TH activity (Fig. 6).

## ANG III Effects on Phosphatidylinositol Hydrolysis in the Presence of AT1 or AT2 Receptor Antagonist or a PLC Inhibitor

In order to determine if the activation of PLC was the intracellular signaling involved in the ANG III–AT1 receptor interaction in the rat hypothalamus, we

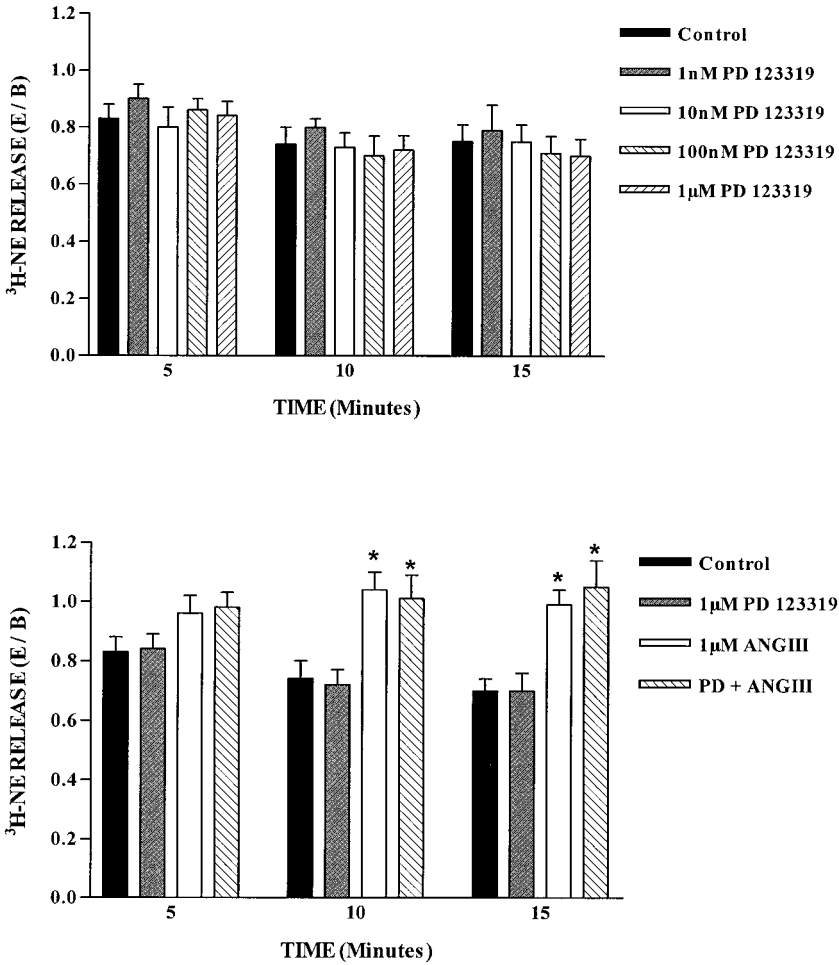


**Fig. 3.** Basal neuronal [$^3$H]norepinephrine ([$^3$H]NE) release in the presence of angiotensin III (ANG III) and/or the AT2 receptor antagonist PD 123319. E/B represents the ratio between tritium release in each experimental period (5, 10, or 15 min) and the basal period. *$p < 0.001$ versus control group. Values are mean ± SEM. Number of experiments is five to seven. Upper panel: Effect of different concentrations of PD 123319 on basal [$^3$H]NE release. Lower panel: Effect of PD 123319 on basal [$^3$H]NE release induced by ANG III.

studied the effect of ANG III on phosphatidylinositol hydrolysis in the presence of LST or Nmc. LST (1 $\mu M$) did not affect phosphatidylinositol hydrolysis, and 5 $\mu M$ Nmc showed a statistically significant tendency to decrease it (Fig. 7). Results showed that 1 $\mu M$ ANG III increased phosphatidylinositol turnover which was inhibited by 1 $\mu M$ LST or 5 $\mu M$ Nmc (Fig. 7). In order to determine whether effects of ANG III were mediated by the stimulation of the AT1 receptor subtype followed by PLC activation and rule out AT2 receptor participation, we investigated the effect of ANG III on phosphatidylinositol hydrolysis in the presence of PD 123319.

