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Effect of Calmodulin on Dopamine-Sensitive Adenylate Cyclase Activity in Rat Striatal Membranes

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SUMMARY

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The effect of an endogenous calcium-binding protein, calmodulin, on basal and dopamine-stimulated adenylate cyclase activity in rat striatum was studied. Basal adenylate cyclase activity in a rat striatal particulate preparation depleted of calmodulin was stimulated by calmodulin as well as the guanyl nucleotides, guanosine triphosphate and guanyl-5′-yl-imidodiphosphate. The presence of guanyl nucleotides was required for dopamine stimulation of adenylate cyclase activity in the membrane preparation. Calcium inhibited dopamine-stimulated adenylate cyclase activity, decreasing the maximal velocity by 50%. When calmodulin was added to the assay with calcium, the V_{max} was restored to that found in the absence of calcium and the K_{act} for dopamine was further decreased more than 2-fold. The effects of calmodulin on basal and dopamine-sensitive adenylate cyclase activity were specific for calmodulin, since these effects were not obtained in the presence of troponin C, a calcium-binding protein from muscle. This work demonstrates that calmodulin is important both for the sensitivity of striatal adenylate cyclase to dopamine and the maximal velocity of the reaction. Furthermore, this work suggests that, in the presence of physiological concentrations of calcium, dopamine stimulation would be curtailed unless calmodulin were present.

INTRODUCTION

There is increasing evidence that an endogenous calcium-binding protein, calmodulin, can modulate the effects of calcium at both pre- and postsynaptic sites in some areas of brain. Calmodulin can modulate the intracellular concentration of cyclic AMP by stimulating membrane-bound adenylate cyclase activity [EC 4.6.1.1.; ATP pyrophosphate-lyase (cyclicizing)] and soluble PDE[1] activity (for review, see ref. 1). Calmodulin is enriched in rat brain synaptic membranes (2) and has been located in postsynaptic densities in mouse basal ganglia (3) and canine cerebral cortex (4). A presynaptic action for calmodulin has been suggested by DeLorenzo *et al.* (5), who have shown that calmodulin can stimulate calcium-mediated NE release from rat brain synaptosomes.

Previous studies have suggested that calmodulin has a role in DA receptor function (6–9) and is involved with DA-sensitive adenylate cyclase activity in rat striatum (6). This enzyme is considered to have a postsynaptic location in the striatum (10). Gnegy *et al.* (6) found that depletion of calmodulin from striatal membranes resulted in a decrease in DA-sensitive adenylate cyclase activity. Furthermore, the calmodulin content in brain can change in response to chronically altered dopaminergic activity. Rats treated chronically with cataleptogenic antipsychotic drugs and then withdrawn from the drugs have increased calmodulin content in their striatal membranes. These animals exhibit behavioral supersensitivity to apomorphine, and their striatal adenylate cyclase has an increased affinity for DA (7, 8). The greater calmodulin content could increase adenylate cyclase activity and thus contribute to dopaminergic supersensitivity. We wished to investigate more directly whether calmodulin could affect DA-sensitive adenylate cyclase activity in rat striatum. We studied the effect of calcium and calmodulin on basal and DA-stimulated adenylate cyclase activity in rat striatal membranes depleted of calmodulin and calcium. Guanyl nucleotides were required for the stimulation of adenylate cyclase by DA in the striatal membranes. We found that calcium was strongly inhibitory to DA stimulation unless calmodulin was present.

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[1] The abbreviations used are: PDE, phosphodiesterase; NE, norepinephrine; DA, dopamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate.

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Calmodulin, when added to a striatal membrane preparation depleted of calmodulin, could increase the maximal velocity of the reaction as well as increase the sensitivity of the adenylate cyclase to DA.

METHODS

Particulate preparation. Striatal particulate preparations were depleted of calmodulin by a modification of the method of Brostrom *et al.* (11). Male Sprague-Dawley rats (150–200 g) were killed by decapitation; the striata were removed and homogenized in 9 volumes of 10 mM Tris maleate buffer (pH 7.5) containing 1 mM $MgSO_4$, 1.2 mM EGTA, and 10 μM GTP. The homogenate was centrifuged at 27,000 × *g* for 20 min, resuspended in the same buffer, and centrifuged a second time at 27,000 × *g*. The pellet resulting from the final centrifugation was resuspended in 10 mM Tris maleate buffer (pH 7.5) containing 1.2 mM EGTA and 1 mM $MgSO_4$. Protein concentration was determined by the method of Lowry *et al.* (12).

