Activation of Tyrosine Hydroxylase in PC12 Cells by the Cyclic GMP and Cyclic AMP Second Messenger Systems

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Abstract: Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, is subject to regulation by a variety of agents. Previous workers have found that cyclic AMP-dependent protein kinase and calcium-stimulated protein kinases activate tyrosine hydroxylase. We wanted to determine whether cyclic GMP might also be involved in the regulation of tyrosine hydroxylase activity. We found that treatment of rat PC12 cells with sodium nitroprusside (an activator of guanylate cyclase), 8-bromocyclic GMP, forskolin (an activator of adenylate cyclase), and 8-bromocyclic AMP all produced an increase in tyrosine hydroxylase activity measured in vitro or an increased conversion of [14C]tyrosine to labeled catecholamine in situ. Sodium nitroprusside also increased the relative synthesis of cyclic GMP in these cells. In the presence of MgATP, both cyclic GMP and cyclic AMP increased tyrosine hydroxylase activity in PC12 cell extracts. The heat-stable cyclic AMP-dependent protein kinase inhibitor failed to attenuate the activation produced in the presence of cyclic GMP. It eliminated the activation produced in the presence of cyclic AMP. Sodium nitroprusside also increased tyrosine hydroxylase activity in vitro in rat corpus striatal synaptosomes and bovine adrenal chromaffin cells. In all cases, the cyclic AMP-dependent activation of tyrosine hydroxylase was greater than that of the cyclic GMP-dependent second messenger system. These results indicate that both cyclic GMP and cyclic AMP and their cognate protein kinases activate tyrosine hydroxylase activity in PC12 cells. Key Words: Tyrosine hydroxylase—Cyclic AMP—Cyclic GMP—Sodium nitroprusside—Forskolin—PC12 cells. Roskoski R. Jr. and Roskoski L. M. Activation of tyrosine hydroxylase in PC12 cells by the cyclic GMP and cyclic AMP second messenger systems. J. Neurochem. 48, 236–242 (1987).

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in the presence of calcium, for example, results in an increase in activity when subsequently measured in vitro (Yanagihara et al., 1984). Treatment of the cells with phorbol esters, dibutyryl cyclic AMP, nerve growth factor, or epidermal growth factor enhances radiophosphate incorporation into tyrosine hydroxylase (Mctigue et al., 1985). We now report that sodium nitroprusside, an activator of guanylate cyclase (Böhme et al., 1978), produced an increase in tyrosine hydroxylase activity in the PC12 cells. Some of these results have been presented in preliminary form (Roskoski, 1986).

MATERIALS AND METHODS

Measurement of tyrosine hydroxylase in vitro

PC12 cells were grown in culture, harvested by trituration, and incubated at 30°C in N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-Ringer solution as described by Baizer and Weiner (1985). In those experiments where tyrosine hydroxylase was measured in vitro following specific treatments in situ, aliquots (38 μl) of PC12 cell suspensions (about 2 mg protein/ml) were dispensed following gentle vortex-mixing into 1.5-ml polyethylene centrifuge tubes. Specific additions were made (2-μl portions) to give the final specified concentration and the incubation was continued for 20 min at 30°C. Then 1 ml of ice-cold HEPES-Ringer solution was added and the cells were harvested by centrifugation (13,000 g; 30 s). The supernatants were aspirated and cells were stored at −70°C (for up to 1 month) or taken for tyrosine hydroxylase activity measurements. After 100 μl of a chilled solution of potassium phosphate (25 mM), NaF (50 mM), EDTA (0.25 mM), and Triton X-100 (0.2%, vol/vol) was added, the cells were disrupted by sonication for 10 s with a Kontes Micro-Ultrasonic Cell Disrupter. The pH of this buffer was the same as that used in the assay. An equal volume of Dowex-50 resin in the same buffer was added to adsorb endogenous catecholamines. Following gentle vortex-mixing, the suspension was briefly centrifuged at 13,000 g and 10-μl portions of the supernatant were taken for tyrosine hydroxylase activity determination as previously described by Vrana et al. (1981). Incubations were performed at 30°C with 0.125 mM 6-methyltetrahydropterin (6-MPH₄) for the time specified. Piperazine-N,N'-bis(2-ethanesulfonic acid) (25 mM) was the buffer used at pH 6.0 and 3-(N-morpholino)propanesulfonic acid (MOPS) (25 mM) was used for those assays performed at pH 7.2. A 5 mM stock solution of 6-MPH₄ was freshly prepared in ice-cold 5 mM HCl; the concentration was determined spectrophotometrically in 100 mM HCl (ambient temperature) at 265 nm using an extinction of 14.7 mM⁻¹.

