Short and Long Term Regulation of Catecholamine Biosynthetic Enzymes by Angiotensin in Cultured Adrenal Medullary Cells

MOLECULAR MECHANISMS AND NATURE OF SECOND MESSENGER SYSTEMS*

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The purpose of this study was to examine the effects of angiotensin on the enzyme activities and gene expression of two catecholamine synthesizing enzymes, tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT), in bovine adrenal medullary (AM) cells. Short term (15 min) incubation of cultured AM cells with 2 μM angiotensin II (s'-AII) did not increase basal secretion of catecholamines; however, longer incubations (3, 24, or 72 h) produced 4-10-fold increases. To determine whether angiotensin affects synthesis of catecholamines, the activities of TH and PNMT were examined. Incubation with s'-AII (15-30 min) decreased the $K_m$ of TH for its biopterin cofactor (6R)-5,6,7,8-tetrahydro-1-biopterin dihydrochloride (BH$_2$) without affecting the $V_{max}$, suggesting activation of TH. After long term incubation (72 h) the $K_m$ value was identical to that of control, while increases in the apparent $V_{max}$ were observed. PNMT activity was unaffected during a 30-min treatment with s'-AII; however, 2-fold increases occurred after a 48-72-h incubation. s'-AII (24 h) increased the relative abundance of TH and PNMT mRNAs, suggesting that the long term increase in enzyme activities reflected increased expression of TH and PNMT genes. Maximal increases were observed at 2 μM s'-AII and the changes were antagonized by saralasin. Induction of TH mRNA by s'-AII was additive to the effects of veratridine or forskolin indicating that effects of angiotensin were not due to membrane depolarization or increased cyclic AMP levels. Incubation with Ca$^{2+}$ ionophore A23187 increased TH and PNMT mRNA levels in AM cells raising the possibility that the increase in cellular Ca$^{2+}$ could mediate effects of angiotensin. Angiotensin-induced increases in TH and PNMT mRNA were inhibited by nifedipine indicating involvement of voltage-dependent Ca$^{2+}$ channels. In addition, the increases in TH, but not PNMT mRNA, were antagonized by dantrolene, which inhibits mobilization of Ca$^{2+}$ from intracellular stores. Calmodulin involvement was suggested by the inhibition of s'-AII induced changes in mRNA with 1 μM calmidazolium. The role of protein kinase C (PKC) was indicated by the following observations: (a) increases in the membrane-bound activity of PKC in cells incubated with s'-AII; (b) induction of TH and PNMT mRNA with PKC-activating phorbol esters; (c) inhibition of the s'-AII effects by pretreatment of cells with 12-O-tetradecanoylphorbol 13-acetate and β-phorbol 12,13-didecanoate; (d) inhibition of the s'-AII effects by treatment of cells with 12-O-tetradecanoylphorbol 13-acetate, which reduced total cellular activity of PKC and attenuated the increase in mRNAs in response to phorbol ester; and (e) inhibition of changes in mRNA by a PKC inhibitor, sphingosine. In conclusion, angiotensin was found to control the expression of genes encoding catecholamine biosynthetic enzymes. The effects of angiotensin seem to be mediated by Ca$^{2+}$-dependent second messenger systems. By these mechanisms angiotensin may exert a long term control of the secretory functions of AM cells.

The catecholamine-secreting cells of adrenal medulla (AM) constitute an important effector system for homeostatic reactions. The afferent information is supplied to those cells through converging neural inputs and a variety of circulating hormones. AM cells respond to this information by releasing norepinephrine, epinephrine, and a variety of neuropeptides, each producing unique physiologic effects. In addition to instantaneous secretory responses, incoming signals may have long-lasting impact on the hormonal output from AM. Mechanisms for such cellular memory may involve changes in the activity of genes encoding catecholamine biosynthetic enzymes and neuropeptides (1-3). Acetylcholine and glucocorticoids increase expression of TH, PNMT (1, 4, 5), and proenkephalin genes (7-8), although only the former can induce direct release of catecholamines and peptides from AM (10).

There is ample evidence indicating that the renin-angiotensin system may play a role in the reflex stimulation of the sympathoadrenal catecholamine cells. Angiotensin has been shown to enhance catecholamine secretion by acting directly on the sympathetic neurons (11-14) and on AM cells (15-18). AM cells contain a particularly high density of angiotensin binding sites (19-21). The purpose of this study was to elucidate mechanisms by which angiotensin controls secretory functions of AM cells. We have found that angiotensin increases the activities of the two major catecholamine biosyn-

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1 The abbreviations used are: AM, adrenal medulla; TH, tyrosine hydroxylase, PNMT, phenylethanolamine N-methyltransferase, PKC, protein kinase C; s'-AII, [Sar]$^2$angiotensin II; TPA, 12-O-tetradecanoylphorbol 13-acetate, BH$_2$, tetrahydrobiopterin (6R)-5,6,7,8-tetrahydro-1-biopterin dihydrochloride.

thetic enzymes TH and PNMT, and examined the molecular mechanisms and the nature of the second messenger systems involved in this regulation. A portion of these studies appeared as an abstract (22).

