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DIFFERENTIAL INDUCTION OF GENE EXPRESSION OF CATECHOLAMINE BIOSYNTHETIC ENZYMES AND PREFERENTIAL INCREASE IN NOREPINEPHRINE BY FORSKOLIN

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Abstract—We examined the effect of forskolin, an adenylate cyclase activator, on gene expression and the activities of the three enzymes specific for catecholamine biosynthesis [tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH) and phenylethanolamine *N*-methyltransferase (PNMT)] and on the amounts of available catecholamines in primary cultured bovine adrenomedullary chromaffin cells. The results showed that TH was increased by 4.7 ± 0.7-fold and 69% in mRNA and activity levels, respectively, compared with the untreated control. DBH was elevated by 3.2 ± 0.2-fold in mRNA and 45% in activity. The increase in PNMT, on the other hand, was smaller: 1.7 ± 0.2-fold in mRNA and 13% in activity. This relatively small increase in PNMT was reflected in the catecholamine levels in that the total epinephrine (EPI) was elevated by only 16% while norepinephrine (NE) was elevated by 99%, which caused a shift in the molar ratio of EPI to NE from 7.0 in the untreated control to 4.1 after forskolin treatment. A large portion of the elevated catecholamines was found in the medium, which represented a 10.1-fold increase for NE and a 6.4-fold increase for EPI compared with the control. Interestingly, this caused the remaining intracellular NE and EPI to be only 117 and 66% of the control, respectively. Thus, forskolin caused coordinate up-regulation of gene expression and enzyme activities of the three catecholamine-synthesizing enzymes but to different degrees, resulting in a relatively larger increase in NE than in EPI, both of which were released dramatically. This large enhancement of catecholamine release, as well as the dramatic shift in their ratio, implicates an important physiological role for cAMP in the regulation of *in vivo* sympathetic activities.

Key words: catecholamines; tyrosine hydroxylase; dopamine β-hydroxylase; phenylethanolamine *N*-methyltransferase; forskolin; cAMP

Catecholamines are synthesized *in vivo* by the sequential reactions of the enzymes tyrosine hydroxylase (TH†; tyrosine 3-monooxygenase; tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2), aromatic L-amino acid decarboxylase (EC 4.1.1.28), dopamine β-hydroxylase (DBH; dopamine β-monooxygenase; 3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase β-hydroxylating, EC 1.14.17.1) and phenylethanolamine *N*-methyltransferase (PNMT, *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase, EC 2.1.1.28). Of these, TH, DBH and PNMT are specific for catecholamine synthesis and are under regulation of a variety of physiological effectors, thereby responding to cellular needs for catecholamines. Of such modulatory mechanisms, short-term regulation occurs primarily on TH by phosphorylation [1, 2], whereas long-term regulation by gene induction often occurs on all three enzymes. For instance, when examined simultaneously, their mRNA levels have been shown to be coordinately up-regulated hormonally by glucocorticoids in PC12 cells [3] and by reserpine-induced neural activities in rats [4]. Such coordinate regulation is thought to ensure effective changes in the rate of catecholamine production in response to their physiological demand.

The second messenger cAMP mediates a variety of intracellular activities by activating protein kinase A. In adrenomedullary chromaffin cells, compounds such as vasoactive intestinal peptide [5, 6], adenosine [7], as well as activation of nicotinic acetylcholine receptors [8, 9] and D_1 dopamine receptors [10], have been shown to elevate cAMP, suggesting an important role for this second messenger in the regulation of functions of these cells.

Of the various roles that cAMP may potentially play in the adrenomedullary cells, the synthesis and release of catecholamines have been of interest to many investigators. Using primary cultured bovine adrenomedullary chromaffin cells, past studies have reported increases in the transcript levels of TH [11], DBH [12] and PNMT [11, 13] by elevated cAMP, whereas others observed no significant effects on PNMT [14]. In rat pheochromocytoma cells, cAMP analogues have been shown to induce TH [15] and DBH mRNA [16]. However, no study has compared

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† Abbreviations: TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase; cAMP, cyclic AMP; NE, norepinephrine; EPI, epinephrine; PCA, perchloric acid; DPBS, Dulbecco's phosphate-buffered saline; and SSC, 0.15 M sodium chloride + 0.015 M sodium citrate.

the extent of changes in the mRNA and activity levels of all three enzymes simultaneously or addressed the consequences of such changes on the availability of catecholamines. Such studies would be necessary in assessing the significance of gene expressional changes in controlling the amounts of catecholamines available for physiological actions *in vivo*.

The present study, therefore, systematically examined the effects of elevated cAMP on the relative changes in mRNA and enzyme activities of TH, DBH and PNMT and on the intracellular and released catecholamine levels, using primary cultured bovine chromaffin cells. Forskolin, an adenylate cyclase activator [17], was used to elevate intracellular cAMP. We show that forskolin treatment caused coordinate induction of these enzymes but to different degrees, and that this caused a preferential increase in the availability of NE over EPI. We also show that cAMP caused an increase not only in the synthesis but also in the release of catecholamines.