Adenylate cyclase assay. Adenylate cyclase activity was measured in an assay (200-μl volume) containing 80 mM Tris maleate buffer (pH 7.5), 5 mM $MgSO_4$, 2 mM cyclic AMP, 4 mM phosphoenolpyruvate, 20 μg of pyruvate kinase, 0.12 mM isobutylmethylxanthine, 100–150 μg of particulate membrane protein, 0.15 mM EGTA (carried over from the particulate preparation), and 1 mM [α-^{32}P]ATP (1–2 × 10^6 cpm/assay) with or without additions such as 10 μM Gpp(NH)p, 10 μM GTP, 125 μM $CaCl_2$, or 500 ng (28 pmoles) of highly purified calmodulin prepared from bovine brain according to the method of Klee (13). Assays were incubated for 3 min and the reaction was stopped by heating for 1 min at 95°; 200 μl of a solution containing 20 mM ATP and 0.7 mM cyclic AMP were then added to the tubes. The particulate material was centrifuged, and the ^{32}P-labeled cyclic AMP in the supernatant was determined by the method of Krishna *et al.* (14). Recovery of the cyclic AMP was measured using ^{3}H-labeled cyclic AMP and was 75–80%.

Determination of calmodulin content. Calmodulin was assayed by its ability to stimulate calmodulin-deficient PDE activity (15) in the PDE assay described previously (2). The calmodulin content (in nanograms) was determined from a standard curve using highly purified calmodulin.

Materials. The drugs (+)- and (−)-butaclamol were kindly supplied by Ayerst Laboratories, Montreal, Canada. Gpp(NH)p and GTP were purchased from International Chemical and Nuclear Corporation, Irvine, Calif. [α-^{32}P]ATP (specific activity 10 Ci/mmole) was purchased from Amersham/Searle Corporation, Arlington Heights, Ill. 3-Hydroxytyramine HCl (DA) and propranolol were purchased from Sigma Chemical Company, St. Louis, Mo.

Calmodulin was purified from bovine brain by the method of Klee (13) and demonstrated a single band on disc gel electrophoresis containing 10% polyacrylamide (16). Troponin C was the generous gift of Dr. John Dedman, Department of Cell Biology, Baylor College of Medicine, Houston, Tex. Both calmodulin and troponin C were prepared in the presence of millimolar concentrations of EGTA and lyophilized against 0.05 M $(NH_4)HCO_3$. The proteins were redissolved in 80 mM Tris maleate buffer, pH 7.4. Protein concentrations were determined by ultraviolet absorption (13) and by the method of Lowry *et al.* (12).

RESULTS

Effect of calmodulin and calcium on basal adenylate cyclase activity. Depletion of most of the calmodulin content from the striatal membranes was necessary to observe the stimulatory effects of this protein on adenylate cyclase activity. The EGTA treatment depleted most, but not all, of the calmodulin from the membrane fraction. As is shown in Table 1, 24% of the calmodulin content in the homogenate remained in the striatal membranes after the EGTA washings. Originally, nearly 50% of the calmodulin content in the brain cell is located in the membranes. Basal adenylate cyclase activity in the striatal homogenates was decreased in the presence of EGTA. Furthermore, EGTA washing decreased the specific activity of the basal adenylate cyclase activity in the particulate fraction by 41% (Table 1).

