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The Role of Cyclic Adenosine 3':5'-Monophosphate in the Regulation of Tyrosine 3-monooxygenase Activity

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SUMMARY

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The activity of tyrosine 3-monooxygenase (TH) was measured in extracts of cell suspensions prepared from a rat pheochromocytoma. Incubation of the cells with cholera toxin (0.1-1 μg/ml) or dibutyryl cAMP (1 mM) leads to an increase in TH activity in the cells. The effects of these agents are not dependent upon the presence of extracellular Ca^{2+}, and are not blocked by diphenylhydantoin, an inhibitor of Ca^{2+} uptake into the cells. The activation of TH by cholera toxin occurs after a lag period of 10-15 min, and is associated with a large rise in cAMP levels in the cells. Cholera toxin and dibutyryl cAMP increase the rate of catecholamine biosynthesis in these cells, but have no effect on the secretion of catecholamine from the cells. Incubation of the cells in media containing 56 mM K^+ also leads to the activation of TH. However, this treatment does not result in a rise in cAMP levels, nor does it increase the rate of formation of [^{3}H]cAMP from [^{3}H]adenine in these cells. These results are consistent with the hypothesis that TH can be regulated by two distinct pathways, one of which involves cAMP, and the other of which is independent of the nucleotide.

INTRODUCTION

A growing body of evidence suggests that cAMP[3] may play a role in the regulation of catecholamine biosynthesis. Dibutyryl cAMP increases catecholamine synthesis in brain slices (1) and in synaptosomes (2), and cAMP activates tyrosine 3-monooxygenase (E.C. 1.14.16.2, TH) *in vitro* (3-7). We have been studying the regulation of catecholamine biosynthesis in cell suspensions prepared from a transplantable rat pheochromocytoma (8). Incubation of these cells in media containing 56 mM K^+ produces an activation of TH, and an increase in catecholamine synthesis. We have now investigated the effects of cholera toxin and of dibutyryl cAMP on TH activity and catecholamine biosynthesis in these cells. These studies provide the first direct correlation between intracellular cAMP levels and TH activity.

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[3] The abbreviations used are: cAMP, cyclic adenosine 3':5'-monophosphate; TH, tyrosine 3-monooxygenase; KRPG, Krebs-Ringer phosphate glucose buffer; $DMPH_4$, 6,7-dimethyltetrahydropterin.

MATERIALS AND METHODS

Cell suspensions were prepared by mechanical disruption of the pheochromocy-

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toma (9), and were pre-incubated for one 30-min period at 37° in KRPG, under an atmosphere of 100% O_2. The cells were collected by centrifugation at 800 × *g* for 40 sec at room temperature, and were resuspended in fresh medium. Aliquots of the cell suspensions were then incubated with the various test agents as described in the text. Following incubation, the cells were chilled, collected by centrifugation, and lysed by freeze-thawing in a dry ice-acetone bath, as previously described (10). Catecholamines were removed by passage of the extracts over Dowex 50 columns (10), and TH activity in the catecholamine-free extracts was assayed by measuring the formation of ^3H_2O from L-[3,5-^{3}H]tyrosine, according to a modification (10) of the method of Shiman *et al.* (11). TH assays were routinely carried out for 15 min at 30° in 0.1 M sodium phosphate, pH 6.8, in the presence of 10 μM L-[3,5-^{3}H]tyrosine and 300 μM 6,7-dimethyltetrahydropterin ($DMPH_4$). Reduced pteridine cofactor was regenerated by dihydropteridine reductase and NADPH (0.5 mM); the dihydropteridine reductase and NADPH are both present in excess. The apparent K_m of TH for $DMPH_4$ was determined in experiments in which the $DMPH_4$ concentration was varied between 50 and 1000 μM; the apparent K_m of the enzyme for tyrosine was determined in experiments in which the tyrosine concentration was varied between 5 and 100 μM, and in which $DMPH_4$ was replaced by tetrahydrobiopterin (300 μM). TH activity was constant for at least 15 min under all of these conditions. The ^3H_2O produced in the TH reaction was collected by passing the samples over Dowex 50 columns, as described by Nagatsu *et al.* (12). TH activity was estimated after subtraction of the radioactivity found in control, enzyme-free incubations, and is expressed as pmole tyrosine converted to dopa/min/mg protein, mean ± SEM of experiments performed in triplicate. Apparent K_ms were estimated by a least-squares analysis of 1/v versus 1/s. Protein was measured by the method of Lowry *et al.* (13), using bovine serum albumin as a standard.