MATERIALS AND METHODS

Cell cultures. Primary cultures of bovine adrenomedullary chromaffin cells were prepared essentially according to the method of Wilson and Viveros [18], as modified by Hwang and Joh [12]. Briefly, adrenal glands obtained fresh from a local slaughterhouse were perfused through the adrenal vein three times with W3 solution containing 145 mM NaCl, 5.4 mM KCl, 1 mM Na_2HPO_4, 11 mM glucose and 15 mM HEPES (pH 7.4). The glands were then perfused with collagenase (2 mg/mL; Boehringer-Mannheim) and incubated for 30 min at 37°. The medullae were dissected free of cortex, minced, and further dissociated in 0.5 mg/mL collagenase at 37° for 40 min. The cells were then treated briefly with DNase (0.01 mg/mL; Sigma) and filtered through four layers of sterile cheesecloth followed by Spectropor membrane 105 μm mesh. After rinsing in W3, chromaffin cells were separated on a Percoll gradient. The cell viability, as estimated by trypan blue exclusion, was greater than 95%, and chromaffin cells as assessed by neutral red staining, over 80%. Five million cells were plated per 60-mm plate in DMEM/F12 containing 10% fetal bovine serum, 50 μg/mL penicillin, 50 μg/mL streptomycin, 5 ng/mL amphotericin B, 250 μM ascorbate, and 5 μg/mL cytosine arabinoside and kept in humidified air at 37° under 5% CO_2. Two days after plating, the medium was replaced with fresh serum-free medium. Forskolin (Sigma) dissolved in 95% ethanol was added 24 hr later, which was considered time 0 in all experiments.

RNA isolation. Cells were washed with ice-cold DPBS and harvested. After pelleting, the cells were lysed by trituration in 140 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.6), 0.5% Nonidet-p40 and 1 mM dithiothreitol followed by incubation on ice for 5 min. The nuclei were pelleted, and to the supernatant was added an equal volume of digestion buffer containing 0.2 M Tris–HCl (pH 8.0), 5 mM EDTA, 300 mM NaCl, and 2% SDS. Proteinase K (Boehringer-Mannheim) was added to a final concentration of 5 μg/mL, and the sample was incubated for 15 min at 50°. After extractions with phenol and chloroform, the final product was precipitated overnight at −20° in ethanol.

Northern blot analysis. Total RNA isolated from 5×10^6 cells was denatured and resolved in 1% agarose gel containing 6% formaldehyde and transferred to Gene Screen Plus membrane (New England Nuclear) by the standard methods [19]. Hybridization was carried out in the presence of 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate, 100 μg/mL salmon sperm DNA and ^{32}P-labeled cDNA probes (5×10^5 cpm/mL) at 42° overnight. The membranes were washed twice in 1× SSC, 1% SDS at 65° for 30 min and subjected to autoradiography. Complementary DNAs for bovine TH [11], bovine PNMT [20] (donated by Dr. T. H. Joh) and bovine DBH cDNA [12] were used as probes. All blots were also hybridized with human α-tubulin cDNA probe [11] as a control for the amount of RNA loaded in each lane of the gel.

Quantitation of extracellular catecholamines. Cells (5×10^6) were maintained in 5 mL of serum-free DMEM/F12 in the presence or absence of 50 μM forskolin for 30 min or 48 hr. For the 30-min samples, 100 μL of the medium was collected and treated with PCA to a final concentration of 0.4 M. After centrifugation, the acid-soluble fraction was diluted 1:10 in HPLC mobile phase [0.1 M sodium acetate, 0.1 M citric acid, 0.5 mM sodium octyl sulfate, 0.15 mM NaEDTA, 1 mM di-*n*-butylamine in H_2O/methanol (90:10)]. For the 48-hr samples, the cells were replenished with fresh medium every 12 hr immediately before which 100 μL of the medium was taken and treated with PCA. After repeating this procedure at 12, 24, 36 and 48 hr, the four samples were pooled, and the acid-soluble fraction was diluted 1:2.5 in the mobile phase, 10 μL of which was subjected to catecholamine determination. Catecholamines were separated by HPLC using a C18 Novapak column in the mobile phase and detected electrochemically by a Waters 460 detector. The amounts of catecholamines were calculated using a Waters 991 computerized integrator system, and a curve was prepared every time.

Determination of intracellular catecholamines. For determination of intracellular catecholamines, the medium was removed after 48 hr, and the cells were washed with ice-cold DPBS twice. The cells were then harvested and evenly suspended in 1 mL DPBS, from which a 20-μL aliquot was taken and treated with PCA to a final concentration of 0.4 M. After precipitating proteins out, the acid-soluble fraction was diluted 1:100 in the mobile phase, 10 μL of which was subjected to HPLC. The concentrations of NE and EPI were determined as described for extracellular catecholamines.

Determination of enzyme activities. Cells (5×10^6) in a 60-mm culture dish were washed twice and harvested in 1 mL DPBS. The cells were pelleted and homogenized in 5 mM potassium phosphate (pH 6.5) containing 0.2% Triton X-100 for enzyme assays. TH activity was assayed at 30° for 30 min as previously described [21]. The reaction mixture (75 μL) contained 100 μM [^{14}C]tyrosine (0.25 μCi), 1 mM 6MPH_4, 200 U catalase, and 100 mM sodium acetate (pH 6.5). DBH activity was measured by a

modification of the method by Molinoff *et al.* [22]. Briefly, tyramine (1 mM) was converted to octopamine in the reaction mixture containing 100 μM $CuSO_4$, 4 mM ascorbate, 33 mM fumarate, 0.6 mM pargyline, 200 U catalase, and 33 mM sodium acetate (pH 5.5) at 37° for 30 min. Octopamine was then converted to synephrine by the addition of partially purified PNMT in the presence of 20 μM *S*-methyl-[^{14}C]adenosyl-L-methionine and 150 mM Tris–HCl (pH 8.6) and 125 μg/mL EDTA. PNMT activity was measured as described by Park [23]. The incubation mixture contained 1 mM phenylethanolamine, 20 μM *S*-methyl-[^{14}C]adenosyl-L-methionine and 150 mM potassium phosphate (pH 8.6).