Neither calcium nor calmodulin individually was able to stimulate basal adenylate cyclase activity in the striatum. Calcium itself did not really affect basal activity (Fig. 1) until high concentrations were added (>200 μM). As Fig. 1 illustrates, calmodulin and calcium added together elicited a biphasic response to adenylate cyclase activity. The effective concentrations of calcium providing stimulation were very low because the assay contained 150 μM EGTA. Free, or effective, concentrations of calcium were calculated according to the method of Nanninga and Kempen (17). These calculations were further verified by a computer program from Potter and Gergely (18). In the presence of 500 ng of calmodulin (1.4 × 10^{-7} M), effective concentrations of calcium between 6.6 × 10^{-9} M and 1.6 × 10^{-6} M (50 μM and 150 μM added calcium) elicited stimulation of adenylate cyclase activity with a maximal effect at 1.2 × 10^{-7} M (125 μM added calcium). Adenylate cyclase activity determined in the presence of calmodulin was inhibited at concentrations of calcium greater than 50 μM (200 μM added calcium). Indirect evidence suggested that the calmodulin remaining in the particulate fraction after EGTA washing significantly contributes to the basal adenylate cyclase activity. The addition of 10^{-4} M trifluoperazine with 125 μM calcium decreased the basal activity 4-fold (from 96 pmoles/min/mg protein to 22 pmoles/min/mg of protein). Trifluoperazine has been shown to bind to calmodulin in the presence of calcium and to inhibit its ability to bind to other enzymes (19). If it is assumed that trifluoperazine is acting in the particulate preparation to inhibit calmodulin binding to adenylate cyclase, the adenylate cyclase in the striatum is actually highly responsive to calmodulin.

As is shown in Table 2, striatal adenylate cyclase activity in the calmodulin-depleted particulate fraction was stimulated 85% by 500 ng of calmodulin at a concen-

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TABLE 1

Calmodulin content and adenylate cyclase activity in fractions from rat striatum

Striatal tissue from one rat was divided into two parts. One part was homogenized in 9 volumes of 10 mM Tris maleate buffer (pH 7.5) containing 1 mM $MgSO_4$. The other part was homogenized in the same manner except that the buffer also contained 1.2 mM EGTA. Membrane fractions (centrifuged at 27,000 × *g*) were prepared from the homogenate fractions as described under Methods. Calmodulin content and adenylate cyclase activity were determined in the homogenate and 27,000 × *g* membrane fractions as described under Methods. Results are given as values ± standard error of the mean for three separate experiments.

Fraction	Washing Condition	Calmodulin content		Adenylate cyclase activity
		Concentration	Total calmodulin	
		μg/mg membrane protein	*mg/g tissue wet wt*	*pmoles/min/mg protein*
Homogenate	Buffer only	6.9 ± 0.6	620 ± 77	331 ± 28
	Buffer + EGTA			233 ± 15[a]
27,000 × *g* membranes	Buffer only	4.9 ± 0.6	290 ± 46	175 ± 7
	Buffer + EGTA	2.9 ± 0.3[b]	150 ± 15	105 ± 17[c]

[a] $p < 0.02$ as compared with the value obtained from homogenates prepared with buffer only.
[b] $p < 0.02$ as compared with the value obtained from membranes prepared with buffer only.
[c] $p < 0.01$ as compared with the value obtained from membranes prepared with buffer only.

tration of 125 μM added calcium (an effective calcium concentration of 1.2×10^{-7} M). The guanyl nucleotides, on the other hand, were able to produce a greater maximal stimulation of adenylate cyclase than did calmodulin. Both GTP and Gpp(NH)p increased the rate of adenylate cyclase activity after lag periods of less than 2 min. A 10-μM Gpp(NH)p concentration stimulated the adenylate cyclase activity by 200% (Table 2). Calcium, at an effective concentration of 1.2×10^{-7} M, significantly inhibited the stimulation by Gpp(NH)p by 42%. The addition of calmodulin reversed the calcium inhibition, and the stimulation by Gpp(NH)p, calcium, and calmodulin was not different from that elicited by Gpp(NH)p alone. Actually, Gpp(NH)p-stimulated adenylate cyclase activity was inhibited at all added concentrations of calcium. The interaction of calcium and calmodulin with GTP stimulation of basal activity was different from that observed with Gpp(NH)p. GTP-stimulated adenylate cyclase activity was not inhibited by calcium but rather seemed to be slightly stimulated (Table 2). GTP stimulation of adenylate cyclase activity was not inhibited until the concentration of free calcium was greater than 2 μM. Stimulation of adenylate cyclase activity by calmodulin was specific because troponin C, another calcium-binding protein, did not stimulate basal adenylate cyclase activity even at 10-fold higher concentrations. Furthermore, as is shown in Table 2, troponin C did not reverse the effects of calcium on Gpp(NH)p stimulation as did calmodulin.