Catecholamine synthesis was estimated by measuring the incorporation of [^{14}C]tyrosine into dopamine and norepinephrine, as previously described (8). In these experiments, cells were pre-incubated for two 30-min periods at 37° in KRPG. During the first 30-min period, this buffer contained 10 μM pargyline; this treatment results in an almost complete inhibition of amine oxidase (flavin containing) (E.C. 1.4.3.4). When the effect of cholera toxin on catecholamine synthesis was studied, this agent was present during the second pre-incubation period, as well as during the subsequent incubation period. Aliquots of the cell suspensions were then incubated for 30 min at 37° in KRPG (or 56 mM K^+-KRPG) containing 10 mM Na ascorbate, 50 μM L-[^{14}C]tyrosine (33 μCi/μmol), and cholera toxin or dibutyryl cAMP, as indicated. Following incubation, the cells were separated from the medium by centrifugation through a layer of silicone oil, catecholamines were separated by high-voltage electrophoresis, and the radioactivity incorporated into dopamine and norepinephrine was determined (8).

Catecholamine secretion was measured as previously described (9).

cAMP levels in the cells were measured by the competitive protein binding assay of Brown *et al.* (14), using a cAMP binding protein isolated from bovine adrenal cortex. The binding-inhibitory activity in the cells diluted out as did authentic cAMP, and was completely destroyed by treatment of the extracts for 30 min with purified cAMP phosphodiesterase. cAMP formation was estimated in cells that had been pre-incubated with [^{3}H]adenine, according to a modification of the method of Kebabian *et al.* (15). In these experiments, cells were pre-incubated for 45 min in KRPG containing 10 μM [2-^{3}H]adenine, 200 μCi/μmol. The cells were then centrifuged, washed twice with KRPG, and resuspended in fresh KRPG for the experimental incubations. Following these incubations, 50 μl of 10 mM cAMP was added to each sample, the cells were placed in a boiling water bath for 10 min, and were then centrifuged for 10 min at 10,000 × *g*. One-half milliliter aliquots of the supernatant fractions were applied to 0.5 ml columns of Dowex 50-X4, 200–400 mesh, H^+ form, and [^{3}H]cAMP was isolated

by sequential chromatography on Dowex 50 and alumina columns, according to the method of Salomon *et al.* (16). Estimates of cAMP formation are corrected for the recovery of cAMP in the isolation procedure, which ranged between 50 and 60%.

KRPG contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 16 mM sodium phosphate, pH 7.4, and 10 mM glucose; 56 mM K^+-KRPG was prepared by substituting the appropriate amount of KCl for NaCl. Ca^{2+}-free buffers were prepared by omitting $CaCl_2$, and adding 0.1 mM ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid. All buffers were equilibriated with 100% O_2 before use. L-[^{14}C]tyrosine (uniformly labeled), L-[3,5-^{3}H]tyrosine, and [2-^{3}H]adenine were obtained from the New England Nuclear Corp., Boston, Massachusetts. The L-[3,5-^{3}H]tyrosine was purified by the method of Creveling and Daly (17) before use. Sheep liver dihydropteridine reductase was a generous gift from Drs. S. Kaufman and S. Milstien, National Institutes of Health, Bethesda, Maryland. Cholera enterotoxin, a product of Schwarz Mann, Orangeburg, New York, was kindly provided by Dr. M. Field, Beth Israel Hospital, Boston, Massachusetts. Tetrahydrobiopterin was a gift from Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, New Jersey. Glass distilled water was used throughout.

