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The Effect of Ethylene Glycol Bis(β-aminoethyl ether)-*N,N'*-tetraacetic Acid and Calcium on Tyrosine Hydroxylase Activity

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SUMMARY

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It has been suggested that calcium plays a role in the regulation of tyrosine hydroxylase [tyrosine 3-monooxygenase; L-tyrosine,tetrahydropteridine oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] activity. We have studied the effects of calcium and the calcium-chelating agent ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) on tyrosine hydroxylase activity *in vitro*. The enzyme was assayed with both optimal and suboptimal concentrations of substrate and cofactor, and with both a chemical and an enzymatic system for maintaining the cofactor in the reduced state. We did not observe any significant stimulation by EGTA of tyrosine hydroxylase in crude extracts prepared from rat striatum, hypothalamus, or adrenal glands. In addition, there was no appreciable effect of calcium on tyrosine hydroxylase in crude striatal extracts. These results do not support the hypothesis that EGTA stimulates tyrosine hydroxylase in dopaminergic neurons.

INTRODUCTION

Recent reports have suggested that tyrosine hydroxylase in dopaminergic, but not noradrenergic, tissue can be activated by the calcium-chelating agent EGTA[3] (1–4). In the corpus striatum and median eminence, which are rich in dopaminergic neurons, addition of EGTA to crude enzyme extracts caused a marked activation of tyrosine hydroxylase, apparently by increasing the affinity of the enzyme for the reduced pterin cofactor (3). This stimulation was specifically reversed by calcium. Conversely, tyrosine hydroxylase from noradrenergic tissue was not affected by EGTA, but was indeed activated by calcium (3, 4). It therefore appeared that calcium might play an important role in regulating neurotransmitter synthesis. In view of the far-reaching implications of such a mechanism, we decided to study this phenomenon further. The findings reported herein fail to substantiate any significant role for calcium in the acute regulation of tyrosine hydroxylase.

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[3] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing about 150 g were killed by decapitation. The corpus striatum and hypothalamus,

representing primarily dopaminergic and noradrenergic tissue-rich brain regions, respectively, were dissected by the method of Glowinski and Iversen (5). Adrenal glands were also removed. The tissues were frozen quickly on Dry Ice and used immediately or stored in liquid nitrogen until the time of the experiment. Immediately before each experiment tissues were homogenized in 0.05 M Tris-acetate, pH 6.0, either with or without 0.2% Triton X-100. The homogenates were centrifuged for 15 min at 40,000 × g, and the supernatant fractions were used as the enzyme preparation.

Tyrosine hydroxylase activity was measured by a tritium release method (6). Each incubation mixture had a total volume of 100 μl and was adjusted to pH 6.0 before the incubation. The concentrations of tyrosine and cofactor (6-methyltetrahydropterine, Calbiochem) are given with each experiment. The cofactor was dissolved in 0.01 N HCl and reduced with hydrogen gas and platinum oxide as described previously (7). The concentration of reduced cofactor in solution was then determined spectrophotometrically, by means of the molar extinction coefficient at 264 nm (1.6×10^4). Either 2-mercaptoethanol or pteridine reductase was used to maintain the cofactor in the reduced state. In addition to cofactor and substrate, the mixture contained 0.2 M sodium acetate, pH 6.0, and 0.5–1.0 μCi of L-[3,5-^{3}H]tyrosine (New England Nuclear). When 2-mercaptoethanol was used as reducing substance, it was present at 0.12 M and the incubation contained 0.1 mM ferrous ammonium sulfate. The alternative reducing system, sheep liver reductase, was purified through the zinc-ethanol precipitation step (8). An amount of the enzyme sufficient to produce optimal activity was added. The incubations which contained reductase also included 1 mM NADPH, 0.1 mM N-methyl-N-(2-hydroxybenzyl)hydrazine (NSD 1039, a gift of Smith Nephew, Ltd.), and about 600 units of catalase. The enzyme reactions were terminated after 15 min of incubation at 37° by the addition of 400 μl of 5% trichloroacetic acid. The tritiated water released was separated from the substrate on a small Dowex 50 column and counted as described previously (7). In all cases, the assays were performed on four separate enzyme preparations, and the results are reported as the mean enzyme activity ± the standard error of the mean. Protein was determined by the method of Lowry *et al.* (9).