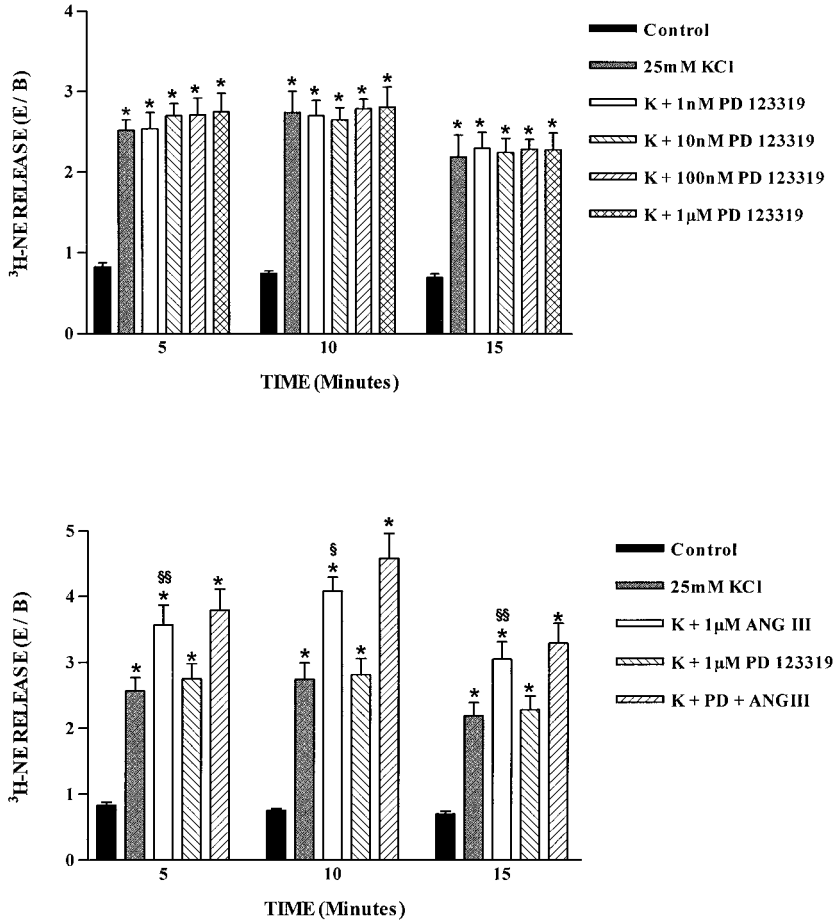


**Fig. 4.** Neuronal [$^3$H]norepinephrine ([$^3$H]NE) release evoked by 25 m*M* KCl in the presence of angiotensin III (ANG III) and/or the AT2 receptor antagonist PD 123319. E/B represents the ratio between tritium release in each experimental period (5, 10, or 15 min) and the basal period. *$p < 0.001$ versus control group; $^{§,§§}p < 0.001$, 0.02, respectively, versus 25 m*M* KCl group. Values are mean ± SEM. Number of experiments is five to eight. Upper panel: Effect of different concentrations of PD 123319 on evoked neuronal [$^3$H]NE release. Lower panel: Effect of PD 123319 on evoked neuronal [$^3$H]NE release induced by ANG III.

Figure 7 (lower panel) shows that 1 $\mu M$ PD 123319 did not modify the increase in phosphatidylinositol hydrolysis elicited by ANG III.

## DISCUSSION

Both brain ANG II and catecholamines play important roles in the central regulation of cardiovascular function, salt and water homeostasis, as well as the control of endocrine and neuroendocrine secretions (Saavedra, 1992; Dampney,