Data analyses. All experiments on enzyme activities and catecholamine quantitation were conducted using triplicate samples for each condition, repeated on four different culture preparations. All specific activity and catecholamine concentration data are expressed as the mean ± SEM per μg or mg of cellular proteins, determined by the method of Bradford [24]. Northern analyses were repeated on three independent culture preparations, and the intensities of hybridization were analyzed by densitometry using a Quantimet 520 image analyzer (Cambridge Instrument Co.) and normalized to corresponding α-tubulin values. The data are expressed as means ± SD in fold increase of the control values. Statistic analyses were performed by Student's *t*-test.

RESULTS

Effects of forskolin on mRNA levels of TH, DBH and PNMT. Primary cultured bovine chromaffin cells were treated with the adenylate cyclase activator forskolin, and the mRNA levels of TH, DBH and PNMT were determined simultaneously by northern blot hybridization. We used 50 μM forskolin, as this concentration was shown previously to be maximally effective in inducing DBH gene expression in these cells [12]. Typical results after 24 hr are shown in Fig. 1. Densitometric analysis of such northern blots from three separate culture preparations revealed that TH was increased the most dramatically (4.7 ± 0.7-fold), while DBH was induced to a smaller degree (3.2 ± 0.2-fold) and PNMT showed only a 1.7 ± 0.2-fold induction. The levels of α-tubulin in the treated and untreated cells were similar, indicating that the effects on TH, DBH and PNMT were specific. Thus, the transcripts of all three genes were increased by forskolin, but their degrees were different, in the order of TH > DBH > PNMT. The time course of the induction pattern between 2 and 48 hr after forskolin treatment is shown in Fig. 2. TH and DBH mRNA levels were induced within 6 hr and stayed maximally elevated until 24 hr, after which slow declines were observed. In comparison, PNMT mRNA was increased more slowly, and reached the maximum around 24 hr after treatment.

Effect of forskolin on TH, DBH and PNMT activities. Whether these differential increases in mRNA levels of TH, DBH and PNMT by forskolin are reflected in their corresponding enzyme activities was investigated. As shown in Fig. 3, after 48 hr

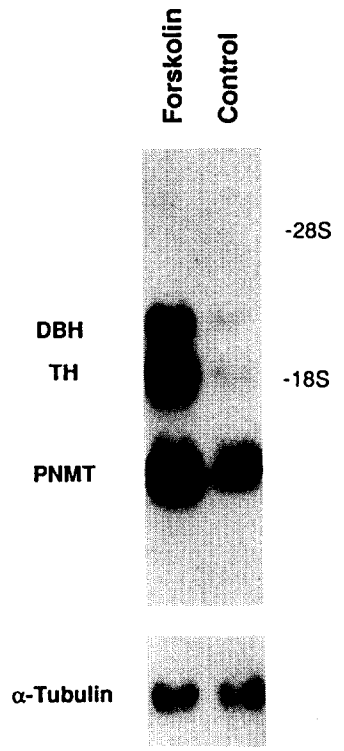

Fig. 1. A typical northern blot of TH, DBH and PNMT after treatment with 50 μM forskolin for 24 hr. Chromaffin cells were prepared and treated for 24 hr with 50 μM forskolin. Total RNA was prepared, and northern blot hybridization was carried out for TH, DBH and PNMT simultaneously (upper panel). The same blot was rehybridized with an α-tubulin cDNA probe (lower panel). Densitometric analysis of northern blots performed on three different culture preparations showed 4.7 ± 0.7, 3.2 ± 0.2, and 1.7 ± 0.2-fold increases for TH, DBH and PNMT, respectively.

with 50 μM forskolin, TH activity was increased from 22.7 ± 2.1 to 38.5 ± 3.6 pmol/100 μg protein/min (69%) and DBH from 19.2 ± 2.8 to 27.8 ± 2.9 pmol/500 μg protein/min (47%). PNMT, on the other hand, showed a small but statistically significant increase from 21.7 ± 2.8 to 24.6 ± 2.8 pmol/mg protein/min (13%). These effects were maximal at 48 hr and were sustained until 72 hr after the forskolin treatment. The relative induction pattern among the three enzymes was similar at other time points, suggesting that this phenomenon is not due to differences in the induction time course (data not shown). Thus, forskolin caused differential increases in the activities as well as in mRNAs of all three enzymes, in the order of TH > DBH > PNMT.