RESULTS

The reported effects of EGTA are on the affinity of the enzyme for its cofactor and substrate. It would therefore be expected that little effect of EGTA would be observed in the presence of saturating amounts of cofactor and substrate, whereas dramatic activation would be apparent if these components were present in subsaturating concentrations. The K_m of striatal tyrosine hydroxylase for 6-methyltetrahydropterine was found to be 0.63 ± 0.08 mM (SEM, n = 9). Therefore 1.0 mM was used as the optimal, and 0.1 mM as the suboptimal, concentration of cofactor. Since the K_m of the enzyme for tyrosine was 77 ± 3 μM (SEM, n = 9), 0.1 mM and 0.01 mM were used as the optimal and suboptimal concentrations of tyrosine, respectively. Another variable which might affect the results is the system used to maintain the cofactor in the reduced state. To allow for potential differences related to the reducing system, the studies were done using either sheep liver pteridine reductase or 2-mercaptoethanol.

The effect of EGTA and calcium on striatal tyrosine hydroxylase was determined in the presence of optimal and suboptimal conditions with both reducing systems (Table 1). When pteridine reductase was used as the reducing agent, neither calcium nor EGTA had a significant effect on tyrosine hydroxylase activity. In the experiments in which 2-mercaptoethanol was used, EGTA actually caused a small but statistically significant inhibition of tyrosine hydroxylase. When the experiment was performed with 6,7-dimethyltetrahydropterine instead of 6-methyltetrahydropterine as cofactor (results not shown), there was again no stimulation of the striatal enzyme by

TABLE 1

Effect of calcium and EGTA on striatal tyrosine hydroxylase

The enzyme was incubated with calcium and/or EGTA for 5 min at 37° before initiation of the reaction by addition of a mixture containing the rest of the ingredients. For optimal conditions, 0.1 mM tyrosine and 1 mM cofactor were used, and for suboptimal conditions, 0.01 mM tyrosine and 0.1 mM cofactor.

Additions	2-Mercaptoethanol		Pteridine reductase	
	Optimal	Suboptimal	Optimal	Suboptimal
	pmoles/mg protein/min ± SEM			
None	884 ± 13	66.1 ± 2.2	1460 ± 100	43.8 ± 3.0
10 μM EGTA	845 ± 21	69.5 ± 2.4	1590 ± 50	43.8 ± 4.0
50 μM EGTA	803 ± 11[a]	61.8 ± 3.0	1510 ± 90	47.8 ± 4.9
100 μM EGTA	726 ± 20[b]	50.4 ± 1.3[b]	1570 ± 50	42.9 ± 5.0
100 μM Ca^{++}	861 ± 23	66.8 ± 4.2	1500 ± 60	42.4 ± 3.4
100 μM Ca^{++} +50 μM EGTA	813 ± 28[c]	63.3 ± 2.2	1490 ± 110	40.6 ± 2.7

[a] Significantly different from control ($p < 0.005$).
[b] Significantly different from control ($p < 0.0005$).
[c] Significantly different from control ($p < 0.05$).

EGTA. Although most experiments were performed on supernatants obtained after centrifugation at 39,000 × *g* for 15 min, several extracts were centrifuged at 100,000 × *g* for 90 min. EGTA did not stimulate the enzyme in the high-speed supernatants. These data indicated that the enzyme from rat striatum was not activated by EGTA. As shown in Table 1, calcium had no effect on striatal tyrosine hydroxylase activity. Preliminary experiments in this laboratory indicated that calcium was also without effect on the enzyme from hypothalamus and adrenal gland.

We performed additional studies on the enzyme with pteridine reductase and suboptimal concentrations of tyrosine and cofactor. Enzyme extracts were prepared with and without Triton X-100 in the homogenizing buffer. EGTA and calcium caused no significant change in tyrosine hydroxylase activity whether or not Triton X-100 was included in the homogenizing buffer (Table 2). We also examined the effect of EGTA on tyrosine hydroxylase from tissues which produce different catecholamines. The enzyme was studied in extracts from striata (primarily dopaminergic), hypothalamus (primarily noradrenergic), and adrenals. As shown in Table 3, 50 μM EGTA did not significantly alter tyrosine hydroxylase activity in any of these tissues.