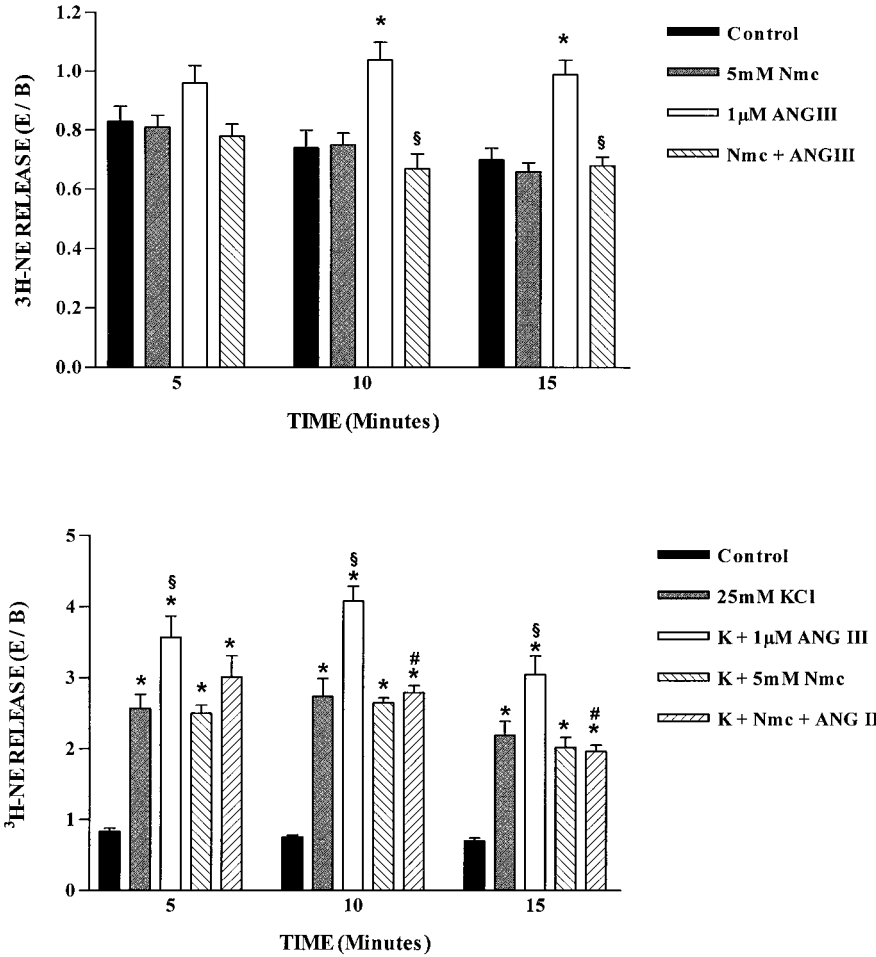


**Fig. 5.** Effect of the phospholipase C inhibitor neomicin (Nmc) on basal (upper panel) or evoked by 25 m*M* KCl (lower panel) neuronal [$^3$H]norepinephrine ([$^3$H]NE) release induced by angiotensin III (ANG III). E/B represents the ratio between tritium release in each experimental period (5, 10, or 15 min) and the basal period. $^*p < 0.001$ versus control groups; $^§p < 0.001$ versus 25 m*M* KCl group: $^\#p < 0.001$ versus 1 $\mu M$ ANG III group. Values are mean ± SEM. Number of experiments is five to seven.

1994; Oparil *et al.*, 1995; Yang *et al.*, 1995; Lenkei *et al.*, 1997; Wright and Harding, 1997). However, little is known about the interaction between ANG III and NE metabolism. Fuxe *et al.* (1984) reported that ANG III diminished the binding affinity of $\alpha_2$-adrenoceptors in the medulla oblongata. Moreover, observations by Yin *et al.* (1990) suggested that ANG III might interact with $\alpha_2$-adrenoceptors located in the nucleus reticularis gigantocellularis in the medulla oblongata. This brain center is involved in the central regulation of cardiovascular function. Weather the interaction between ANG III and NE occurs at the pre and/or postsynaptic level is still a controversial question. Some authors suggest that this interaction occurs at the