Effect of forskolin on intracellular catecholamines. Whether the differential induction of the three enzymes by forskolin ultimately influences the intracellular concentration of their final product, catecholamines, was also tested. Cells were treated with 50 μM forskolin for 48 hr, and the intracellular catecholamine contents were compared with those

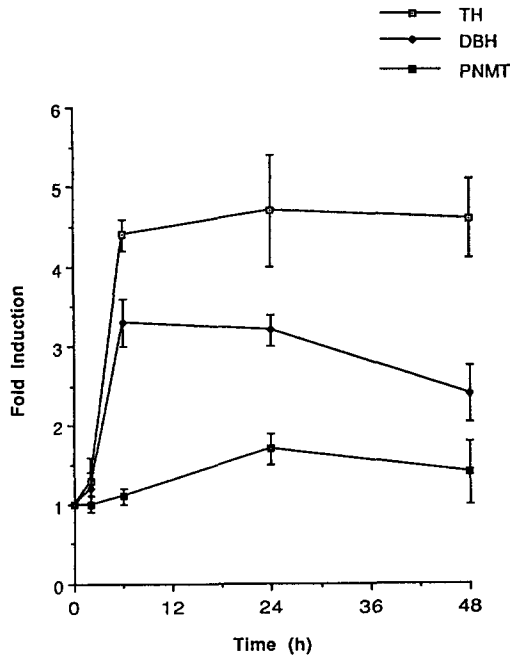

Fig. 2. Densitometric quantification of northern blot autoradiograms. TH, DBH and PNMT mRNA levels were measured by northern blot analysis. TH, DBH and PNMT bands at 0, 2, 6, 24, and 48 hr from appropriately exposed autoradiograms were individually quantified by densitometry and subsequently normalized against corresponding α-tubulin mRNA levels. All experiments were performed on three independent culture preparations, and data are means ± SD values in fold increase of control values.

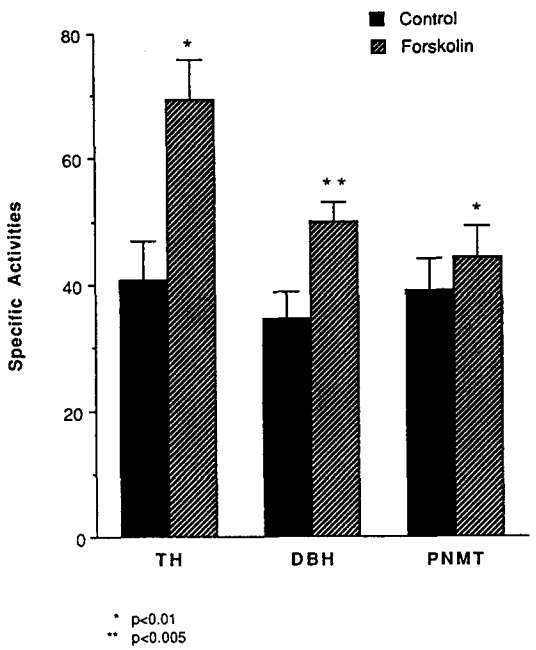

Fig. 3. Activities of TH, DBH and PNMT in bovine adrenal chromaffin cells treated with 50 μM forskolin for 48 hr. Specific activities of TH (pmol/100 μg/min), DBH (pmol/500 μg/min) and PNMT (pmol/mg/min) were measured by the radioenzymatic methods described in Materials and Methods. Enzyme assays were done in duplicate on triplicate samples for each condition, repeated on four different culture preparations. Data represent the means ± SEM.

of the untreated controls. As shown in Fig. 4, surprisingly, the forskolin treatment caused only a small but statistically important elevation in the NE level (117% of the control) and a reduction in the EPI level (66% of the control). Thus, the degree of increase in the intracellular NE was much smaller than that of the activities of their synthesis enzymes, TH and DBH. Furthermore, whereas PNMT activity was shown to be increased to a small extent, the intracellular EPI was reduced compared with the untreated control, raising the possibility that the efficiency of EPI release may be enhanced as well.

Effect of forskolin on catecholamine release. As the change in the intracellular catecholamine levels did not correspond to the increases in the activities of their synthesis enzymes, we tested the possibility that forskolin might influence catecholamine release as well. For this, we quantitated the amount of catecholamines present in the medium after the forskolin treatment. As catecholamines may be unstable once released into the medium, we first performed a set of preliminary experiments where we added into the cell-free medium amounts of catecholamines known to be comparable to those attained in the medium after treating the cells with forskolin (0.5 and 1 μg/mL for NE; 3 and 6 μg/mL for EPI); the medium was incubated under the same conditions, and the time course of the degradation of the catecholamines was monitored between 30 min and 48 hr. We found that catecholamines were stable during the first 12 hr in the medium after which degradation was observed (data not shown). Thus, for the actual experiments we treated the cells with forskolin, collected the medium every 12 hr, and pooled all four samples. As shown in Fig. 5, when the cells were treated short term (30 min), forskolin had little effect on catecholamine secretion. When they were treated long term (48 hr), however, the concentrations of the catecholamines in the medium were dramatically higher for both NE (10.1-fold) and EPI (6.4-fold) compared with those of the untreated control. It is unlikely that this large efflux of catecholamines is due to cytotoxicity because (i) no lactate dehydrogenase activity was found in the medium, and (ii) the number of viable cells, as determined by trypan blue staining, did not change after the forskolin treatment (data not shown).