TABLE 2

Effect of Triton X-100, calcium, and EGTA on striatal tyrosine hydroxylase

Striata were homogenized in 0.05 M Tris-acetate, pH 6.0, with or without 0.2% Triton X-100. The assay was performed in the pteridine reductase system, using suboptimal concentrations of tyrosine (0.01 mM) and cofactor (0.1 mM).

Additions	Without Triton X-100	With Triton X-100
	pmoles/mg protein/min ± SEM	
None	32.3 ± 8.0	36.4 ± 11.1
50 μM EGTA	31.1 ± 6.5	38.4 ± 7.7
100 μM Ca^{++}	33.0 ± 6.8	36.4 ± 9.2
100 μM Ca^{++} +50 μM EGTA	31.0 ± 6.6	40.0 ± 7.6

Dopamine is known to be an effective inhibitor of tyrosine hydroxylase, and striatal tissue contains a significant amount of dopamine. Therefore a change in the K_I for dopamine could be an important regulatory mechanism for the striatal enzyme. If EGTA caused an increase in the K_I for dopamine, EGTA would make the enzyme less sensitive to feedback inhibition by dopamine. This possibility was investigated by studying striatal extracts from which most of the endogenous dopamine had been removed by gel filtration. When crude striatal extracts were passed over a Sephadex G-25 column as described in Table 4, more than 90% of the dopamine was removed from the en-

TABLE 3

Effect of EGTA on tyrosine hydroxylase from various brain regions and adrenal glands

The enzyme extracts were assayed using the sheep liver pteridine reductase system. For optimal conditions, 0.1 mM tyrosine and 1 mM cofactor were used, and for suboptimal conditions, 0.01 mM tyrosine and 0.1 mM cofactor.

Enzyme source	Optimal		Suboptimal	
	Control	50 μM EGTA	Control	50 μM EGTA
	pmoles/mg protein/min ± SEM			
Striatum	753 ± 67	847 ± 70	20.0 ± 1.5	19.6 ± 1.6
Hypothalamus	105 ± 13	110 ± 13	3.64 ± 0.27	3.91 ± 0.30
Adrenal gland	940 ± 35	971 ± 36	40.7 ± 1.5	38.9 ± 1.3

zyme fraction. In some cases dopamine was added to crude or Sephadex-treated extracts in order to study the inhibition of the enzyme. The K_I for dopamine in crude striatal extracts was found to be approximately 1.3 μM. The studies reported in Table 4 were performed with pteridine reductase and suboptimal concentrations of cofactor. Gel filtration brought about an increase in tyrosine hydroxylase activity, as reported previously (10). When dopamine was added back to the Sephadex G-25-treated enzyme, there was a decrease in enzyme activity. EGTA caused a small but statistically significant increase in the activity of the Sephadex G-25-treated enzyme. However, there was no suggestion that EGTA significantly altered the K_I for dopamine.

TABLE 4

Effect of EGTA on feedback inhibition of tyrosine hydroxylase

These experiments were performed with an optimal concentration of tyrosine (0.1 mM) and a suboptimal concentration of cofactor (0.1 mM) in the pteridine reductase system. Rat striata were homogenized in 4 volumes of 0.05 M Tris-acetate, pH 6.0, containing 0.2% Triton X-100 and centrifuged at 40,000 × *g* for 15 min. The supernatant fraction was used directly as the crude extract. A portion of the supernatant was chromatographed on a 0.9 × 24 cm Sephadex G-25 (coarse) column equilibrated with the homogenizing buffer. Fractions of 0.75 ml were collected, and the fraction with the highest protein concentration was used as the Sephadex G-25-treated enzyme.