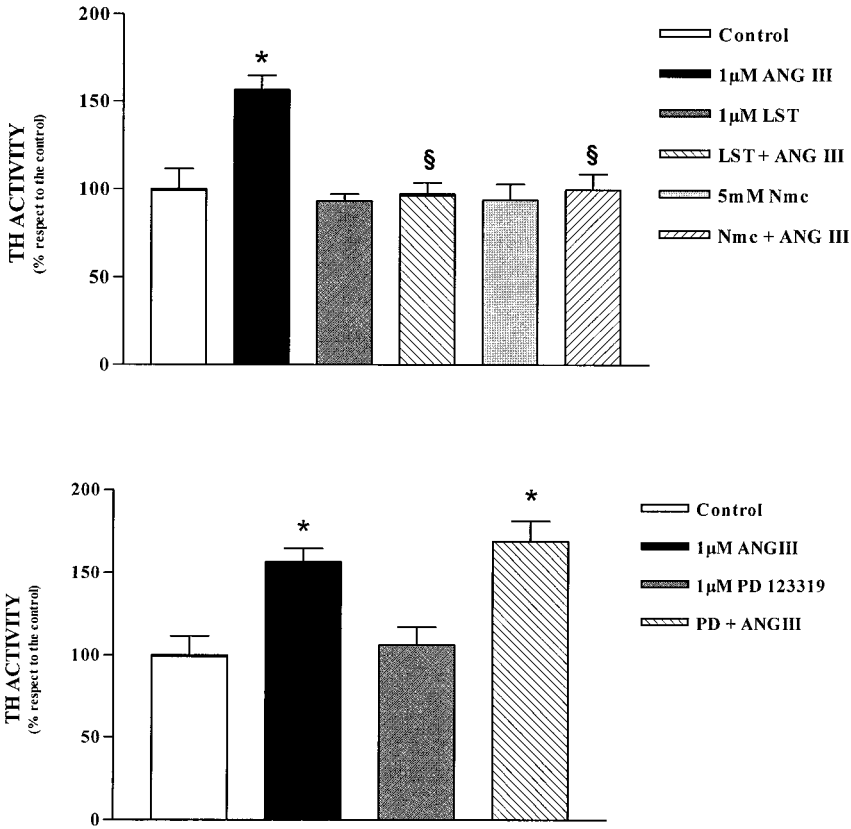


**Fig. 6.** Effect of angiotensin III (ANG III) on tyrosine hydroxylase (TH) activity in the presence of losartan (AT1 antagonist) (LST) or neomicin (phospholipase C inhibitor) (Nmc) (upper panel) or PD 123319 (AT2 antagonist) (lower panel). $*p < 0.005$ versus control group; $^{\S}p < 0.001$ versus 1 $\mu M$ ANG III group. Values are mean ± SEM. Number of experiments is five or six.

postsynaptic level, while others propose the involvement of a G-protein in NE modulation by presynaptic adrenoceptors (Yin *et al.*, 1990; Fuxe *et al.*, 1984; Gelband *et al.*, 1998). Results obtained in our laboratory demonstrated that ANG III modified both NE uptake and release in rat hypothalamus and medulla oblongata (Vatta *et al.*, 1991; Papouchado *et al.*, 1995). These data strongly support that ANG III acts at the presynaptic level besides the possible postsynaptic effect described by other authors.

The present results show that ANG III increases both basal and evoked neuronal NE release in the rat hypothalamus mediated by the stimulation of the AT1 receptor and PLC activation. Exocytosis is the final step of a complex process that involves transport of vesicles, and synthesis and maturation of neurotransmitters and diverse cytoeskeleton proteins such as synapsin I and II, fodrin, actin, scinderin, and gelsolin (Kono *et al.*, 1975; Trifaro and Vitale 1993; Trifaro *et al.*, 1993). When a neuron is depolarized by a high $K^+$ concentration, calcium channels are opened

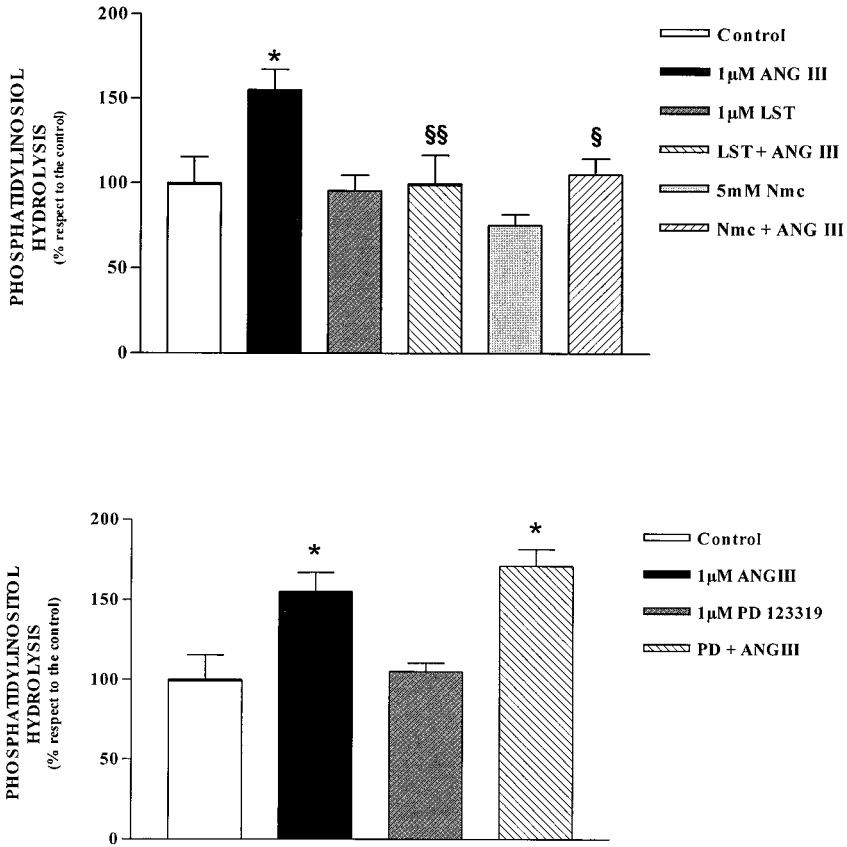


**Fig. 7.** Effect of angiotensin III (ANG III) on phosphatidylinositol hydrolysis in the presence of losartan (AT1 antagonist) (LST) or neomicin (phospholipase C inhibitor) (Nmc) (upper panel) or PD 123319 (AT2 antagonist) (lower panel). $^{*}p < 0.02$ versus control group; $^{\S,\S\S}p < 0.01, 0.025$, respectively, versus 1 $\mu M$ ANG III group. Values are mean ± SEM. Number of experiments is five or six.