RESULTS

We have previously shown that incubation of pheochromocytoma cells in media containing 56 mM K^+ results in an activation of TH. This effect of 56 mM K^+ is dependent upon the presence of extracellular Ca^{2+}, and is blocked by diphenylhydantoin, which inhibits the stimulus-coupled uptake of Ca^{2+} into the cells (10). Cholera toxin and dibutyryl cAMP also activate TH in these cells (Table 1). These agents, like 56 mM K^+, produce approximately a two-fold increase in TH activity. However, the effects of cholera toxin and dibutyryl cAMP are not dependent upon extracellular Ca^{2+}, and are not inhibited by diphenylhydantoin. In this experiment, cholera toxin was used at a concentration of 1 μg/ml, and dibutyryl cAMP was used at a concentration of 1 mM. The toxin produces a maximal effect at a concentration of 100 ng/ml, and causes a partial activation of TH at 10 ng/ml; dibutyryl cAMP at a concentration of 0.1 mM does not activate TH (not shown). Table 1 reports the TH activity in cells that had been incubated under control conditions for 10 min. TH activity in cells incubated under these conditions remains constant for at least 60 min.

TABLE 1

Activation of tyrosine 3-monooxygenase by cholera toxin and dibutyryl cAMP

Pheochromocytoma cells were incubated at 37° in Krebs-Ringer phosphate glucose buffer, containing the additions and modifications indicated. Cells were incubated for 10 min in control medium, in medium containing 56 mM K^+, and in the presence of dibutyryl cAMP; cells were incubated for 30 min with cholera toxin. Following incubation, the cells were collected by centrifugation, and TH activity was measured as described. Data are presented as mean ± SEM of triplicate determinations.

Incubation conditions	Tyrosine 3-monooxygenase activity		
	1.25 mM Ca^{2+}	0 Ca^{2+} 0.1 mM EGTA[a]	1.25 mM Ca^{2+} 0.1 mM DPH[b]
	pmole/min/mg protein		
Control	13.6 ± 0.3	12.1 ± 2.0	15.9 ± 0.8
56 mM K^+	30.4 ± 2.1	13.6 ± 0.5	17.8 ± 1.0
Cholera toxin (1 μg/ml)	30.0 ± 0.5	29.9 ± 1.1	32.6 ± 0.8
Dibutyryl cAMP (1 mM)	23.0 ± 0.6	25.9 ± 0.2	23.4 ± 1.6

[a] Ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetraacetic acid.

[b] Diphenylhydantoin.

There is a lag period of about 10–15 min between exposure of the cells to cholera toxin and the activation of TH (Fig. 1). The enzyme is rapidly activated after this lag period, and appears to be fully activated within about 20 min after addition of the toxin. Cholera toxin also causes a large increase in cAMP levels in the pheochromocytoma cells (Fig. 1). In this experiment, cAMP levels in control cells were approximately 80 pmole/mg protein, and cholera toxin produced a six-fold increase in these levels. The activation of TH is closely correlated with this rise in cAMP levels.

The activation of TH by 56 mM K^+ is

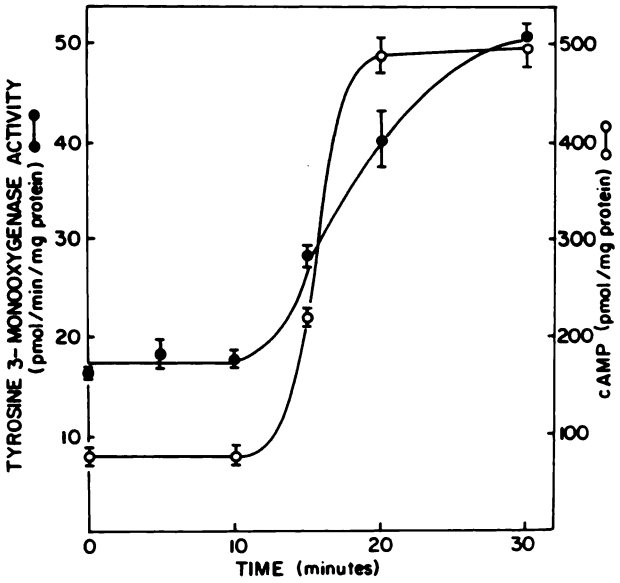

FIG. 1. *Effect of cholera toxin on tyrosine 3-monooxygenase activity and cAMP levels in pheochromocytoma cells*