Measurement of catecholamine biosynthesis in situ

 Portions (18 μl) of PC12 cell suspensions (about 2 mg protein/ml) in HEPES-Ringer's solution containing 25 μM paraglyline were dispensed in 1.5-ml polyethylene tubes. Twenty minutes after 1-μl aliquots of the specified addition were made, a 1-μl portion of 400 μM uniformly labeled [¹⁴C]tyrosine was added followed by gentle vortex-mixing. Following the incubations at 30°C, 10 μl of ice-cold stop solution containing 100 mM formic acid, 1.5 mM 3,4-dihydroxyphenylalanine (DOPA), and 3 mM dopamine was added. The cells were subjected to two freeze/thaw cycles (−20°C; +30°C) and 10-μl portions were taken for low-voltage paper electrophoresis as previously described by Vrana et al. (1981). After drying at 110°C, the zones containing comigrating dopamine/norepinephrine and tyrosine/DOPA were visualized by UV light. The catecholamine and standard [¹⁴C]tyrosine zones were excised and radioactivity was measured by liquid scintillation spectrometry. Blank values of catecholamine formation were determined by incubating the cells at 0°C on ice. Blank radioactivity values were about 70 cpm in the catecholamine zone and were resolved from 80,000 cpm of radioactivity in the tyrosine zone. Most experimental values for catecholamine formation were 1,000 cpm or greater. Protein was measured by the procedure of Bradford (1976).

RESULTS

Sodium nitroprusside pretreatment of PC12 cells activates tyrosine hydroxylase activity in vitro

Erny and Wagner (1984) reported that rat PC12 cells contain an adenosine receptor that activates adenylyl cyclase. They included adenosine deaminase in their incubation medium to minimize the effects of endogenous adenosine. We obtained similar results and also examined the tyrosine hydroxylase activity in vitro in response to pretreatment of intact cells with theophylline. This compound is a potent adenosine receptor antagonist with a Kᵦ in the micromolar concentration range (Snyder, 1985). We found that 0.1 mM theophylline also produced a significant 40% decrease in basal tyrosine hydroxylase activity measured in vitro (not shown). On the basis of these results, we included either adenosine deaminase or theophylline as specified with the PC12 cells in HEPES-Ringer's solution during preincubation with the other agents.

To test for the potential involvement of the cyclic GMP second messenger system in regulating tyrosine hydroxylase activity in the physiological situation, PC12 cells were treated with sodium nitroprusside. This agent activates guanylate cyclase activity and enhances cyclic GMP biosynthesis (Böhme et al., 1978). Following a 20-min preincubation, sodium nitroprusside enhanced the activity of tyrosine hydroxylase measured in vitro (Fig. 1). The maximal response was seen at concentrations of 0.1–1 mM; a 1 μM concentration, however, produced a statistically significant increase in tyrosine hydroxylase activity.