EXPERIMENTAL PROCEDURES

Materials

Dulbecco's modified Eagle's medium, fetal calf serum, salmon sperm DNA, [32P]adenosine II, saralasin, 12-0-tetradecanoylphorbol 13-acetate (TPA), 4a-phorbol 12,13-didecanoate, 4b-phorbol 12,13-didecanoate, forskolin, sphingosine, calmidazolium, histone Type III-S, EDTA, EDTA, phenylmethylsulfonyl fluoride, 1,2-sn-diacylglycerol (DG), acetylcholine, physostigmine, nicotine, veratridine, catalase (C-100) dithiothreitol, normetanephrine HCI. Per-
coll, and tyrosine were purchased from Sigma. Triton X-100 was a
product of Research Products International Corp., IL, and phospha-
tidyl serine was from ICN Biochemicals.

(6f)-5,6,7,8-Tetrahydro-1-biopeterin dihydrochloride (BHL) was
obtained from Research Biochemicals Inc. (Natick, MA). Collagenase
type B was obtained from Boehringer Mannheim. Whatman P-81
phosphocellulose paper was purchased from Fisher. Other chemicals
were of molecular biology grade and were obtained from Boehringer Mannheim or Belliesa Research Laboratories. [32P]dCTP, 5000
Ci/mmol, [α-32P]ATP, 11 Ci/mmol, 1-(ring-3,5-3H)tyrosine (55 Ci/
m mol), and [3-methyl-3H]adenosine-1-methionine (15 Ci/mmol) were purchased from Du Pont-New England Nuclear.

Cell Culture

Primary cultures of bovine adrenal medullary cells were prepared
according to the method of Wilson and Viveros (23), as described previously. Briefly, bovine adrenal medullae were digested with col-
lagenase and cells were isolated by centrifugation on Percoll gra-
dients. Cells were plated in 90-mm wells (0.5-6 x 10^5 cells/cm^2 RNA isolation) or in 10-mm wells (1-1.2 x 10^5 cells/cm^2, catechol-
amine and enzyme assays) in Dulbecco's modified Eagle's medium/
F-12 medium containing 10% fetal calf serum, penicillin (100 units~
ml), streptomycin (100μg/ml), and gentamycin (45μg/ml), and then
treated in 3% CO_2 and 97% air at 37 °C for 2-6 days. The drug
treatments were initiated after cells were in serum-free medium for
1-3 days.

Isolation of RNA and mRNA Assays

Total AM cell RNA was isolated from two combined wells by a
guanidinium thiocyanate/cesium chloride gradient procedure (24).2
Relative abundance of TH and PNMT mRNAs was assayed by dot
blot and Northern blot analyses as described previously (1, 5) Bovine
PNMT cDNA (600 base pairs) (25) and cDNA TH cDNA (400 base
pairs) (26) were used as hybridization probes. Bacterial clones con-
taining recombinant plasmids with PNMT and TH cDNAs were obtained from B. B. Kaplan (Department of Psychiatry, Univer-
sity of Pittsburgh) and Dr. C. M. Chikarashii (Department of Neu-
rology, Tufts Medical Center, Boston, MA), respectively. cDNA
probes were isolated and nick-translated with [α-32P]dCTP as de-
scribed previously (1, 5). The intensity of hybridization signals in
each assay was expressed in relation to the intensity obtained with
RNA preparations from control cultures which were simultaneously
processed and assayed. Results are presented as the mean ± S.E. of
the indicated number (n) of RNA preparations.

Catecholamine Assay

The norepinephrine and epinephrine content of the culture media and
cells was determined by high performance liquid chromatography
with electrochemical detection as described previously.

Measurement of TH and PNMT Activities

Protein Extraction—Cells were washed with ice-cold calcium-free
salts solution (145 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, 11.2 mM
glucose, 15 mM HEPES, pH 7.4) and frozen on dry ice. Following
thawing and refreezing on dry ice, cells (2 x 10^7) were briefly sonicated
(1 s) in 2 ml of ice-cold lysis buffer (5 mM Tris-HCl, pH 7.0, 5 mM
Dithiothreitol, 50 mM sodium fluoride, and 1 mM EDTA). Low
molecular weight endogenous inhibitors of TH activity were removed
by chromatography on Sephadex G-25 PD-10 columns (Pharmacia,
Uppsala, Sweden). Protein was eluted with 3 ml of lysis buffer and
used for TH and PNMT assays. Protein concentration was estimated
using a Bio-Rad assay kit and the procedure suggested by the manu-
facturer, using bovine serum albumin as the standard.

TH Assay—TH activity was determined in 60-μl aliquots of extract
(0.5-2 μg of protein), using the [H]catecholamine as substrate and
a procedure described by Reinhard et al. (27). Aliquots of cell extracts
were added to tubes containing 30 μl of 0.05 μM tyrosine (~2 x 10^6
dpm), 0.33 mM Tris-HCl (pH 7.0), 15 mM dithiothreitol, and 180 μg of
catalase. Reaction was initiated by addition of 10 μl of BH_4, and tubes
were incubated 20 min at 37 °C. In blank samples, BH_4 was omitted.
The reactions were terminated by adding 1.0 ml of a stirred suspension
of 7.5% (w/v) charcoal slurry in 1.0 M HCl. Mixtures were mixed and
centrifuged at 15,000 x g for 4 min. Aliquots of 0.5 μl of supernatant
were mixed with 6.5 ml of 0.07-3 μg/100 μl. For the determination of the apparent K_M
and V_max values for TH, the concentration of the BH_4 cofactor varied
between 0.1 and 2.1 μM (5-7 concentrations/experiment), while the
concentration of tyrosine remained constant. The kinetic constants
were calculated using a regression fitted to the data on a double-
reciprocal plot. Due to variability in TH activity, from different cell
preparations, enzyme activity was expressed as percent of control
values obtained in the same cell preparation.