Cells were incubated at 37° in KRPG containing 1 μg/ml cholera toxin. At the times indicated, samples were removed for TH assay (●), and for measurement of cAMP levels (○). TH activity is expressed as pmole tyrosine converted to dopa/min/mg protein, mean ± SEM of triplicate determinations. cAMP levels are expressed as pmole/mg protein, mean ± SEM of triplicate determinations.

characterized by an increase in the V_{max} of enzyme activity, and not by changes in the apparent K_ms of the enzyme for its pteridine cofactor or for tyrosine (18). It was of interest to determine the effects of cholera toxin on the kinetic properties of TH. Under our assay conditions, TH activity in extracts of control cells has an apparent K_m for tyrosine of 26 ± 3 μM (n = 4), and an apparent K_m for $DMPH_4$ in the range of 140–200 μM (n = 4). Treatment of the cells with 56 mM K^+ or cholera toxin causes an increase in the V_{max} of TH, but neither treatment results in a significant change in the apparent K_m of the enzyme for tyrosine or for $DMPH_4$. Figure 2 presents the results of a typical estimation of the apparent K_m of TH for $DMPH_4$. In this experiment, the apparent K_m of the enzyme for $DMPH_4$ was 117 μM in extracts from control cells, 161 μM in 56 mM K^+-treated cells, and 159 μM cholera toxin-treated cells. It appears that cholera toxin and 56 mM K^+ produce similar changes in the kinetic properties of TH. Attempts to determine the apparent K_m of TH for tetrahydrobiopterin were frustrated

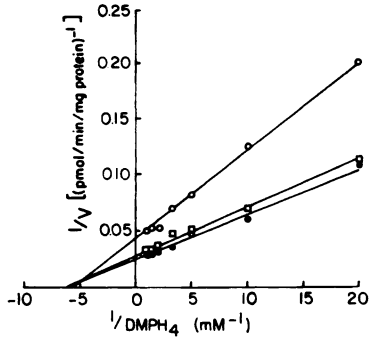

FIG. 2. *Apparent K_m of tyrosine 3-monooxygenase for $DMPH_4$*

Cells were incubated for 30 min at 37° in KRPG (○), 56 mM K^+-KRPG (□), or KRPG containing 1 μg/ml cholera toxin (●). Following incubation, the cells were chilled and collected by centrifugation, and the apparent K_m of TH for $DMPH_4$ was determined. The values shown are for one representative experiment; three other experiments produced comparable results.

because graphs of 1/v versus 1/s were not linear for this cofactor. Numata (Sudo) *et al.* have reported that TH from bovine adrenal medulla exhibits two apparent K_ms

for tetrahydrobiopterin (19). Although our data are in general agreement with the results of these workers, we were unable to determine reproducible apparent K_ms of TH for tetrahydrobiopterin.

Because cholera toxin and dibutyryl cAMP activate TH, it was reasonable to suspect that these agents might also stimulate catecholamine synthesis in pheochromocytoma cells. As shown in Table 2, both cholera toxin and dibutyryl cAMP, like 56 mM K^+, do increase the rate of catecholamine synthesis in these cells. The increase in catecholamine synthesis produced by these treatments is approximately two-fold, and is thus comparable in magnitude to the activation of TH that they produce. Under all incubation conditions, the accumulation of [^{14}C]norepinephrine is greater than the accumulation of [^{14}C]dopamine. Treatment of the cells with cholera toxin or with dibutyryl cAMP has no effect on the uptake of tyrosine into the cells, or on the specific activity of the intracellular tyrosine pool. As previously reported, however, the specific activity of the [^{14}C]tyrosine pool in K^+-stimulated cells is about ½ that found in cells incubated under control conditions (8).