Effect of forskolin on the amounts of total catecholamines. Based on the finding that forskolin caused catecholamine release into the medium, it was possible that the sum of intracellular and released catecholamines was actually elevated by forskolin, correlating with the observed increases in their specific enzyme activities. To assess the overall changes in the available catecholamines, the total (the intracellular plus extracellular) catecholamines were calculated. As shown in Fig. 6, the total NE

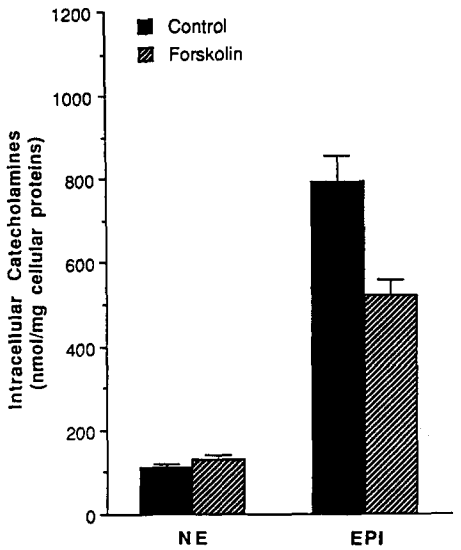

Fig. 4. Effects of forskolin on the levels of intracellular catecholamines in bovine adrenal chromaffin cells. Cells were treated with 50 μM forskolin for 48 hr and harvested in 1 mL DPBS. An aliquot (20 μL) was subjected to protein precipitation in 0.4 M PCA, and the acid-soluble fraction was diluted 1:100. Ten microliters of the diluted sample was subjected to catecholamine determination by HPLC coupled with an electrochemical detector, as described in Materials and Methods. Experiments were done on triplicate samples for each condition, repeated on four different culture preparations. Data represent the means ± SEM of nmol NE or EPI per mg cellular proteins. Values for the forskolin-treated cells were significantly ($P < 0.01$) different from those of the control for NE and EPI.

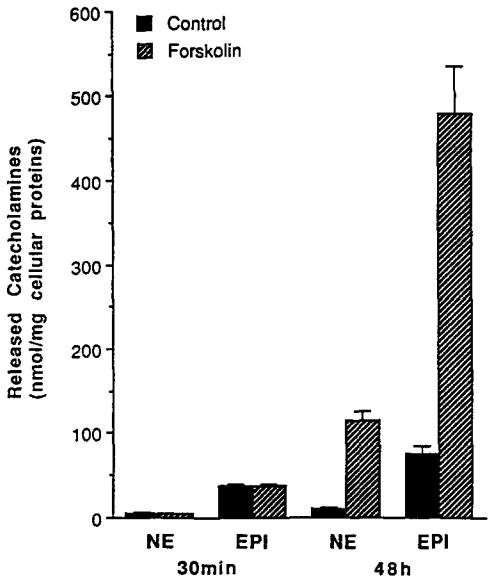

Fig. 5. Effects of forskolin on the amounts of extracellular catecholamines. Bovine adrenal medullary cells were fed with fresh DMEM/F12 without serum, and forskolin was added to a final concentration of 50 μM at time 0. After an appropriate time interval, the medium was collected and treated with PCA to a final concentration of 0.4 M. For the 48-hr sample, the cells were replenished with fresh medium every 12 hr immediately before which an aliquot of the medium (100 μL) was taken and treated with PCA. The procedure was repeated at 12, 24, 36, and 48 hr, and the four samples were pooled. The acid-soluble fractions of the 30-min and 48-hr samples were diluted appropriately and subjected to HPLC coupled with an electrochemical detector. Experiments were done on triplicate samples for each condition, repeated on four different culture preparations. The data represent the means ± SEM of nmol NE or EPI per mg cellular proteins. Values for the forskolin-treated cells were significantly ($P < 0.01$) different from those of the control for NE and EPI at 48 hr.

and EPI were increased to 199 and 115% of the control, respectively, which corresponded better with the increases in the activities of their synthesis enzymes. Furthermore, while the molar ratio between EPI and NE in the untreated cells was 7.0, the forskolin treatment caused a shift in this ratio to 4.1. Thus, similar to the differential pattern of gene expression among TH, DBH and PNMT, forskolin caused a greater increase in the synthesis of NE than EPI.

DISCUSSION

The results of the present study demonstrate that in bovine adrenal chromaffin cells, the mRNA levels and activities of the three catecholamine-synthesizing enzymes, TH, DBH and PNMT, were increased by forskolin, but to different degrees, in the order of TH > DBH > PNMT. This was also reflected in the amounts of total catecholamines available in that the degree of NE increase was much larger than that of EPI. In addition, forskolin not only influenced the synthesis of catecholamines, but also had a stimulatory long-term effect on their release.

A cAMP-response element (CRE) has been found in rat, human, bovine, mouse and quail TH [25–29] and in rat and human DBH [30, 31], and shown to mediate cAMP responsiveness for rat and quail TH [29, 32, 33] as well as rat and human DBH [30, 34, 35] by functional analysis of fusion gene constructs transiently expressed in rat, human, and quail cell lines. This suggests that the increases in bovine TH and DBH mRNAs shown in the present study are likely mediated by this element as well. In light of the recent finding that CRE is also important in conferring tissue-specific basal expression of rat TH [33, 36] and human DBH [34, 35], the CRE motif seems to be critical in the regulation of expression of these genes. On the other hand, no CRE has been found in the PNMT gene thus far [37–39]. The observed increase in PNMT expression by cAMP may be mediated by a different and weaker mechanism than through CRE, and this may partially explain its relatively small and slow induction.