PNMT Assay—PNMT activity was determined by modification of
the method of Axelrod (28). Aliquots of cell extract (0.6-2.0 μg of
protein) were added to tubes containing 50 μl of 3 μM Tris-
HCl (pH 8.5). Tubes were kept on ice for 20 min, and the reaction
was initiated by the addition of 30 μl of 5 mM normetanephrine HCl,
and 0.25 μl [S-methyl-3H]adenosine-1-methionine (15 Ci/mmole)
and transferred to a 37 °C water bath. In the blank samples
normetanephrine was omitted. Samples were incubated 40 min, and
the reactions were terminated by the addition of 0.5 μl of 0.5 M borate
buffer (pH 10.1). The resulting radioactive product was extracted
with toluene/isomyl alcohol (3:2) and estimated by liquid scintilla-
tion counting.

Measurement of Protein Kinase C Activity

Activity of Ca^2+-activated phospholipid-dependent PKC was
measured according to Kikkawa et al. (29), and Roskowski (30), with
minor modifications. Cells were washed 3 times in ice-cold Ca^2+-free
HEPES-buffered salt solution (pH 7.4), and then sonicated 6 s in ice-
cold lysis buffer containing 30 mM Tris-HCl (pH 7.5), 2 mM EDTA,
1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml leupeptin. Hol-
mogenates were centrifuged for 60 min at 100,000 x g at 4 °C and
resulting supernatant was used as a source of soluble PKC activity.
The 100,000 x g pellet was washed with lysis buffer and extracted with
same buffer containing 0.1% Triton X-100. The pellet was
incubated on ice for 30 min and then centrifuged at 15,000 x g for
90 min, and the resulting supernatant was used to measure membrane-bound activity. The protein content in soluble fraction and pellet extract were measured according to Lowry et al. (30). The final reaction mixture (200 μl) contained 35 mM Tris-HCl, 0.25 mM EDTA, 0.5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride, 12.5 μg/ml leupeptin, 1 μM CaCl_2, 0.5 μg/ml histone type III-S, 40 μg/ml phosphatidylserine (PS), 10 μg/ml 1,2-sn-diacylglycerol-(DG), protein (1-4 μg), and [α-32P]ATP (100-200
cpm/μl) (pH 7.5). The reaction was started by adding a known amount
of protein. Protein was omitted in blank samples. Samples were
incubated for 5 min at 30 °C, and the reaction was terminated by
spotting 25-μl aliquots of incubation mixtures onto strips of
phosphocellulose paper (Whatman P-81). The papers were washed
3 times with 75 μM phosphoric acid (30, air-dried, and transferred to
scintillation vials. Radioactivity was counted in Ultrafluor. PKC
activity was calculated as a difference between activity in the presence
and absence of CaCl_2/phosphatidylserine + DG. The activity in
the absence of CaCl_2/phosphatidylserine + DG was the same as that
obtained when only phosphatidylserine + DG were omitted. The
results were expressed in nanomoles of histone-incorporated phos-
phate/min protein/min. Protein was determined by the method of
Lowry et al. (31).

Statistics

One way analysis of variance was used to test for overall statistical
significance. Comparisons between groups were made using Fisher's
least significant difference test (32).
RESULTS

Effects of Angiotensin on Catecholamine Output from Adrenal Medullary Cells—The concentration of epinephrine and norepinephrine in the medium after a 15 min incubation of the nontreated AM cells was 2305 ± 593 and 1624 ± 474 pmol/ml (n = 3), respectively. Incubation with the stable analog of angiotensin, s'-AII (2 nM) for 15 min did not increase extracellular catecholamine concentration above control levels. However, after 3, 24, and 72 h of continuous incubation with 2 nM s'-AII, increases of 4.8-, 5.2-, and 3.5-fold in epinephrine and 1.72-, 23.7-, and 4.7-fold in norepinephrine were found extracellularly.

Effect of Angiotensin on TN and PNMT Activities—Incubation of cells with s'-AII (0.1 μM) for 10 min increased TH activity from 0.21 ± 0.07 (control) to 0.38 ± 0.17 (pmol/min/μg protein), when assayed in a subsaturating concentration (0.1 μM) of BH₄ cofactor (p < 0.01, n = 3). No significant changes were found at saturating concentration (2 μM) of cofactor (2.35 ± 0.34 and 3.05 ± 0.17 pmol/min/μg protein for control and s'-AII, respectively). The ratio of TH activities at 0.1:9 μM BH₄, increased significantly in cells treated with s'-AII (73.6%, p > 0.01) suggesting activation of TH. To confirm this, enzyme kinetics were examined in the presence of varying concentrations of BH₄ (0.1-2.0 μM) and the substrate [3H]noradrenaline (50 nM). The Michaelis constant, Kₘ, and maximum velocity, Vₘₐₓ, for the reaction were determined as described under “Experimental Procedures.” A total of 9 experiments were carried out using six independent cell preparations. Mean control values were: Vₘₐₓ, 3.34 ± 0.98 pmol/min/mg protein; Kₘ, 0.63 ± 0.11 μM (n = 3). Results are shown as percent of control values (% of control) ± S.E. from n experiments or results of the individual experiments (48 h).