TABLE 2

Stimulation of catecholamine synthesis by cholera toxin and dibutyryl cAMP

Pheochromocytoma cells were incubated for 30 min at 37° in Krebs-Ringer phosphate glucose buffer containing 50 μM L-[^{14}C]tyrosine (33 μCi/μmol), 10 mM Na ascorbate, and the other modifications indicated. Following incubation, the cells were collected by centrifugation through silicone oil, and the radiaoctivity incorporated into dopamine and norepinephrine was determined. Calculations of dopamine and norepinephrine synthesis represent the sum of the radioactivity in the cells and in the medium. Data are presented as mean ± SEM of triplicate determinations.

Incubation conditions	Product accumulated		
	Dopamine	Norepinephrine	Total catecholamine
	dpm/min/mg protein		
Control	356 ± 7	581 ± 15	937
56 mM K^+	603 ± 7	1031 ± 7	1634
Cholera toxin (1 μg/ml)	624 ± 44	1024 ± 44	1648
Dibutyryl cAMP (1 mM)	726 ± 73	987 ± 29	1713

The effects of cholera toxin and dibutyryl cAMP on catecholamine secretion were also determined (Table 3). Under the conditions of our experiments, neither cholera toxin nor dibutyryl cAMP stimulate catecholamine secretion from the pheochromocytoma cells. In this respect, the effects of these agents differ from the effects of 56 mM K^+ on the cells.

Because TH can be activated by cholera toxin and by dibutyryl cAMP, it was important to determine whether the activation of the enzyme by 56 mM K^+ was associated with a rise in cAMP levels in the cells. Figure 3 shows the time course of the effect of 56 mM K^+ on TH activity and on cAMP levels in pheochromocytoma cells. Enzyme activity increases without a detectable lag period, and the enzyme appears to be fully activated within 3–5 min after exposure of the cells to 56 mM K^+. However, as shown in Fig. 3, 56 mM K^+ does not cause a measurable rise in cAMP levels in the cells.

Because of the high cAMP levels in the pheochromocytoma cells, we were concerned that we might not be able to detect small increments in these levels. We therefore decided to measure the effects of 56 mM K^+ and cholera toxin on the synthesis of [^{3}H]cAMP in cells that had been preincubated with [^{3}H]adenine. As shown in Table 4, treatment of the cells with cholera

TABLE 3

Effect of cholera toxin and dibutyryl cAMP on catecholamine secretion

Pheochromocytoma cells were incubated for 30 min at 37° in Krebs-Ringer phosphate glucose buffer, containing the modifications indicated. Following incubation, the cells were removed by centrifugation, and the catecholamine content of the incubation medium was determined. Catecholamine secretion was calculated after subtraction of the small amount of catecholamine found in the medium of cell suspensions that had been maintained at 0°. Data are presented as mean ± SEM of triplicate determinations.

Incubation conditions	Catecholamine secretion
	nmole/mg protein
Control	0.2 ± 0.1
56 mM K^+	2.5 ± 0.3
Cholera toxin (1 μg/ml)	0.2 ± 0.1
Dibutyryl cAMP (1 mM)	0.3 ± 0.1

toxin increases the accumulation of [^{3}H]-cAMP in these cells. In contrast, 56 mM K^+ does not increase the formation of [^{3}H]-cAMP under these conditions. In the experiment reported in Table 4, cells were incubated with cholera toxin or 56 mM K^+ for 30 min, in the presence of the cyclic nucleotide phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (0.1 mM). Essentially similar results ware obtained in the absence of this compound. In other experiments, we found no effect of 56 mM K^+ on the accumulation of [^{3}H]cAMP after a 10 min incubation period (not shown).

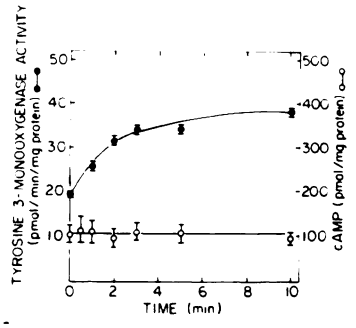

FIG. 3. *Effect of 56 mM K^+ on tyrosine 3-monooxygenase activity and cAMP levels in pheochromocytoma cells*

Cells were incubated at 37° in 56 mM K^+-KRPG. At various times, samples were removed for TH assay (●), and for measurement of cAMP levels (○). TH activity is expressed as pmole tyrosine converted to dopa/min/mg protein, mean ± SEM of triplicate determinations. cAMP levels are expressed as pmole/mg protein, mean ± SEM of triplicate determinations.