The enzyme activities were elevated in the same general fashion as the corresponding mRNA changes, although not as dramatically. The fact that the increase in the activities was lower than that in mRNA levels probably represents the presence of

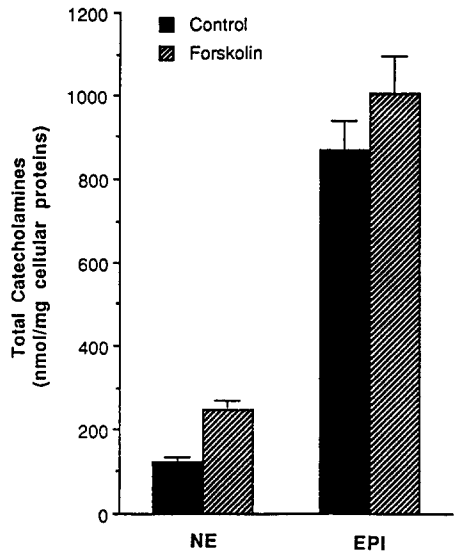

Fig. 6. Amounts of total catecholamines in bovine adrenal chromaffin cells treated with 50 μM forskolin for 48 hr. Total (intracellular plus extracellular) catecholamines were calculated from experiments done on triplicate samples for each condition, repeated on four different culture preparations. The data represent the means ± SEM of nmol NE or EPI per mg cellular proteins. Values for the forskolin-treated cells were significantly ($P < 0.01$) different from control for NE and EPI.

translational control; a similar phenomenon has been observed for TH, for instance, by dexamethasone in PC12 [3] and in transgenic mice harboring human TH [40]. Although TH has been shown to undergo short-term activation by phosphorylation [1, 2], most of the activity increase after 48 hr shown in the present study is likely due to new TH protein synthesis. Activities of DBH and PNMT, which do not undergo such activation, were also raised correspondingly to their mRNA increases. This differential induction of the three catecholamine enzymes was reflected in the synthesis of their final product, catecholamines. While both NE and EPI were elevated in their total amounts, the increase was much greater in NE than in EPI, resulting in a large shift in the molar ratio between the two catecholamines.

The effect of cAMP in secretion has been unclear due to the contradictory reports thus far. Cyclic AMP analogues have been reported to have a marginal stimulatory effect [41–43], no effect [44, 45] or an inhibitory effect [46]. In our hands, whereas forskolin had little effect over the short term (30 min), it caused a large accumulation of extracellular catecholamines in the long term (48 hr) suggesting that the cAMP pathway may be linked to the secretory process, possibly via influencing gene expression of the proteins involved. The fact that EPI is released to a degree that depletes its intracellular content to a level lower than the control suggests that the release may be an independent and active process, rather than a passive mechanism for efflux of excess catecholamines. Although one cannot eliminate the possibility that forskolin may cause an inhibition of the catecholamine reuptake system as well, recent studies showing that [Met^5]enkephalin, the neuropeptide coreleased with catecholamines from, but not taken up by, the bovine adrenal medullary cells, is also accumulated in the medium after forskolin treatment [47, 48] support the hypothesis that the catecholamine efflux observed in the present study is probably due to increased release to a large extent.

That cAMP elicits both synthesis and release of catecholamines implicates an important physiological role for the extracellular effectors whose signals are transduced via the adenylate cyclase coupled mechanism. *In vivo*, chronic exposure to the agents that elevate cAMP in adrenal chromaffin cells, such as vasoactive intestinal peptide, dopamine (via D_1 receptor) and acetylcholine (via nicotinic receptor), may change catecholamine synthesis and release patterns and thus influence the sympathetic activities. In addition, the fact that a shift in the ratio between the released NE to EPI occurs, although the latter still represents the majority, implicates a possibility that a shift in physiological responses may also occur *in vivo*. Although EPI has been thought to be the primary hormone released by the adrenal medulla and responsible for augmentation of sympathetic activities, it is possible that NE may play a rather significant role in some physiological conditions where intracellular cAMP is chronically elevated.

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REFERENCES

1. Joh TH, Park DH and Reis DJ, Direct phosphorylation of brain tyrosine hydroxylase by cAMP-dependent protein kinase: Mechanism of enzyme activation. *Proc Natl Acad Sci USA* **75**: 4744–4748, 1978.
2. Campbell D, Hardie DG and Vuillet PR, Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. *J Biol Chem* **261**: 10489–10492, 1986.
3. Kim K-T, Park DH and Joh TH, Parallel up-regulation of catecholamine biosynthetic enzymes by dexamethasone in PC12 cells. *J Neurochem* **60**: 946–951, 1993.
4. Wessel TC and Joh TH, Parallel upregulation of catecholamine biosynthetic enzymes in the rat brain and adrenal gland. *Mol Brain Res* **15**: 349–360, 1992.
5. Tischler AS, Perlman RL, Costopoulos D and Horwitz J, Vasoactive intestinal peptide increases tyrosine hydroxylase activity in normal and neoplastic chromaffin cell cultures. *Neurosci Lett* **61**: 141–146, 1985.
6. Waymire JC, Craviso GL, Lichteig K, Johnston JP, Baldwin C and Aigmond RE, Vasoactive intestinal peptide stimulates catecholamine biosynthesis in isolated adrenal chromaffin cells: Evidence for a cyclic

AMP-dependent phosphorylation and activation of tyrosine hydroxylase. *J Neurochem* **57**: 1313–1324, 1991.