Effect of Angiotensin on TH and PNMT mRNA Levels—To gain information on the molecular mechanisms of the long term regulation of TH and PNMT activities by angiotensin, total RNA was isolated from control and s'-AII-treated cells and subjected to Northern and dot blot analysis using specific hybridization probes (25, 26). As found earlier these probes hybridized with major RNA species similar to well characterized TH and PNMT mRNA in bovine AM cells. While the incubation with 0.2 nM s'-AII (24 h) had no effect on mRNA levels (Fig. 1), higher concentrations of the s'-AII increased both TH and PNMT mRNA levels. Changes in TH mRNA levels were maximal (2 nM s'-AII) while PNMT mRNA observed at 2 nM s'-AII were not significantly different from those produced by higher concentrations of s'-AII. The mean increases produced by 2 nM to 10 μM s'-AII were 174% (TH) and 350% (PNMT) above control (Fig. 1). s'-AII induced increases in mRNA levels were reproducibly antagonized in three independent experiments by the angiotensin antagonist saralasin. In the experiment shown on Fig. 2, a 24-h incubation with saralasin did not affect TH mRNA levels in AM cells. Small increases in PNMT mRNA in saralasin-treated cells (22 and 42%) were observed. Saralasin completely abolished effects of s'-AII on TH mRNA levels, and reduced by 70% increases in PNMT mRNA (Fig. 2).

Role of Membrane Depolarization in Angiotensin-induced Changes in mRNA Levels—Veratridine depolarizes cellular membrane by increasing sodium permeability (33) resulting in the stimulation of the secretion of AM hormones (33, 34) and the expression of TH and PNMT genes (35). To examine whether angiotensin may act through similar mechanisms additivity of the effects of angiotensin and veratridine was investigated. The increases in TH mRNA produced by 24 h incubations with s'-AII or veratridine or by combined treatment with two drugs are shown in Fig. 3. Treatment with s'-AII and veratridine produced additive increases in TH mRNA levels. Additivity was observed at maximal (2 nM, Fig. 3A) and supramaximal (5 μM, Fig. 3B) concentrations of s'-AII. Statistical analysis of the combined results of these two experiments revealed that treatment with s'-AII and veratridine was significantly more effective (p < 0.01) in raising TH mRNA levels than with either agent alone. In both experiments treatment with s'-AII and veratridine appeared to have a synergistic effect on TH mRNA (Fig. 3, A and B), indicating...
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I. CONTROL
2Gₘ sₕ gₛ [S'M'] ANGIOTENSIN II
2x10⁻³
3x10⁻³
4x10⁻³
5x10⁻³
TH PNMT
TH - PNMT 9-a
8 lb-lo
10-G 10-e
10⁻⁷ 10-G 10⁻⁵
S'-ANGIOTENSIN [M] J

FIG. 1. Effect of angiotensin on tyrosine hydroxylase and phenylethanolamine N-methyltransferase mRNA in adrenal medullary cells. Relative abundance of TH and PNMT mRNA was estimated by dot blot and Northern blot procedures using specific hybridization probes (see "Experimental Procedures"). A, cells were incubated for 24 h without (control) or with the indicated concentrations of s'-AII. Data are shown as the ratio of the peak area of densitometric scan to the amount of total RNA and mRNA levels are presented in arbitrary optical density units. 100 units were assigned to mRNA levels in cultures incubated with 200 nM s'-AII. Individual points represent an average from two or the mean ± S.E. from four (control and 200 nM s'-AII) RNA preparations. Two separate cell preparations were used in this experiment.

FIG. 2. Inhibition of the effects of angiotensin on mRNA levels by saralasin. Cells were incubated for 24 h with s'-AII (20 nM) or saralasin (2 µM). In experiments in which cells were exposed to saralasin and s'-AII, saralasin was added 15 min before s'-AII. Bars represent average numbers from two RNA preparations.

FIG. 3. Effects of coincubation with veratridine on changes in TH mRNA produced by angiotensin. Cells were incubated for 24 h with 2 nM (A) or 5 µM (B) s'-AII, 2 µM veratridine, or veratridine and s'-AII together. Bars represent average numbers from two preparations.

FIG. 4. Effects of Ca²⁺ ionophore on TH and PNMT mRNA levels. Cells were incubated 12 h with 0.1 µM A23187. Bars represent mean ± S.E. from three RNA preparations.

that these agents acted through separate, yet interacting, pathways.