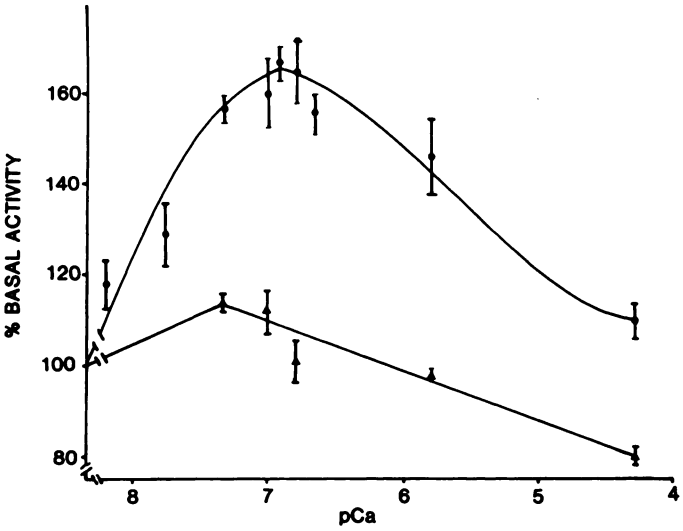

FIG. 1. *Effects of calcium and calmodulin on basal adenylate cyclase activity in rat striatal membranes*

Membranes were depleted of calcium and calmodulin by EGTA washing as described under Methods. Adenylate cyclase assays were performed as described under Methods with (●) and without (▲) 500 ng of highly purified calmodulin. All assays contained 150 μM EGTA. Each point is the average of triplicate determinations from three separate experiments.

Effect of calmodulin on DA-sensitive adenylate cy-

TABLE 2

Stimulation of adenylate cyclase activity in rat striatum by calmodulin and guanyl nucleotides

Addition[a]	Adenylate cyclase activity[b]
	pmoles/min/mg protein
Experiment I (*N* = 9)	
None	96 ± 9
Calmodulin + Ca^{2+}	176 ± 17
Gpp(NH)p	289 ± 12
Gpp(NH)p + Ca^{2+}	170 ± 12[c]
Gpp(NH)p + Ca^{2+} + calmodulin	285 ± 16
Experiment II (*N* = 12)	
None	87 ± 8
Calmodulin + Ca^{2+}	143 ± 9
GTP	180 ± 20
GTP + Ca^{2+}	192 ± 21
GTP + Ca^{2+} + calmodulin	216 ± 22
Experiment III (*N* = 4)	
None	96 ± 7
500 ng troponin C + Ca^{2+}	115 ± 4
5000 ng troponin C + Ca^{2+}	120 ± 10
Gpp(NH)p	240 ± 18
Gpp(NH)p + Ca^{2+} + 500 ng troponin C	167 ± 12[c]
Gpp(NH)p + Ca^{2+} + 5000 ng troponin C	171 ± 16[c]

[a] Concentrations of additions were as follows: 10 μM Gpp(NH)p and GTP; 500 ng of calmodulin; 125 μM $CaCl_2$.
[b] Values are means ± standard error of the mean.
[c] $p < 0.01$ as compared with the value for Gpp(NH)p alone.

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clase activity. Adenylate cyclase activity in the striatal particulate preparation that had been washed repeatedly with EGTA could not be stimulated by DA. The addition of calcium, calmodulin, or both agents together was insufficient to restore DA sensitivity. The inclusion of 10 μM Gpp(NH)p or GTP in the assay was necessary to achieve a dose-dependent response to DA in the formation of cyclic AMP. Adenylate cyclase activity was stimulated 1.4- to 1.5-fold by DA in the presence of 10 μM Gpp(NH)p and 1.7- to 1.9-fold in the presence of 10 μM GTP. As is illustrated in Table 2, this actually represented a large stimulation over the original basal activity. DA stimulation is expressed as the number of picomoles per minute per milligram of protein of ^{32}P-labeled cyclic AMP formed over the basal values for the guanyl nucleotide alone or the guanyl nucleotide and calmodulin together. The increase in adenylate cyclase activity in the presence of 10 μM Gpp(NH)p reached a maximum at 5 μM DA (Fig. 2A). The activation constant (K_{act}) for DA was determined directly from the dose-response curve and corroborated by double reciprocal plots for adenylate cyclase activation. K_{act} is defined as the concentration of DA that stimulated adenylate cyclase activity by 50% of maximum. The K_{act} for DA in the presence of Gpp(NH)p was 1.1 μM and the V_{max} was 85 pmoles/min/mg protein (Table 3). The addition of 125 μM calcium (1.2×10^{-7} M free calcium) brought about a 50% decrease in V_{max} with no change in sensitivity for DA (Fig. 2A). When 500 ng of calmodulin were added with the calcium, the maximal velocity was restored to the value found with Gpp(NH)p alone, and the dose-response curve for DA was shifted to the left (Fig. 2A). The K_{act} for DA was decreased 3-fold to 0.35 μM (Table 3). The average kinetic parameters of five separate experiments are shown in Table 3.