and intracellular calcium is increased; in consequences, $Ca^{2+}$/calmodulin-dependent kinase II activity is enhanced and synapsin I is phosphorylated. This event leads to dissociation among synapsin I, actin filaments, and synaptic vesicles, resulting in a disassembly of the actin filament networks and in vesicle migration at the active zone of the nerve endings (Trifaro and Vitale, 1993). The actin filament network is regulated by second messengers such as protein kinase C, but this messenger is not enough to induce catecholamine secretion (Trifaro *et al.*, 1993). An increase of intracellular calcium level is essential to produce neurotransmitter secretion, with protein kinase C acting as modulator of the neurotransmission process (Trifaro *et al.*, 1993). ANG III binds mainly to two subtypes of ANG receptors, AT1 and AT2. Brain AT1 receptors mediate classic ANG actions such as regulation of cardiovascular function and water and salt homeostasis and are located in the

catecholaminergic neurons (Lenkei *et al.*, 1997; Wright and Harding, 1997; Gelband *et al.*, 1998). This receptor mediates ANG II–NE neuromodulated increase of amine release and TH and dopamine $\beta$-hydroxilase mRNA transcription (Saavedra, 1992; Gelband *et al.*, 1998). AT1 receptor is coupled to a G-protein complex and its stimulation produces an increase of PLCactivity, resulting in the formation of inositol-1,4,5-triphosphate and 1,2-diacylglycerol. These messengers enhance intracellular calcium concentration and protein kinase C activity, which then act in a synergistic manner (Yu *et al.*, 1996; Gelband *et al.*, 1998). The activation of this pathway through the AT1 receptor is associated with evoked NE release (Gelband *et al.*, 1998). Present and other findings strongly support that ANG III, upon AT1 receptor stimulation, increases PLC activity and inositol-1,4,5-triphosphate and 1,2-diacylglycerol formation, resulting in an increase of intracellular calcium and protein kinase C activity, essential mediators in neurotransmission. Since both ANG II and ANG III increase neuronal NE release through AT1 receptor activation and not via the AT2 receptor, the reduction of evoked NE release (25 m*M* KCl) produced by LST (Fig. 3, upper panel) could result from blockade of endogenous ANGs binding to AT1 receptor (Kumagai and Reid, 1994).

Biosynthesis is another important step in the metabolic pathway of catecholamines. TH is a specific marker of catecholaminergic neurons and the enzymatic key in the biosynthesis of catecholamines (Icard-Liepkalns *et al.*, 1993; Kumer and Vrana, 1996). This enzyme regulates the conversion of L-tyrosine to L-dopa. Several mechanisms regulate TH activity. Two different feedback mechanisms modulate the activity of the enzyme; one is the classic kinetic modulation by final product, acting as a sensor of the intracellular control of the amines, and the second mechanism is the conversion into a less active, but stable form of the enzyme (Kumer and Vrana, 1996). TH is also allosterically regulated by polyanions. Nelson and Kaufman (1987) demonstrated that TH activity is increased by low concentrations of RNA and inhibited by high levels of RNA. It is currently accepted that nucleic acids play an important role in the regulation of TH activity (Kumer and Vrana, 1996). On the other hand, phosphorylation is an important short-term mechanism in the regulation of enzymatic activity. Thus, in the rat, TH is phosphorylated *in vitro* by protein kinases such as cAMP and cGMP-dependent protein kinases, $Ca^{2+}$/calmodulin-dependent protein kinase, and protein kinase C. In addition, $Ca^{2+}$ is a trigger of phosphorylation and/or activation of TH (Icard-Liepkalns *et al.*, 1993; Kumer and Vrana, 1996).