Roles of Calcium and Calmodulin in the Regulation of mRNA Levels by Angiotensin—Stimulation of angiotensin receptors on AM cells elicits influx of extracellular Ca²⁺ and mobilizes intracellular Ca²⁺ stores (36). Incubation of AM cells with calcium ionophore A23187 (0.1 µM) for 12 h resulted in a statistically significant 49% increase in TH mRNA (Fig. 4). A similar increase in PNMT mRNA levels was observed. This change was close to statistically significant level (p = 0.06) (Fig. 4). To further elucidate the role of Ca²⁺ in the regulation of TH and PNMT mRNA by angiotensin, the effects of calcium channel antagonists nifedipine, and an inhibitor of intracellular Ca²⁺ release, dantrolene (36-41) were examined. Basal TH and PNMT mRNA levels were not significantly affected by nifedipine or dantrolene when compared to drug-free control cultures (Fig. 5). Nifedipine (20 µM) prevented induction of PNMT mRNA levels by s'-AII (Fig. 5). The levels of TH mRNA in cells treated with s'-AII and nifedipine were not significantly different from the levels in cells incubated with nifedipine alone. Thus the influx of Ca²⁺ through voltage-dependent channels appears to mediate induction of TH and PNMT genes by s'-AII. Induction of TH mRNA was also inhibited by dantrolene (Fig. 5), indicating that the mobilization of the intracellular calcium reserves participates in the regulation of TH gene by angiotensin (Fig. 5).

To examine whether calmodulin may serve as an effector protein for the Ca²⁺ effects observed in angiotensin-treated cells, calmidazolium, a calmodulin antagonist (42-44), was
Kinase C Activity in Adrenal Medullary Cells—PKC is another calcium-regulated protein which could mediate effects of Ca**+ on mRNA levels. Activities of the soluble and membrane-bound enzyme were examined in AM cells treated with s'-AII and TPA. Short term treatment (30 min) with 0.2 μM TPA or s'-AII did not significantly affect total (soluble plus membrane) activity of phospholipid-diacylglycerol-dependent calcium-activated kinase (Table III). In contrast, cells incubated with a higher concentration of TPA (1 μM) for 20 h showed approximately 80% reduction in the total activity of PKC (Table III). In control cells, the majority of the activity was recovered in the soluble fraction. Short term treatment with TPA reduced the activity of the soluble enzyme and produced nearly a 3-fold increase in the membrane-bound activity (Table III). Similarly, in cells treated with TPA for 20 h, a larger portion of the residual PKC activity was recovered in the membrane fraction (Table III). Incubation of AM cells with s'-AII reproducibly increased the activity of the membrane-bound PKC. As shown in Table III, s'-AII increased the fraction of the membrane-bound enzyme activity by 43%.

Role of Protein Kinase C in Angiotensin-induced Changes in TH and PNMT Gene Expression—To determine whether expression of TH and PNMT genes could be regulated by PKC, cells were incubated with PKC activators. TPA produced increases both in TH and PNMT mRNA levels (Fig. 6). The induction of mRNA levels was detectable already after 3 h, and was maximal after 6 h. After 24 h incubation with TPA PNMT mRNA declined to the levels not significantly different from control, whereas TH mRNA remained elevated. The levels of TH and PNMT mRNAs were also significantly increased by 4β-phorbol 12,13-didecanoate, an analogue of phorbol ester which stimulates PKC. The inactive phorbol ester 4α-phorbol 12,13-didecanoate on the other hand had no effect.

Increases in the activity of particulate PKC in angiotensin-treated cells and the ability of phorbol esters to induce adrenal medullary TH and PNMT genes, suggested that PKC may mediate effects of calcium on mRNA levels in angiotensin-treated cells. Therefore the additivity of the effects of s'-AII and TPA was examined. No additivity in the changes in TH mRNA was found, suggesting that phorbol ester and angiotensin may act through a similar mechanisms. A 24-h incubation with TPA did not affect PNMT mRNA levels (see also Fig. 6A), but when added together with s'-AII, it prevented an increase in mRNA produced by s'-AII (Fig. 7). These observations were produced in two separate experiments using different cell preparations (data not shown).

To reduce PKC activity in AM cells and examine its effect on the induction of mRNAs by s'-AII, two procedures were used, down-regulation of PKC with TPA, and the incubation of AM cells with the PKC inhibitor sphingosine (45). Cells were preincubated for 48 h with 0.1 μM TPA (experiment I, Table IV) or for 24 h with 1 μM TPA (experiment II, Table IV) followed by a 24-h incubation in control medium, with 0.1 μM TPA or with 20 nM s'-AII. Preincubation with TPA reduced PNMT mRNA levels by over 60% (experiments I and II, Table IV). In the same cultures, TH mRNA levels remained unchanged (experiment I) or increased (experiment II). The 48-h preincubation with 0.1 μM TPA reduced responses to subsequent treatment with TPA by 63% for PNMT and 58% for TH. In cells pretreated with TPA increases in PNMT mRNA in response to s'-AII were abolished (Table IV). Preincubation with TPA also inhibited angiotensin-induced increase in TH mRNA levels (Table IV, experiment II). Incubation of AM cells for 24 h with sphingosine (37.5 μM) did not affect basal levels of TH or PNMT mRNA (TH mRNA 106.5 ± 18.4%, n = 6, PNMT mRNA 83.9 ± 15.4% of control, n = 5). However, sphingosine reduced the effects of TPA on TH by 88.1%, indicating an inhibition of PKC in sphingosine-treated cells (Fig. 8). In this experiment, PNMT mRNA remained increased above control only during a 24-h incubation with TPA. This increase was also reduced by 69.8% with sphingosine (Fig. 8). Sphingosine also significantly reduced increases in TH and PNMT mRNAs produced by s'-AII (Fig. 8), further supporting the role of PKC in this regulation.