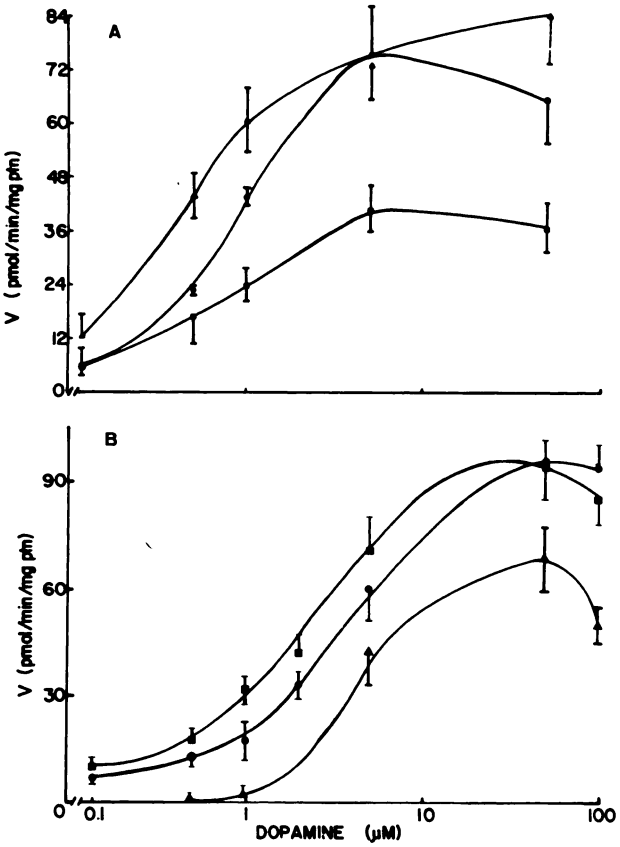

FIG. 2. *Effects of calcium and calmodulin on dopamine-stimulated adenylate cyclase activity in the presence of Gpp(NH)p and GTP*

A. In the presence of Gpp(NH)p. Striatal membranes were prepared and adenylate cyclase assays were performed as described under Methods. Each assay contained 10 μM Gpp(NH)p in the absence (●) or presence of 125 μM $CaCl_2$ (■) or 125 μM $CaCl_2$ + 500 ng of calmodulin (▲). Each point is the average of triplicate determinations from five separate experiments.

B. In the presence of GTP. Striatal membranes were prepared and adenylate cyclase assays were performed as described under Methods. Each assay contained 10 μM GTP in the absence (●) or presence of 125 μM $CaCl_2$ (▲) or 125 μM $CaCl_2$ + 500 ng of calmodulin (■). Each point is the average of triplicate determinations from eight separate experiments.

The same pattern was seen for the effects of calcium and calmodulin on DA-stimulated activity in the presence of 10 μM GTP (Fig. 2B). The K_{act} for DA stimulation of adenylate cyclase was 3.8 μM (Table 4), nearly 3 times greater than that found in the presence of Gpp(NH)p. However, the V_{max} of the reaction (122 pmoles/min/mg of protein) was slightly greater than that obtained with Gpp(NH)p. As is shown in Fig. 2B, the addition of 125 μM calcium to the assay again decreased DA stimulation, in spite of the fact that calcium did not decrease GTP stimulation of basal activity. Calcium (1.2×10^{-7} M) reduced the V_{max} 2-fold with no significant change in the K_{act} for DA. The addition of calmodulin with the calcium elicited an increase in V_{max} to the level found with GTP alone and resulted in an increased sensitivity for DA (K_{act} for DA = 1.8 μM). Kinetic constants for DA stimulation of adenylate cyclase in eight different experiments are shown in Table 4.