Our results clearly demonstrate that ANG III increases TH activity through AT1 receptor stimulation and PLC pathway activation. In view of these results, we can hypothesize that ANG III, through PLC activation, increases phosphatydilinositol hydrolysis and in consequence enhances, via inositol-1,4,5-triphosphate, the intracellular calcium concentration that triggers TH activation. However, we cannot rule out that ANG III stimulates the AT1 receptor–PLC-1,2-diacylglycerol–protein kinase C pathway, which can in turn modulate TH activity.

ANG III diminishes neuronal uptake and enhances the release of NE, suggesting a depletion of NE stores (Vatta *et al.*, 1991; Papouchado *et al.*, 1995). We reported that ANG III diminishes endogenous content of NE in the hypothalamus (Papouchado *et al.*, 1994). The depletion of NE content in neurons stimulates NE

synthesis. Therefore, ANG III may also increase TH activity by the classic feedback mechanism of kinetic modulation by final product. Our results are in line with previous studies showing that the modulation of neuronal activity is mediated by the AT1-Gq/11$\alpha$, PLC, or PKC pathway (Gelband *et al.*, 1998). Other reports also show that the AT1 receptor is negatively coupled to adenylate cyclase (Saavedra, 1992; Wright and Harding, 1997). Present results show that ANG III-induced NE release and TH activity are mediated by PLC activation. It is unlikely that inhibition of cAMP formation would be the intracellular signal in ANG III-induced NE release and TH activity modulation, since the output of catecholamines (Trifaro and Vitale, 1993) and TH activation (Icard-Liepkalns *et al.*, 1993; Kumer and Vrana, 1996) are mainly associated with enhancement of adenylate cyclase activity followed by cAMP generation.

It is well known that the stimulation of catecholamine release produces an increase in the biosynthesis of biogenic amines. Agents that modify catecholamine release may also alter the biosynthetic process (Ungar and Phillips, 1983). Our results show that ANG III increases both neuronal NE release and TH activity. The hypothalamus contains more dopaminergic neurons than noradrenergic cell bodies, but ANG III effects on neuronal uptake and release of NE and stimulation of TH clearly suggest the activation of noradrenergic neurons.

Several centrally regulated physiological actions might be controlled by the interaction between ANG III and noradrenergic neurotransmission. In the median preoptic nucleus the increased neuronal release of ANG II and/or ANG III stimulates AT1-A receptors and acts synergistically with noradrenergic afferents arising from the A1, A2, and A6 noradrenergic cell groups to facilitate neuronal output to higher order neurons or regions, increasing water intake (Lenkei *et al.*, 1997). However, in the hypothalamic paraventricular and supraoptic nucleus, ANGs do not act directly on the anteroventral preoptic nucleus through the AT1 receptor (Lenkei *et al.*, 1997). Some authors propose that ANGs effects might be indirectly mediated by neurotransmitters such as NE (Yang *et al.*, 1995; Lenkei *et al.*, 1997). The infusion of $\alpha_1$- and $\alpha_2$-adrenergic receptor antagonists into the hypothalamic paraventricular nucleus inhibits ANG-induced release of vasopressin (Saavedra, 1992; Lenkei *et al.*, 1997). Furthermore, the central ANG system regulates blood arterial pressure through different mechanisms such as vasopressin release, baroreflex inhibition, sympathetic nerve activity induction, and catecholamine metabolism stimulation (Saavedra, 1992; Steckelings *et al.*, 1992; Lenkei *et al.*, 1997; Wright and Harding, 1997; Gelband *et al.*, 1998). Both ANG II and ANG III directly and/or indirectly affect the synthesis and release of several hypothalamic neurohormones. The indirect actions occur by the facilitation of NE release at different hypothalamic levels such as for GnRH and vasopressin secretion (Ganong, 1989; Saavedra, 1992; Lenkei *et al.*, 1997).

In conclusion, to our knowledge this is the first study to report an effect of ANG III on TH activity and on neuronal NE release regulation mediated by the activation of the AT1–PLC pathway in rat hypothalamus. These and previous (Vatta *et al.*, 1991; Papouchado *et al.*, 1994, 1995) findings permit us to conclude that ANG III may act as a neuropeptide modulator of noradrenergic transmission at the hypothalamic level, leading to an increase in noradrenergic activity. ANG

III enhancement of the hypothalamic noradrenergic activity may be related to the origin and/or development and/or maintenance of diverse cardiovascular disorders such as hypertension.

## ACKNOWLEDGMENTS

This work was supported by grants from the University of Buenos Aires (UBACYT, TB-28) and CONICET (PIP 4566).

## REFERENCES

Abdelrahman, A., and Pang, C. C. Y. (1992). Competitive antagonism of pressor responses to angiotensin II and angiotensin III by the angiotensin II-1 receptor ligand losartan. *Can. J. Physiol. Pharmacol.* **70:**716–719.