Effects of Forskolin on Angiotensin-produced Changes in mRNA Levels—The levels of TH and PNMT mRNAs in
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TABLE III

Effects of phorbol ester and angiotensin on distribution of protein kinase C activity in adrenal medullary cells

Cells were treated with s1-AII (200 nM, 30 min), or TPA (0.2 µM, 30 min, or 1 µM, 20 h). PKC activity was measured in soluble and membrane fractions of adrenal medullary cells as described under "Experimental Procedures." Values are mean ± S.E. (n) of individual culture wells. Total activity (membrane + soluble) in control cultures was 7.5 ± 1.25 nmol/mg protein/min (mean ± S.E. from six experiments using three cell preparations). Total activity in s1-AII- or TPA-treated cultures is expressed as percent of control from the same cell preparation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Control (mg protein)</th>
<th>s1-AII (200 nM, 30 min)</th>
<th>TPA (0.2 µM, 30 min)</th>
<th>TPA (1 µM, 20 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total activity (% of control)</td>
<td>100</td>
<td>94.6 ± 1.5</td>
<td>128.8 ± 24.2</td>
<td>22.2 ± 2.0*</td>
</tr>
<tr>
<td>Membrane (% of total)</td>
<td>26.9 ± 3.7 (6)</td>
<td>38.2 ± 1.54* (4)</td>
<td>78.7 ± 12.1* (3)</td>
<td>61.0 ± 4.0* (3)</td>
</tr>
</tbody>
</table>

* p < 0.001 different from control.

A, cells were treated for 12 h with 0.1 µM 4a-phorbol 12,13-didecanoate (4a-PDD), 0.1 µM 4α-phorbol 12,13-didecanoate (4α-PDD), or incubated without phorbol esters (control). B, cells were harvested at the same time. Insets, examples of Northern blot analysis of phorbol ester action (12 h) on mRNA levels. Data represent the mean ± S.E. from (n) RNA preparations. Statistical analyses: * p < 0.05, ** p < 0.01, *** p < 0.001 with respect to control; +, p < 0.05 with respect to 4α-phorbol 12,13-didecanoate-treated cultures.

Fig. 6. Effects of phorbol esters on TH and PNMT mRNA.

A, cells were treated for 12 h with 0.1 µM 4a-phorbol 12,13-didecanoate (4a-PDD), 0.1 µM 4α-phorbol 12,13-didecanoate (4α-PDD), or incubated without phorbol esters (control). B, cells were harvested at the same time. Insets, examples of Northern blot analysis of phorbol ester action (12 h) on mRNA levels. Data represent the mean ± S.E. from (n) RNA preparations. Statistical analysis: * p < 0.05, ** p < 0.01, *** p < 0.001 with respect to control; +, p < 0.05 with respect to 4α-phorbol 12,13-didecanoate-treated cultures.

Fig. 7. Effect of coincubation with phorbol ester on changes in TH mRNA levels produced by angiotensin. Cells were incubated for 4 h with 2 nM s1-AII or 0.1 µM TPA. To some cells s1-AII was added together with TPA. Data represent average numbers from two or mean ± S.E. from four RNA preparations.

FIG. 6. Effects of phorbol esters on TH and PNMT mRNA. A, cells were treated for 12 h with 0.1 µM 4α-phorbol 12,13-didecanoate (4α-PDD), 0.1 µM 4α-phorbol 12,13-didecanoate (4α-PDD), or incubated without phorbol esters (control). B, cells were harvested at the same time. Insets, examples of Northern blot analysis of phorbol ester action (12 h) on mRNA levels. Data represent the mean ± S.E. from (n) RNA preparations. Statistical analyses: * p < 0.05, ** p < 0.01, *** p < 0.001 with respect to control; +, p < 0.05 with respect to 4α-phorbol 12,13-didecanoate-treated cultures.

Bovine AM cells could be increased by cyclic AMP analogs or forskolin. To examine, whether the effects of s1-AII on mRNA were mediated through the cyclic AMP responses to s1-AII were studied in the presence and absence of maximally effective concentrations of forskolin. s1-AII and forskolin increased TH mRNA level in an additive fashion, suggesting independent mechanisms for the effect of s1-AII and forskolin (Fig. 9).

Fig. 7. Effect of coincubation with phorbol ester on changes in TH mRNA levels produced by angiotensin. Cells were incubated for 24 h with 2 nM s1-AII or 0.1 µM TPA. To some cells s1-AII was added together with TPA. Data represent average numbers from two or mean ± S.E. from four RNA preparations.

DISCUSSION

These studies demonstrate that the stimulation of catecholamine secretion from AM cells by angiotensin is accompanied by increases in the activities of two catecholamine biosynthetic enzymes, TH and PNMT. This finding is consistent with an earlier report (46) on the increased catecholamine biosynthesis in sympathetically innervated tissues treated with angiotensin.