Additions	Crude extract	Sephadex G-25-treated enzyme
	pmoles/mg protein/min ± SEM	
None	107 ± 4	463 ± 23
50 μM EGTA	108 ± 8	616 ± 35
20 μM dopamine	38.6 ± 1.1	355 ± 8
50 μM EGTA + 20 μM dopamine	42.3 ± 1.3	373 ± 8

DISCUSSION

The current study clearly shows that EGTA does not exert major regulatory effects on the activity of tyrosine hydroxylase in striata, hypothalami, or adrenals. Our results differ from a recent report (3) which suggested that EGTA causes marked stimulation of tyrosine hydroxylase from dopaminergic tissue. We examined various incubation conditions in an attempt to explain these conflicting results. Tyrosine hydroxylase activity was measured with and without detergent in the homogenizing buffer, with both optimal and suboptimal concentrations of tyrosine and cofactor, and with both a chemical and an enzymatic reducing system. We did not observe stimulation of tyrosine hydroxylase by EGTA under any of these conditions. When striatal extracts which were reduced in dopamine content by gel filtration were examined, it was found that EGTA caused a small but statistically significant increase in tyrosine hydroxylase activity. However, the magnitude of this increase was very much less than that reported previously for the crude extract (3). The stimulation which we observed under these conditions was so small that it probably does not reflect a physiologically significant mode of regulation for tyrosine hydroxylase.

The assay conditions in these experiments were very similar to those described in the report on the stimulatory

effect of EGTA on tyrosine hydroxylase in dopaminergic neurons (3). Both groups homogenized the tissues in 0.05 M Tris-acetate, pH 6.0. In some experiments we also included Triton X-100 in the homogenizing buffer. Morgenroth *et al.* (3) prepared their enzyme extracts by centrifuging homogenates at 104,000 × *g* for 90 min. We used centrifugation at 100,000 × *g* for 90 min in some experiments, and a lower centrifugal force (39,000 × *g* for 15 min) in other studies. Each of the two groups measured tyrosine hydroxylase activity by a tritium release method. The incubation conditions were also quite similar, with acetate buffer, pH 6.0, a dopa decarboxylase inhibitor, catalase, pteridine reductase, and NADPH in the assays performed in the two laboratories. We also used mercaptoethanol, rather than pteridine reductase, as the reducing agent in some experiments. We investigated the regulation of the enzyme with both 6,7-dimethyltetrahydropterine, as used by Morgenroth *et al.*, and 6-methyltetrahydropterine. In both laboratories, the enzyme was incubated with the calcium or EGTA for 5 min at 37° before the tyrosine hydroxylase reaction was started. Thus the experimental conditions we used were generally quite similar to those described in the literature (3), and we also examined the effects produced by altering some of these conditions. There were, however, several minor differences in the techniques used in the two laboratories, and there is a possibility that these procedural variations might account for the differences in the results obtained. For example, the dopa decarboxylase inhibitor varied in the two systems. We used 0.1 mM *N*-methyl-*N*-(2-hydroxybenzyl)hydrazine, while Morgenroth *et al.* used 5 μM 3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate (3). The pterin cofactor used in our studies was freshly reduced with hydrogen gas and platinum oxide, and the authors of the previous studies (3, 4) utilized commercially available reduced pterin cofactor. The catalase concentrations were also different, 6000 units/ml in this work and 3300 units/ml in the earlier reports (3, 4). We used sodium acetate buffer for enzyme incubations. The earlier papers (3, 4) referred to the use of "acetate buffer," without specifying the cation. Since Tris-acetate was the homogenizing buffer, it might also have been used for the enzyme assays. Two other differences which could be particularly important were the method of preparation of pteridine reductase and the assay incubation time. Our pteridine reductase, which was purified through the zinc-ethanol precipitation step, was more extensively purified than the reductase preparations (3, 4), which were only carried through the first ammonium sulfate fractionation. We assayed tyrosine hydroxylase by incubation at 37° for 15 min, rather than 45 min (3, 4), because we found the reaction rate to be linear during the shorter time.

Regulation of tyrosine hydroxylase activity is an important physiological mechanism for modulating tissue levels of catecholamine neurotransmitters. It is well established that end product inhibition (11) and changes in the affinity of the enzyme for its pterin cofactor (12) are important factors in the short-term regulation of tyrosine hydroxylase. Although at this point it is not possible to understand the reason for the conflicting results observed by ourselves and others, it is likely that future studies to resolve this problem will shed considerable light on the regulatory properties of tyrosine hydroxylase.

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