Because the cyclic GMP second messenger system has not been previously characterized in the PC12 cell line, additional experiments were performed to determine its properties more fully. We first examined the
Activation of tyrosine hydroxylase by cyclic GMP-dependent phosphorylation in vitro

Cell-free extracts of PC12 cells were next used to determine whether cyclic GMP-dependent protein kinase phosphorylation mediated by its endogenous protein kinase would alter tyrosine hydroxylase activity. Cyclic AMP and cyclic GMP exhibit greater affinity for their cognate enzyme, but will activate the alternative enzyme when present at a higher concentration (Nestler and Greenberg, 1984). We used the heat stable cyclic AMP-dependent protein kinase inhibitor to help in determining the identity of endogenous protein kinases which mediate the activation of tyrosine hydroxylase. This inhibitor is specific for cyclic AMP-dependent protein kinase and lacks an inhibitory effect with the cyclic GMP-dependent protein kinase (Traugh et al., 1974).

Cyclic GMP-dependent phosphorylation conditions produced a 41% increase in tyrosine hydroxylase activity (Table 2). This increase was unaffected by the cyclic AMP-dependent protein kinase inhibitor. Cyclic AMP-dependent phosphorylation conditions

| TABLE 1. Effects of various agents on relative cyclic nucleotide synthesis in PC12 cells |
|-----------------|-----------------|-----------------|
|                  | [3H]Cyclic GMP | [3H]Cyclic AMP  |
| Control          | 625 ± 31        | 1,880 ± 94      |
| Sodium nitroprusside, 1 mM | 2,940 ± 38*    | 1,930 ± 84      |
| Forskolin, 0.5 μM     | 606 ± 43        | 10,200 ± 141*   |
| Methacholine, 50 μM     | 640 ± 49        | 1,770 ± 87      |

Cells were cultured, harvested, and incubated in Ringer's solution as described in Materials and Methods. The prelabeling procedure of Keaban et al. (1972) was used (40 μM labeled base, 45 min, 30°C). The cells were harvested by centrifugation at 3,000 g for 1 min at ambient temperature, resuspended in Ringer's solution, and pelleted again. The cells were suspended in Ringer's solution and incubated with the designated component for 20 min at 30°C in the presence of 0.1 mM theophylline. After the cells were centrifuged for 20 s at 13,000 g, the supernatants were aspirated and 20-μl portions of 25 mM H3PO4 (pH 3.5) containing 3 mM each of 5′AMP, cyclic AMP, and adenine or 5′GMP and cyclic GMP were used for cyclic AMP and cyclic GMP determinations, respectively. The cells were lysed by three freeze/thaw cycles and portions were applied to polyethylenimine plates with fluorescent indicator. Chromatography was performed with 25 mM H3PO4 (pH 3.5) containing 100 mM NaCl. The cyclic nucleotides were well resolved from the nucleoside phosphates, the purines, and purine ribosides by this procedure. After UV visualization, the marker zones were cut out, dark-adapted for 48 h, and then radioactivity was measured by liquid scintillation spectrometry. The data represent the means ± SEM of triplicates. Similar results were obtained in three other independent experiments.

*Significantly different from the corresponding control (p < 0.001).

| TABLE 2. Activation of tyrosine hydroxylase by phosphorylation in vitro |
|-----------------|-----------------|-----------------|
|                  | Activity (pmol/min/mg) |
| None                  | 244 ± 21         |
| ATP                  | 264 ± 15         |
| ATP + cyclic AMP     | 341 ± 22         |
| ATP + cyclic AMP + heat-stable inhibitor | 330 ± 23         |
| ATP + cyclic AMP + heat-stable inhibitor | 449 ± 23         |
| Heat-stable inhibitor | 257 ± 14         |
| Cyclic AMP          | 233 ± 12         |
| Cyclic AMP          | 244 ± 18         |
| ATP (minus Mg2⁺)    | 237 ± 17         |

PC12 cells were harvested, incubated in Ringer's solution containing 0.10 mM theophylline for 20 min at 30°C, and disrupted by sonication (0°C). Then 15-μl portions containing about 5 mg/ml protein were incubated in 30 μl of a final solution containing 25 mM MOPS (pH 7.2), 10 mM MgCl2, 0.1 mM EDTA, and 0.1 mM EGTA for 10 min at 30°C. This solution contained the following components where indicated: 0.1 mM ATP, 2 μM cyclic AMP, 2 μM cyclic AMP, and 50 μg of cyclic AMP-dependent protein kinase inhibitor. The means ± SEM of triplicate determinations are given. Similar results were obtained in four other experiments. Significantly different from the control: *p < 0.05; †p < 0.01.
Role of cyclic nucleotides in altering tyrosine hydroxylase activity in vitro and catecholamine biosynthesis in situ