Increases in TH activity resulted from two temporarily distinct events. Initial increase in TH activity was readily observed after a 10-15 min incubation with an angiotensin II analog. This increase was detectable only under subsaturating concentrations of biopterin cofactor and was associated with a decrease in Km of the enzyme for the cofactor. Similar changes in TH kinetics were previously observed with TH activated by cyclic AMP or forskolin (47). In our laboratory, changes in TH kinetics induced by angiotensin were not additive with the changes produced by forskolin. Thus, the early changes in TH kinetics induced by angiotensin may represent enzyme activation attributable to some modification of its molecules. Since intracellular concentration of endogenous biopterin cofactor is at subsaturating levels (47), an

3 M. K. Stachowiak, unpublished observation.
Experiment I (48/24 h)

Cells were pretreated 48 h with 0.1 μM TPA (experiment I), 24 h with 1 μM TPA (experiment II), or were incubated in TPA-free medium (-) for the equal periods of time. Subsequently cells were washed and incubated 24 h with one of the following agents: 0.1 μM TPA, 20 nM s'-AII, or in the drug-free medium. Data represent mean ± S.E. of n RNA preparations, or results from individual RNA preparations.

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<td>100.0 ± 15</td>
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<td>-/TPA (2)</td>
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TABLE IV

Effect of preincubation with TPA on the induction of tyrosine hydroxylase and phenylethanolamine N-methyltransferase genes by phorbol ester and angiotensin

Cells were pretreated 48 h with 0.1 μM TPA (experiment I), 24 h with 1 μM TPA (experiment II), or were incubated in TPA-free medium (-) for the equal periods of time. Subsequently cells were washed and incubated 24 h with one of the following agents: 0.1 μM TPA, 20 nM s'-AII, or in the drug-free medium. Data represent mean ± S.E. of n RNA preparations, or results from individual RNA preparations.

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Fig. 8. Effect of sphingosine on the changes in TH and PNMT mRNA levels produced by TPA and angiotensin. Cells were preincubated with or without sphingosine (97.5 μM) for 15 min. Subsequently, media were replaced with sphingosine or sphingosine-free media, containing either 0.1 μM TPA, or 20 nM s'-AII, or neither compound. The incubations were continued for 24 h. The increases produced by s'-AII and TPA in the absence of sphingosine were 154.5 ± 36.2 and 188 ± 19.7% for TH, and 693 ± 261 and 134.5 ± 23.0% for PNMT, respectively. Data are expressed as percent of control responses which were set at 100% for each cell preparation. Bars represent mean ± S.E. of (n) RNA preparations. Statistical analysis: *, **, *** p < 0.05, p < 0.01 different from the responses in the absence of sphingosine.

Fig. 9. Effect of coincubation with forskolin on changes in TH mRNA produced by angiotensin. Cells were treated for 24 h with s'-AII (see legend to Fig. 5), 5 μM forskolin, or with forskolin and s'-AII together. Data are presented in arbitrary optical density units. Two separate cell preparations were used in this experiment, in each 100 units were assigned to mRNA levels in cells treated simultaneously with s'-AII and forskolin. Bars represent the mean ± S.E. from n RNA preparations. Statistical analysis: *, **, *** p < 0.05, p < 0.01, p < 0.001 with respect to control; +, ++, + p < 0.05, p < 0.01.

activity under the influence of angiotensin and the evidence that TH is a substrate for PKC in AM cells (50, 51) suggest that activation of TH could be mediated by PKC.

While the early changes in TH activity reflected post-translational modifications, long term increases in the activities of TH and PNMT produced by angiotensin could result from their enhanced synthesis, as indicated by the increases in relative abundances of TH mRNA and PNMT mRNA. Low nanomolar concentrations of angiotensin analog were sufficient to elicit those changes. The inhibition of the effects of s'-AII with saralasin indicated that the effects of s'-AII were mediated by angiotensin receptors. Concentrations of s'-AII required for the long term increases in catecholamine secretion and in mRNA levels were lower than those required to elicit short term secretory response (36). Similarly, short term stimulation of enkephalin release from AM cells occurred at angiotensin concentrations approximately 2 orders of magnitude higher than those inducing increase in proenkephalin mRNA and long term peptide secretion. Thus, induction of genes encoding catecholamine biosynthetic enzymes and enkephalin peptides could be a primary effect of angiotensin in AM cells, whereas increased secretion of AM hormones would reflect their enhanced synthesis.

Previous studies suggested that acetylcholine-induced increases in the expression of TH and PNMT genes in AM are mediated by cyclic AMP.2 depolarization of the cellular membrane (35) or depletion of intracellular catecholamines.2 Although angiotensin was found to increase cAMP synthesis in AM cells (52), this may not play a role in the changes in mRNA levels. Induction of TH mRNA by forskolin and angiotensin appeared to proceed through independent cellular pathways, as indicated by the additivity of forskolin and angiotensin in increasing TH mRNA levels. In contrast to the nicotinic stimulation, low concentrations of angiotensin did not deplete cellular catecholamine contents4 and did not


appear to depolarize AM cells. The latter was suggested by the absence of catecholamine and enkephalin secretion from cells incubated with s'-AlI for the short time period. In addition the effects of membrane depolarizing agent veratridine, and angiotensin on TH mRNA levels were additive, suggesting participation of different signaling systems. Combination of veratridine and angiotensin was synergistic for TH mRNA indicating that signaling pathways for these two agents may not be strictly independent. The cross-talk between signaling systems operating in depolarized and angiotensin stimulated cells may provide a fine gain control mechanism in regulation of TH gene activity in neurally and hormonally stimulated AM cells.