7. Chern YJ, Kim KT, Slakey LL and Westhead EW, Adenosine receptors activate adenylate cyclase and enhance secretion from bovine adrenal chromaffin cells in the presence of forskolin. *J Neurochem* **50**: 1484–1493, 1988.
8. Guidotti A and Costa E, A role for nicotinic receptors in the regulation of adenylate cyclase of adrenal medulla. *J Pharmacol Exp Ther* **189**: 655–675, 1974.
9. Eiden LE, Giraud P, Dave JR, Hotchkiss AJ and Affolter H-U, Nicotinic receptor stimulation activates enkephaline release and biosynthesis in adrenal chromaffin cells. *Nature* **312**: 661–663, 1984.
10. Artalejo CR, Ariano MA, Perlman RL and Fox AP, Activation of facilitation calcium channels in chromaffin cells by D_1 dopamine receptors through a cAMP/protein kinase A-dependent mechanism. *Nature* **348**: 239–245, 1990.
11. Carroll JM, Evinger MJ, Goodman HM and Joh TH, Differential and coordinate regulation of TH and PNMT mRNAs in chromaffin cell cultures by second messenger system activation and steroid treatment. *J Mol Neurosci* **3**: 75–83, 1991.
12. Hwang O and Joh TH, Effects of cAMP, glucocorticoids and calcium on dopamine β-hydroxylase gene expression in bovine chromaffin cells. *J Mol Neurosci* **4**: 173–183, 1993.
13. Stachowiak MK, Hong JS and Viveros OH, Coordinate and differential regulation of phenylethanolamine *N*-methyltransferase, tyrosine hydroxylase, and proenkephalin mRNAs by neural and hormonal mechanisms in cultured adrenal medullary cells. *Brain Res* **510**: 277–288, 1990.
14. Wan DC and Livett BG, Induction of phenylethanolamine *N*-methyltransferase mRNA expression by glucocorticoids in cultured bovine adrenal chromaffin cells. *Eur J Pharmacol* **172**: 107–115, 1989.
15. Lewis EJ, Tank AW, Werner N and Chikaraishi DM, Regulation of tyrosine hydroxylase mRNA by glucocorticoid and cyclic AMP in a rat pheochromocytoma cell line. Isolation of a cDNA clone for tyrosine hydroxylase mRNA. *J Biol Chem* **258**: 14632–14637, 1983.
16. McMahon A and Sabban EL, Regulation of expression of dopamine β-hydroxylase in PC12 cells by glucocorticoids and cyclic AMP analogues. *J Neurochem* **59**: 2040–2047, 1992.
17. Morita K, Dohi T, Kitayama S, Koyama Y and Tsujimoto A, Enhancement of stimulation-evoked catecholamine release from cultured bovine adrenal chromaffin cells by forskolin. *J Neurochem* **48**: 243–247, 1987.
18. Wilson SP and Viveros OH, Primary culture of adrenal medullary chromaffin cells in a chemically defined medium. *Exp Cell Res* **133**: 159–169, 1981.
19. Thomas P, Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* **77**: 5201–5204, 1980.
20. Baetge EE, Suh YH and Joh TH, Complete nucleotide and deduced amino acid sequence of bovine phenylethanolamine *N*-methyltransferase: Partial amino acid homology with rat tyrosine hydroxylase. *Proc Natl Acad Sci USA* **83**: 5454–5458, 1986.
21. Abate C and Joh TH, Limited proteolysis of rat brain tyrosine hydroxylase defines an N-terminal region required for regulation of cofactor binding and directing substrate specificity. *J Mol Neurosci* **2**: 203–215, 1991.
22. Molinoff PB, Weinshilboum RW and Axelrod J, A sensitive enzymatic assay for dopamine β-hydroxylase. *J Pharmacol Exp Ther* **178**: 425–431, 1971.
23. Park DH, Monoamine methyltransferase: phenylethanolamine *N*-methyltransferase. In: *Neuromethods* (Eds. Boulton AA, Baker GB and Yu PH), Vol. 5, pp. 117–145. Humana Press, Clifton, NJ, 1986.
24. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* **72**: 248–254, 1976.
25. Lewis EJ, Harrington CA and Chikaraishi DM, Transcriptional regulation of the tyrosine hydroxylase gene by glucocorticoids and cyclic AMP. *Proc Natl Acad Sci USA* **84**: 3553–3554, 1987.
26. Coker GT III, Vinnedge L and O'Malley K, Characterization of rat and human tyrosine hydroxylase genes: Functional expression of both promoters in neuronal and non-neuronal cell types. *Biochem Biophys Res Commun* **157**: 1341–1347, 1988.
27. D'Mello SR, Turzai LM, Gioio AE and Kaplan BB, Isolation and structural characterization of the bovine tyrosine hydroxylase gene. *J Neurosci Res* **23**: 31–41, 1989.
28. Iwata N, Kobayashi K, Sasaoka T, Hidaka H and Nagatsu T, Structure of the mouse tyrosine hydroxylase gene. *Biochem Biophys Res Commun* **182**: 348–354, 1992.
29. Fauguet M and Boni C, The quail tyrosine hydroxylase gene promoter contains an active cyclic AMP-responsive element. *J Neurochem* **60**: 274–281, 1993.
30. Shaskus J, Greco D, Asnan LP and Lewis EJ, A bifunctional genetic regulatory element of the rat dopamine β-hydroxylase gene influences cell type specificity and second messenger-mediated transcription. *J Biol Chem* **267**: 18821–18830, 1992.
31. Kobayashi K, Kurosawa Y, Fujita K and Nagatsu T, Human dopamine β-hydroxylase gene: Two mRNA types having different 3′-terminal regions are produced through alternative polyadenylation. *Nucleic Acids Res* **17**: 1089–1102, 1989.
32. Fader D and Lewis EJ, Interaction of cyclic AMP and cell–cell contact in the control of tyrosine hydroxylase RNA. *Mol Brain Res* **8**: 25–29, 1990.
33. Kim KS, Lee MK, Carroll J and Joh TH, Both the basal and inducible transcription of the tyrosine hydroxylase gene are dependent upon a cAMP response element. *J Biol Chem* **268**: 15689–15695, 1993.
34. Ishiguro H, Kim KT, Joh TH and Kim KS, Neuron-specific expression of the human dopamine β-hydroxylase gene requires both the cAMP response element and a silence region. *J Biol Chem* **268**: 17987–17994, 1993.
35. Lamouroux A, Houhou I, Biguet NF, Serck-Hanssen G, Guibert B, Icard-Liepkalns C and Mallet J, Analysis of the human dopamine β-hydroxylase promoter: Transcriptional induction by cyclic AMP. *J Neurochem* **60**: 364–367, 1993.
36. Kim KS, Park DH, Wessel TC, Song B, Wagner JA and Joh TH, A dual role for the cAMP-dependent protein kinase in tyrosine hydroxylase gene expression. *Proc Natl Acad Sci USA* **90**: 3471–3475, 1993.
37. Ross ME, Evinger MJ, Hyman SE, Carroll JM, Mucke L, Comb M, Reis DJ, Joh TH and Goodman HM, Identification of a functional glucocorticoid response element in the phenylethanolamine *N*-methyltransferase promoter using fusion genes introduced into chromaffin cells in primary culture. *J Neurosci* **10**: 520–530, 1990.
38. Morita S, Kobayashi K, Hidaka H and Nagatsu T, Organization and complete nucleotide sequence of the gene encoding mouse phenylethanolamine *N*-methyltransferase. *Mol Brain Res* **13**: 313–319, 1992.
39. Sasaoka T, Kaneda N, Kurosawa Y, Fujita K and Nagatsu T, Structure of human phenylethanolamine *N*-methyltransferase gene: Existence of two types of