TABLE 4

Effect of 56 mM K^+ and cholera toxin on cAMP accumulation

Pheochromocytoma cells were pre-incubated for 45 min at 37° Krebs-Ringer phosphate glucose buffer containing 10 μM [2-^{3}H]adenine, 200 μCi/μmole. The cells were centrifuged, washed, and then incubated for 30 min at 37° in fresh medium containing 0.1 mM 3-isobutyl-1-methylxanthine, and the modifications indicated. Following this incubation, 50 μl of 10 mM cAMP was added, the cells were placed in a boiling water bath for 10 min, and [^{3}H]cAMP was isolated. Calculations of cAMP accumulation are corrected for the recovery of cAMP during the isolation procedure. Data are presented as mean ± SEM of triplicate determinations.

Incubation conditions	cAMP accumulation
	dpm/mg protein
Control	185 ± 18
56 mM K^+	202 ± 35
Cholera toxin (100 ng/ml)	603 ± 26

DISCUSSION

Dibutyryl cAMP, and other cAMP analogues, can activate TH in brain slices (1), in synaptosomes (3, 7), and in peripheral adrenergic neurons (20). TH activity in both central (21) and peripheral (20, 22) adrenergic nerves is also increased by electrical stimulation. However, because of the cellular heterogeneity of these experimental systems, it has not previously been possible to correlate enzyme activity with cAMP levels in TH-containing cells. Cholera toxin causes a large increase in the concentration of cAMP in pheochromocytoma cells, and produces a concurrent activation of TH is these cells. Because the activation of TH by cholera toxin is associated with a rise in cAMP levels, and because it can be mimicked by exogenous dibutyryl cAMP, it is likely that this action of cholera toxin is mediated by a rise in intracellular cAMP.

Incubation of pheochromocytoma cells in media containing 56 mM K^+ results in a coupled increase in catecholamine synthesis and catecholamine secretion. In contrast, cholera toxin and dibutyryl cAMP stimulate catecholamine synthesis, but do not increase catecholamine secretion. In the presence of these two agents, there is a dissociation in the regulation of these processes.

There is considerable controversy concerning the effects of cAMP and of electrical stimulation on the kinetic parameters of TH activity. Most workers have found that the activation of TH is characterized by a decrease in the apparent K_m of the enzyme for its pteridine cofactor (3, 5–7). The report that the activation of TH is also accompanied by a decrease in its apparent K_m for tyrosine (3) has not been confirmed (5–7). In our experiments, the activation of TH by cholera toxin and by 56 mM K^+ is characterized by an increase in the V_{max} of the enzyme; activation of the enzyme does not change its apprent K_ms for tyrosine or for pteridine cofactor. This discrepancy be-

tween our results and those of other workers presumably reflects differences in the TH assay conditions, or differences in the composition of the crude extracts used for TH assay. Whatever the explanation, it is noteworthy that electrical stimulation of peripheral adrenergic nerves has been reported to cause an increase in the V_{max} of catecholamine synthesis, but not to alter the apparent K_ms of this process for tyrosine or for pteridine cofactor (23). Thus, the behavior of TH in our experiments may fortuitously mimic the properties of the enzyme *in situ*.

The fact that 56 mM K^+ and cholera toxin produce similar changes in the kinetic properties of TH is consistent with the hypothesis that cAMP may also mediate the activation of TH by high K^+. However, stimulation of pheochromocytoma cells by 56 mM K^+ does not increase the concentration of cAMP in the cells, nor does it increase the formation of [^{3}H]cAMP in cells that have been pre-incubated with [^{3}H]adenine. Although these experiments do not exclude the possibility that 56 mM K^+ causes a change in cAMP levels in some sub-cellular compartment, they do not provide support for the hypothesis that cAMP mediates the activation of TH by 56 mM K^+. We suggest that TH can be activated by two distinct pathways, one of which involves cAMP, and the other of which is independent of the nucleotide. Additional work will be required to determine the role of cAMP in the physiological regulation of TH activity.

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