Since treatment of AM cells with Ca$^{2+}$ ionophore mimicked qualitatively effects of s'-AlI on TH and PNMT mRNA, one candidate for coupling the changes in mRNA levels with the stimulation of angiotensin receptors appeared to be calcium. Angiotensin was shown to elicit increases in the cytosolic [Ca$^{2+}$] in AM cells (36, 53, 54) which could be attenuated by nifedipine (38). Changes in mRNA levels induced by angiotensin were also antagonized by nifedipine in concentrations required to effectively inhibit angiotensin induced influx of Ca$^{2+}$ in intact cells (51). Thus, induction of mRNAs appeared to involve transmembrane movement of Ca$^{2+}$ through a voltage dependent Ca$^{2+}$ channels. However, unlike the complete inhibition of the increase in PNMT mRNA, increase in TH mRNA levels was only partially reduced by nifedipine. A portion of angiotensin induced increase in the cellular [Ca$^{2+}$] is derived from intracellular stores. This was indicated by the numerous observations that increases in intracellular calcium could be detected despite blockage of Ca$^{2+}$ channels (36, 39–41, 56, 57), and was consistent with the increases of synthesis of Ca$^{2+}$-releasing inositol phosphates in angiotensin-stimulated AM cells (58). Inhibition of angiotensin-induced mobilization of intracellular calcium by dantrolene has been documented in several laboratories (36–41). Hence, we have used this agent to examine the role of intracellular Ca$^{2+}$ on the induction of TH and PNMT mRNA levels by angiotensin. Inhibition of the induction of TH mRNA by dantrolene suggests that Ca$^{2+}$ released from such stores may also participate in the regulation of the TH gene activity by angiotensin. Apparently intracellular Ca$^{2+}$ did not mediate the induction of PNMT mRNA, as shown by lack of the inhibition by dantrolene; only extracellular Ca$^{2+}$ mediated induction of PNMT mRNA.

The flow of information in the calcium messenger system may proceed by two distinct branches: calmodulin and PKC. To inhibit calmodulin action we used calmidazolium, a potent antagonist of calmodulin-regulated enzymes (43, 44). Attenuation of the angiotensin-induced increases in mRNA levels by calmidazolium suggest that effects of calcium on gene expression could be mediated by calmodulin. There are several reasons to believe that the increases in TH and PNMT mRNAs also involve PKC. First, TH and PNMT mRNA levels could be increased by TPA and other kinase C stimulating phorbol esters. Prolonged incubation with TPA, however, decreased the basal level of PNMT mRNA (Table IV). This inhibitory effect of TPA is likely related to the down-regulation of PKC. Taken together, results of the experiments with phorbol esters indicate that PKC is involved in the regulation of TH and PNMT gene expression as it was previously shown for the cyclic AMP-dependent pathway (26). Second, angiotensin produced an apparent translocation of PKC from the soluble fraction to cellular membranes. A similar phenomenon was also observed with the short term incubation of cells with TPA. Since only membrane PKC is enzymatically active (59), translocation should lead to an increase in functional PKC. The character and magnitude of changes in PKC activity by angiotensin were similar to the changes found in AM cells treated with nicotinic agonists (60). Thus, in addition to neural regulation by acetylcholine, activity of PKC appears to also be controlled by hormonal input to adrenal medullary cells. Third, involvement of PKC was indicated by attenuation of the effects of angiotensin on TH and PNMT mRNAs in cells preincubated with TPA. Prolonged incubation of AM cells with TPA reduced total PKC activity by nearly 80%, and reduced response of TH and PNMT mRNAs to the stimulation with TPA. These effects occurred without apparent changes in cell viability, or cellular catecholamine content, and with only slightly increased resting release of catecholamines. Regulation of TH gene expression by PKC is consistent with the presence of TPA regulatory element in bovine TH gene promoter (55). Fourth, the involvement of PKC was further indicated by inhibition of the effects of angiotensin by sphingosine, a potent competitive inhibitor of PKC (45). Sphingosine appeared to inhibit PKC in AM cells as indicated by the attenuation of the effects of TPA on TH and PNMT mRNA levels. Sphingosine neither affected total RNA content in AM cultures (data not shown), nor altered relative abundance of mRNA for TH, PNMT, nor inhibited increases in mRNA levels in veratridine-treated AM cells. These findings indicate that angiotensin increased TH and PNMT mRNA levels by acting through PKC activity.

The findings presented in this study may shed new light on the mechanism by which angiotensin may exert its physiologic function in the sympatho-adrenal system. Regulation of the activities of genes encoding catecholamine biosynthetic enzymes may permit angiotensin to control secretory responses of AM in a long term manner. Through this mechanism, homeostatic information could be stored in AM cells and have long-lasting impact on their function. The prolonged effects of the stimulation of angiotensin receptors, which outlast ligand-receptor interactions may also underlie pathologic effects of angiotensin. These effects could involve hypertrophy of the vascular musculature (6), and increased expression of genes involved in the secretory function of AM cells. Interestingly, these diverse effects of angiotensin could be mediated by similar cellular mechanisms including calcium, PKC, and the c-fos protooncogene (61).

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