The effect of calmodulin on DA-sensitive adenylate cyclase activity was specific for calmodulin in the capacity of a calcium-binding protein. Troponin C, a calcium-binding protein found in muscle cells (Mr = 17,800 daltons), was unable to substitute completely for the effects of calmodulin on basal (Table 2) or DA-stimulated activities (Fig. 3) even at a concentration nearly 10-fold higher than that of calmodulin. As is shown in Fig. 3, 5 μg of troponin C could begin to restore the V_{max} of the reaction but could not approximate the shift in K_{act} for DA, as did calmodulin.

TABLE 3

Kinetic constants for stimulation of striatal dopamine-sensitive adenylate cyclase by Gpp(NH)p, calcium, and calmodulin

Kinetic constants were determined by Lineweaver-Burke analyses in five separate experiments and averaged. Values are means ± standard error of the mean.

Addition	K_{act}	V_{max}
	μM	*pmoles/min/mg protein*
Gpp(NH)p	1.1 ± 0.3	85 ± 17
Gpp(NH)p + 125 μM Ca^{2+}	0.83 ± 0.1	45 ± 5[a]
Gpp(NH)p + 125 μM Ca^{2+} + calmodulin	0.35 ± 0.09[b]	85 ± 12

[a] $p \leq 0.02$ as compared with Gpp(NH)p alone or Gpp(NH)p, 125 μM Ca^{2+}, and calmodulin.

[b] $p \leq 0.02$ as compared with Gpp(NH)p alone or Gpp(NH)p + 125 μM Ca^{2+}.

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TABLE 4

Kinetic constants for stimulation of striatal dopamine-sensitive adenylate cyclase activity in the presence of GTP, calcium, and calmodulin

Kinetic constants were determined by Lineweaver-Burke analyses in eight separate experiments and averaged. Values are means ± standard error of the mean.

	K_{act}	V_{max}
	μM	*pmoles/min/mg protein*
GTP ($N = 8$)	3.8 ± 0.6	122 ± 9.6
GTP + 125 μM Ca^{2+} ($N = 5$)	5.2 ± 0.1	68 ± 8[a]
GTP + 125 μM Ca^{2+} + calmodulin	1.8 ± 0.4[b]	112 ± 10

[a] $p \leq 0.01$ as compared with GTP alone or GTP, 125 μM Ca^{2+}, and calmodulin.

[b] $p \leq 0.04$ as compared with GTP alone or GTP + 125 μM Ca^{2+}.

The stimulation by DA in the presence of guanyl nucleotides and calmodulin exhibited the characteristics and specificity expected of a DA receptor. As is shown in Fig. 4, NE was much less potent than DA in stimulating the adenylate cyclase activity in the presence of calmodulin and Gpp(NH)p. The K_{act} for NE was 7.8 μM as compared with a K_{act} of 0.35 μM for DA, but the V_{max} of 100 pmoles/min/mg of protein was the same as that obtained for DA stimulation (Fig. 4). The addition of calmodulin did not elicit a decrease in K_{act} for NE as was shown for DA.

The stimulation of DA in the presence of calmodulin and Gpp(NH)p could be blocked stereospecifically by 1 μM (+)-butaclamol, an active antipsychotic drug and DA antagonist, but not by (−)-butaclamol, its inactive isomer (Table 5). Propranolol, a *beta*-adrenergic antagonist, was also inactive in blocking the DA stimulation at a concentration of 1 μM. As described by Schramm (20), the order of addition of the reagents was important to obtain receptor blockade in the presence of Gpp(NH)p. Striatal