To gain additional information on the role of cyclic nucleotides in regulating catecholamine biosynthesis, the effect of several agents on this process was examined. 8-Bromocyclic GMP, 8-bromocyclic AMP, and dibutyryl cyclic AMP pretreatment produced an increase in tyrosine hydroxylase activity measured in vitro (Table 3). Sodium nitrite, a less effective activator of guanylate cyclase than sodium nitroprusside (Böhmé et al., 1978), also increased tyrosine hydroxylase activity. Pretreatment of cells with dibutyryl cyclic GMP, cyclic GMP, or cyclic AMP, however, failed to alter tyrosine hydroxylase activity. The inability of the first agent to alter tyrosine hydroxylase activity is most likely related to its relatively poor capacity to activate cyclic GMP-dependent protein kinase (Lincoln and Corbin, 1983). The inability of the underivatized cyclic AMP or cyclic GMP to activate tyrosine hydroxylase may be due to their relatively poor permeation through the PC12 cell plasma membrane or to their rapid hydrolysis by phosphodiesterases. This result also indicates that the activation of tyrosine hydroxylase observed requires the intact cell and does not occur following cellular disruption.

An optimal concentration of forskolin, an activator of adenylyl cyclase, produced a greater increase in tyrosine hydroxylase activity than did optimal concentrations of sodium nitroprusside. The derivatives of cyclic AMP also produced a greater degree of activation of tyrosine hydroxylase than 8-bromocyclic GMP (Table 3).

In addition to measuring tyrosine hydroxylase activity in vitro, we examined the effect of these agents on catecholamine biosynthesis in situ. After pretreatment of PC12 cells in a specific fashion for 20 min, uniformly labeled [14C]tyrosine was added to give a final concentration of 20 μM. After transport, dopamine and portions were taken for paper electrophoresis to resolve dopamine/norepinephrine from labeled precursor as described in Materials and Methods. The points represent the means of triplicate determinations; the SEM was 7% or less of the mean. Similar results were obtained in three independent experiments. (C), 10 μM forskolin; (D), 0.10 mM sodium nitroprusside; (E), control; (V), 0.10 mM idoyltyrosine.

![Graph](image-url)

**FIG. 2.** Time course of catecholamine biosynthesis in situ. PC12 cells were harvested and preincubated in Ringer's solution containing 0.10 mM theophylline and the specified agent for 20 min. Then [14C]tyrosine was added to give a final concentration of 20 μM. The reaction was quenched with formic acid containing carrier dopamine and portions were taken for paper electrophoresis to resolve dopamine/norepinephrine from labeled precursor as described in Materials and Methods. The points represent the means of triplicate determinations; the SEM was 7% or less of the mean. Similar results were obtained in three independent experiments. (C), 10 μM forskolin; (D), 0.10 mM sodium nitroprusside; (E), control; (V), 0.10 mM idoyltyrosine.

The results give the means ± SEM of triplicate determinations. Similar results were obtained in four other experiments.

The specific activity does not take into account endogenous, unlabeled tyrosine.

Significantly different from the corresponding control (p < 0.01).