Berridge, R., Downes, C. P., and Hauley M. R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol response in brain and salivary glands. *Biochem. J.* **206:**587–595.

Blair-West, J. P., Coghlin, J. P., Denton, D. A., Fei, D. T. W., Hardy, K. J., Scoggins, B. A., and Wright, R. D. (1980). A dose–response comparison of the actions of angiotensin II and angiotensin III in sheep. *J. Endocrinol.* **87:**409–417.

Blair-West, J. R., Coglhlin J. P., Denton D. A., Funder J. W., Scoggins B. A., and Wright E. D. (1971). Effect of the heptapeptide (2–8) and hexapeptide (3–8) fragments of angiotensin II on aldosterone secretion. *J. Clin. Endocrinol. Metab.* **32:**575–578.

Campbell, W. B., and Pettinger, W. A. (1976). Organ specificity of angiotensin II and desaspartyl angiotensin II in the conscious rat. *J. Pharmacol. Exp. Ther.* **198:**450–456.

Dampney, R. A. L. (1994) Functional organization of central pathways regulating the cardiovascular system. *Physiol. Rev.* **74:**323–364.

Fitzsimons J. T. (1998). Angiotensin, thirst, and sodium appetite. *Physiol. Rev.* **78:**583–686.

Fuxe, K., Agnati, L. F., Harfstrand, A., Martire, M., Goldstein, M., Grimaldi, R., Bernardi, P., Zini, I., Takemoto, K., and Mutt, V. (1984). Evidence for a modulation by neuropeptide Y of the $\alpha_2$-adrenergic transmission line in central adrenaline synapses. New possibilities for treatment of hypertensive disorders. *Clin. Exp. Hypertens.* **A6:**1951–1956.

Ganong, W. F. (1989). Angiotensin II in the brain and pituitary: Controling roles in the regulation of adenohypophyseal secretion. *Hormone Res.* **31:**24–31.

Gelband, C. H., Sumners, C., Lu, D., and Raizada, M. K. (1998). Angiotensin receptors and norepinephrine neuromodulation: Implications of functional coupling. *Regul. Pept.* **73:**141–147.

Gelband, C. H., Zhu, M., Lu, D., Reagan, L. P., Fluharty, S. J., Posner, P., Raizada, M. K., and Sumners, C. (1997) Functional interactions between neuronal AT1 and AT2 receptors. *Endocrinology* **138:**2195–2198.

Hendry, I. V., and Iversen, L. L. (1971). Effect of nerve growth factor and its antiserum on tyrosine hydroxylase activity in mouse superior cervical sympathetic ganglion. *Brain Res.* **29:**159–162.

Icard-Liepkalns, C., Berrard, S., Faucon-Biguet, N., Lebourdelles, B., Ravassard, P., Robert J. J., and Mallet, J. (1993). Tyrosine hydroxylase regulation in neurotransmission and neuroplasticity. *J. Physiol* (*Paris*) **87:**153–157.

Jahn, R., and Sudhof, T. C. (1994). Synaptic vesicles and exocytosis. *Annu. Rev. Neurosci.* **17:**19–46.

Kono, T., Oseko, F., Shimpo, S., Nanno, M., and Endo, J. (1975). Biological activity of desasp$^1$-angiotensin II (angiotensin III) in man. *J. Clin Endocr. Metab.* **41:**1174–1177.

Kumagai, K., and Reid, I. A. (1994). Losartan inhibits sympathetic and cardiovascular responses to carotid occlusion. *Hypertension* **23(Part 2):**827–831.

Kumer, S. C., and Vrana, K. E. (1996). Intricate regulation of tyrosine hydroxylase activity and gene expression. *J. Neurochem.* **67:**443–462.

Lenkei, Z., Palkovits, M., Corvol, P., and Lorens-Cortes, C. (1997). Expression of angiotensin type-1 (AT1 and type-2 (AT2) receptor mRNAs in the adult rat brain: A functional neuroanatomical review. *Frontiers Neuroendocrinol.* **18:**383–439.

Lowry, O. H., Rosebrough, N. J., Farra, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265–275.

Nelson, T. J., and Kaufman, S. (1987). Interaction of tyrosine hydroxylase with ribonucleic acid and purification with DNA-cellulose or poly (A)-sepharose affinity chromatography. *Arch. Biochem. Biophys.* **257:**69–84.