mRNA with different transcription initiation sites. *Neurochem Int* **15**: 555–565, 1989.
40. Kaneda N, Sasaoka T, Kobayashi K, Kiuchi K, Nagatsu I, Kurosawa Y, Fujita K, Yokoyama M, Nomura T, Katsuke M and Nagatsu T, Tissue-specific and high-level expression of the human tyrosine hydroxylase gene in transgenic mice. *Neuron* **6**: 583–594, 1991.
41. Greenberg A and Zinder O, α- and β-Receptor control of catecholamine secretion from isolated adrenal medulla cells. *Cell Tissue Res* **226**: 655–665, 1982.
42. Peach MJ, Stimulation of release of adrenal catecholamine by adenosine 3′:5′-cyclic monophosphate and theophylline in the absence of extracellular Ca^{2+}. *Proc Natl Acad Sci USA* **69**: 834–836, 1972.
43. Morita K, Dohi T, Kitayama S and Tsujimoto A, Enhancement of acetylcholine-evoked catecholamine release from perfused dog adrenals by elevating cyclic AMP levels. *Arch Int Pharmacodyn Ther* **275**: 208–216, 1985.
44. Kumakura K, Guidotti A and Costa E, Primary cultures of chromaffin cells: Molecular mechanisms for the induction of tyrosine hydroxylase mediated by 8-Br-cyclic AMP. *Mol Pharmacol* **16**: 865–876, 1979.
45. Hochman J and Perlman RL, Catecholamine secretion by isolated adrenal cells. *Biochim Biophys Acta* **421**: 168–175, 1976.
46. Baker EM, Cheek TR and Burgoyne RD, Cyclic AMP inhibits secretion from bovine adrenal chromaffin cells evoked by carbamylcholine but not by high K^+. *Biochim Biophys Acta* **846**: 388–393, 1985.
47. Suh HH, Hudson PM, McMillian MK and Hong JS, Pertussis toxin stimulates the secretion of [Met^5]-enkephalin and the expression of proenkephalin A mRNA in bovine adrenal medullary chromaffin cells. *Biol Signals* **1**: 257–265, 1992.
48. Suh HH, McMillian MK, Hudson PM, Pennypacker KR and Hong JS, Expression of the proenkephalin A gene and [Met^5]enkephalin secretion induced by prostaglandin E_2 in bovine adrenal medullary chromaffin cells: Involvement of second messengers. *Mol Cell Neurosci* **4**: 113–120, 1993.