**TABLE 3.** Effect of treatment of PC12 cells with various agents on tyrosine hydroxylase activity in vitro and catecholamine biosynthesis in situ

<table>
<thead>
<tr>
<th>Activity (pmol/min/mg)</th>
<th>In vitro</th>
<th>In situ</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>223 ± 17</td>
<td>2.42 ± 0.12</td>
</tr>
<tr>
<td>8-Bromocyclic GMP, 1 mM</td>
<td>363 ± 211</td>
<td>4.11 ± 0.15</td>
</tr>
<tr>
<td>8-Bromocyclic AMP, 1 mM</td>
<td>452 ± 246</td>
<td>7.56 ± 0.21</td>
</tr>
<tr>
<td>Dibutyryl cyclic GMP, 1 mM</td>
<td>217 ± 14</td>
<td>2.19 ± 0.15</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP, 1 mM</td>
<td>471 ± 183</td>
<td>7.42 ± 0.24</td>
</tr>
<tr>
<td>Sodium nitroprusside, 100 μM</td>
<td>397 ± 240</td>
<td>4.60 ± 0.21</td>
</tr>
<tr>
<td>Sodium nitrite, 1 mM</td>
<td>318 ± 175</td>
<td>4.04 ± 0.18</td>
</tr>
<tr>
<td>Forskolin, 10 μM</td>
<td>482 ± 152</td>
<td>7.72 ± 0.29</td>
</tr>
<tr>
<td>Cyclic GMP, 1 mM</td>
<td>212 ± 19</td>
<td>2.49 ± 0.18</td>
</tr>
<tr>
<td>Cyclic AMP, 1 mM</td>
<td>231 ± 14</td>
<td>2.55 ± 0.14</td>
</tr>
</tbody>
</table>

PC12 cells were harvested and incubated in Ringer's solution containing 0.10 mM theophylline for 20 min with the specified concentration of each agent, collected, disrupted by sonication, and taken for activity determinations (pH 7.2, 0.125 mM 6-MPH₄, 8 min, 30°C).

PC12 cells were harvested and incubated with the specified concentration of each agent for 20 min. Then 1/20th volume of 400 μM [14C]tyrosine (yielding a final concentration of 20 μM) was added. After 15 min, formic acid with carrier dopamine was added and samples were processed as described in the legend to Fig. 2. The specific activity does not take into account endogenous, unlabeled tyrosine.

Significantly different from the corresponding control (p < 0.01).

Produced a 80% increase in tyrosine hydroxylase activity. This increase, on the other hand, was attenuated by the cyclic AMP-dependent protein kinase inhibitor (Table 2). These results provide evidence for the existence of both cyclic GMP- and cyclic AMP-dependent protein kinases in PC12 cells. They also suggest that the cyclic GMP-dependent protein kinase activates tyrosine hydroxylase activity. This has also been demonstrated with purified tyrosine hydroxylase and purified cyclic nucleotide-dependent protein kinases (Roskoski, 1986).
dase activity and a resulting decrease in degradation of radiolabeled catecholamines. The relative increase was greater in the presence of stimulatory agents. These experiments provide a measure of the validity of this approach in studying catecholamine biosynthesis in situ.

Using this methodology, we found a good correlation of the relative efficacy for cyclic nucleotides in augmenting catecholamine biosynthesis in situ and tyrosine hydroxylase in vitro (Table 3). The percentage increase in situ was somewhat greater than that observed in vitro. The greater apparent activity observed in vitro when compared with that in situ is probably a result of several phenomena. First, radioactive tyrosine must be transported into the cell before hydroxylation occurs. Second, endogenous tyrosine in the PC12 cells decreases the specific activity of radioactive tyrosine. Third, the concentration and relative effect of the reducing cosubstrate in vitro (0.125 mM) is probably much greater than that of the natural cosubstrate in situ (Kaufman and Kaufman, 1985).

**Sodium nitroprusside stimulates tyrosine hydroxylase activity in rat corpus striatal synaptosomes and in bovine adrenal chromaffin cells**

Although guanylate cyclase and cyclic GMP-dependent protein kinase occur in essentially all tissues in which they have been sought (Lincoln and Corbin, 1983), their activities may not be sufficient to activate tyrosine hydroxylase in all cell types. We therefore examined the effect of sodium nitroprusside pretreatment on tyrosine hydroxylase activity in vitro in rat corpus striatal synaptosomes. We found that it produced a 50% increase in enzyme activity (Table 4). Forskolin and dibutyryl cyclic AMP produced a 130–140% increase in enzyme activity in this system. Sodium nitroprusside pretreatment also produced a 75% increase in enzyme activity in bovine adrenal chromaffin cells (Table 5). Forskolin and dibutyryl cyclic AMP were more potent activators of tyrosine hydroxylase activity in both systems.