Oparil, S., Chen, Y. F., Berek, K. H., Calboun, D. A., and Wyss, J. M. (1995). The role of the central nervous system in hypertension. In Laragh, J. H., and Brenner, B. M. (eds.), *Hypertension: Pathophysiology, Diagnosis and Management,* Raven Press, New York, pp. 713–740.

Papouchado, M., Vatta, M., Zarrabeitia, V., Dominguez, A., Fernandez, B., and Feldstein, C. (1994). Effects of angiotensin III on noradrenergic neurotransmission in the rat hypothalamus [Abstract]. *Am. J. Hypertens.* **7(Part 2):**139A.

Papouchado, M. L., Vatta, M. S., Escalada, A., Bianciotti, L. G., and Fernandez, B. E. (1995). Angiotensin III modulates noradrenaline uptake and release in the rat hypothalamus. *J. Auton. Phamacol.* **15:**1–8.

Radhakrishnan, R., and Sim, M. K. (1994). Enhanced pressor response to angiotensin III in spontaneously hypertensive rats: Effects of losartan. *Eur. J. Pharmacol.* **259:**87–90.

Saavedra, J. M. (1992). Brain and pituitary angiotensin. *Endocrine Rev.* **13:**329–380.

Steckelings, U., Lebrun, C., Qadri, F., Veltmar, A., and Unger, T. (1992). Role of brain angiotensin in cardiovascular regulation. *J. Cardiovasc. Pharmacol.* **19(Suppl. 6):**S72–S79.

Taylor, C. W., Merrit, N. A., Putney, J. W., and Rubin, R. P. (1986). Effects of $Ca^{2+}$ on phosphoinositide breakdown in exocrine pancreas. *Biochem. J.* **238:**765–772.

Trifaro, J. M., and Vitale, M. L. (1993). Cytoeskeleton dynamics during neurotransmitter release. *Trends Neurosci.* **16:**466–472.

Trifaro, J. M., Vitale, M. L., and Rodriguez del Castillo, A. (1993). Scinderin and chromaffin cell actin network dynamics during neurotransmitter release. *J. Physiol. (Paris)* **87:**89–106.

Ungar, A., and Phillips, J. H. (1983). Regulation of the adrenal medulla. *Physiol. Rev.* **63:**787–843.

Vatta, M. S., Bianciotti, L. G., Papouchado, M. L., Locatelli, A. S., and Fernandez, B. E. (1991). Effects of atrial natriuretic peptide and angiotensin III on the uptake and intracellular distribution of norepinephrine in medulla oblongata of the rat. *Comp. Biochem. Physiol.* **99C:**293–297.

Vatta, M., Peña, C., Fernandez, B. E., and Rodriguez de Lores Arnaiz, G. (1999a). A brain $Na^+/K^+$-ATPase inhibitor (Endobain E) enhances norepinephrine release in rat hypothalamus. *Neuroscience* **90:**573–579.

Vatta, M. S., Rodriguez-Fermepin, M., Durante, G., Bianciotti, L. G., and Fernandez, B. E. (1999b). Atrial natriuretic factor inhibits norepinephrine biosynthesis and turover in the rat hypothalamus. *Regul. Pept.* **85:**101–107.

Wright, J. W., and Harding, J. W. (1997). Important roles for angiotensin II and III in the brain renin–angiotensin system. *Brain Res. Rev.* **25:**96–124.

Yang, C. C. H., Kuo, T. B. J., Chan, S. H. H., and Chan, J. Y. H. (1995). Neurobiology of central angiotensin III and dipsogenesis. *Biol. Signals* **4:**59–71.

Yin, D.-C., Chan, J. Y. H., and Chan, S. H. H. (1990). Interaction between angiotensin III and $\alpha_2$-adrenoceptors of the medulla oblongata involved in cardiovascular regulation in the rat. *Regul. Pept.* **29:**59–72.

Yu, K., Lu, D., Rowland, N. E., and Raizada, M. K. (1996). Angiotensin II regulation of tyrosine hydroxilase gene expression in the neuronal cultures of normoensive and spontaneously hypertensive rats. *Endocrinology* **137:**3566–3576.

Zigmond, R. E., and Chalazonitis, A. (1979). Long-term effects of preganglionic nerve stimulation on tyrosine hydroxylase activity in the cat superior cervical ganglion. *Brain Res.* **164:**137–152.

Zini, S., Fournie-Zaluski, M.-C., Chauvel, E., Roques, B. P., Corvol, P., and Llorens-Cortes, C. (1996). Identification of mtabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors. Predominant role of angiotension III in the control of vasopressin release. *Proc. Natal. Acad. Sci. USA* **93:**11968-11973.