**DISCUSSION**

PC12 cells contain an adenosine receptor that stimulates cyclic AMP biosynthesis (Erny and Wagner, 1984). This leads to an increase in tyrosine hydroxylase activity and an increase of DOPA formation in situ. In addition to adenosine deaminase (Erny and Wagner, 1984), we find that theophylline also decreases basal tyrosine hydroxylase activity. Convenience, stability, and cost favor the use of theophylline in the absence of other considerations.

The main finding in this report is that PC12 cells synthesize cyclic GMP and that this leads to an increase in catecholamine formation in situ and to an increase in tyrosine hydroxylase activity measured in vitro. Sodium nitroprusside increases relative cyclic GMP formation and cyclic GMP content (as determined by radioimmunoassay). Sodium nitrite, a less potent activator of guanylate cyclase, increases tyrosine hydroxylase activity in PC12 cells. 8-Bromocyclic GMP treatment of PC12 cells also increases tyrosine hydroxylase activity. The lack of a stimulatory effect by dibutyryl cyclic GMP is most likely related to its poor efficacy in activating cyclic GMP-dependent protein kinase (Lincoln and Corbin, 1983). The relative activity of cyclic GMP-dependent protein kinase in most mammalian tissues is 5–10% that of cyclic AMP-dependent protein kinase (Nestler and Green-gard, 1984). This is probably the explanation for the finding that tyrosine hydroxylase activity is increased to a greater extent by agents that activate the cyclic AMP second messenger system when compared to activation of the cyclic GMP system.

There are at least two possible mechanisms by which cyclic GMP could activate tyrosine hydroxylase through protein phosphorylation. The traditional...
view is that cyclic GMP activates its cognate protein kinase which in turn would bring about protein phosphorylation and activate tyrosine hydroxylase. A second possibility is that the elevated cyclic GMP activates cyclic AMP-dependent protein kinase. That the former mechanism pertains in the PC12 cells is best shown by the effects of the heat stable cyclic AMP-dependent protein kinase inhibitor. It failed to block tyrosine hydroxylase activation in the presence of cyclic GMP, but it was effective in the presence of cyclic AMP. The present experiments do not demonstrate that cyclic GMP-dependent protein kinase activates tyrosine hydroxylase activity by direct phosphorylation. This has been shown, however, through the use of purified rat pheochromocytoma tyrosine hydroxylase and purified bovine lung cyclic GMP-dependent protein kinase (Roskoski and Wilgus, 1985; Roskoski, 1986).

The activation of adenylyl cyclase by a large number of hormones and neurotransmitters is well documented (Nestler and Greengard, 1984; Ip et al., 1985). In contrast, our knowledge of the identity and mechanism of activation of guanylate cyclase by bioactive agents is meager (Nestler and Greengard, 1984). An increase in cyclic GMP mediated by muscarinic receptor activation by acetylcholine is probably the best example of a receptor-mediated effect on this second messenger system (George et al., 1973). Although PC12 cells contain a cholinergic muscarinic receptor (Cross et al., 1984), its activation does not increase cyclic GMP formation in this cell line (Table 1).

Our results indicate that cyclic GMP and cyclic AMP are acting in parallel to activate tyrosine hydroxylase activity. This is in contrast to the hypothesis that these two cyclic nucleotides mediate opposite biological effects (Goldberg et al., 1973). Tyrosine hydroxylase is an enzyme subject to regulation by cyclic nucleotide as well as calcium second messengers. The degree and extent that each second messenger may play in regulating tyrosine hydroxylase activity in the physiological situation has introduced an unforeseen complexity in studying this process.

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REFERENCES