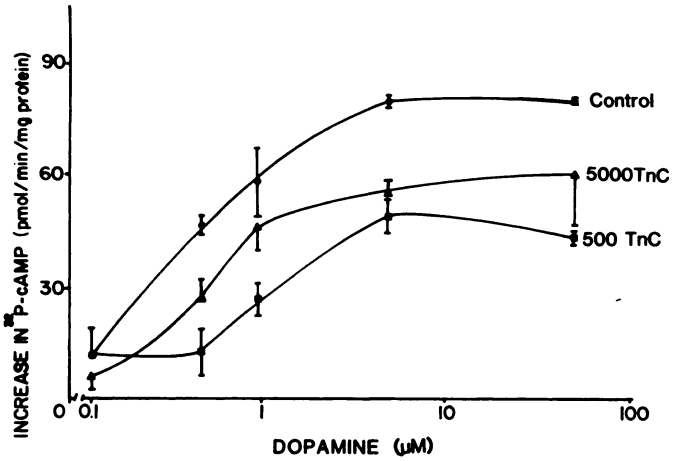

FIG. 3. *Effects of calcium and troponin C on dopamine-sensitive adenylate cyclase activity in the presence of Gpp(NH)p*

Striatal membranes were depleted of calcium and calmodulin as described under Methods. Adenylate cyclase assays were performed as described under Methods. Each assay contained 10 μM Gpp(NH)p in the absence (●) and presence of 125 μM $CaCl_2$ + 500 ng troponin C (*TnC*) (■) or 125 μM $CaCl_2$ + 5000 ng troponin C (▲). Each point is the average of triplicate determinations from three separate experiments.

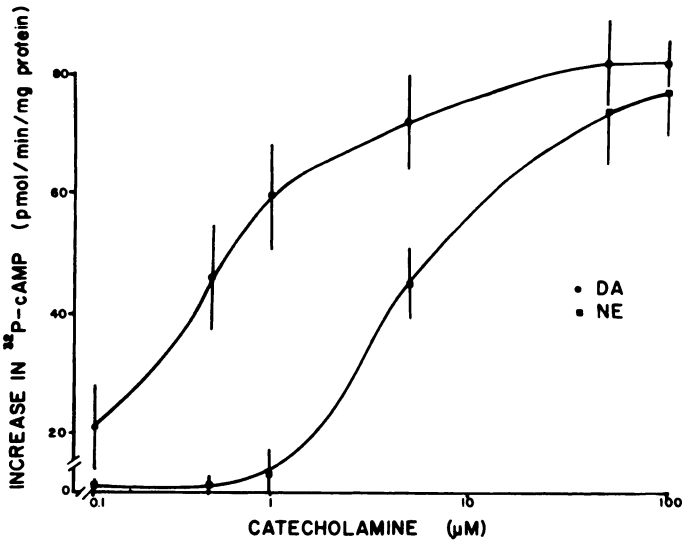

FIG. 4. *Stimulation of adenylate cyclase activity in rat striatal membranes by dopamine and norepinephrine*

Striatal membranes were prepared and adenylate cyclase assays were performed as described under Methods. Each assay contained 10 μM Gpp(NH)p, 125 μM $CaCl_2$, 500 ng of calmodulin, and various concentrations of DA or NE. Each point is the average of triplicate determinations from three separate experiments.

particulate preparations were incubated at 37° for 5 min with the receptor-blocking agents, then Gpp(NH)p and calmodulin were added to the assay. We found specific receptor blockade at 0.1 μM concentration of the drugs as well. Similar results were obtained using GTP (data not shown).

DISCUSSION

Studies concerning the effects of calcium on catecholamine-stimulated adenylate cyclase activity in the brain

TABLE 5

Effect of dopaminergic and adenergic receptor blocking agents on dopamine stimulation of striatal adenylate cyclase activity

Addition	Adenylate cyclase activity[a]	
	Gpp(NH)p	Calmodulin + Gpp(NH)p[b]
	pmoles/min/mg protein	
None	142 ± 4.3 (4)	141 ± 2.8 (5)
5 μM DA	193 ± 15[c]	198 ± 11[d]
5 μM DA + 1 μM (+)-butaclamol[e]	137 ± 11	138 ± 14
5 μM DA + 1 μM (−)-butaclamol	189 ± 17	186 ± 12
5 μM DA + 1 μM propranolol	189 ± 18	186 ± 12

[a] Values are means ± standard error of the mean.

[b] The concentration of Gpp(NH)p was 10 μM; 125 μM $CaCl_2$ was always added with 500 ng of calmodulin.

[c] $p < 0.02$ as compared with adenylate cylase activity in the presence of Gpp(NH)p.

[d] $p < 0.001$ as compared with adenylate cyclase activity in the presence of Gpp(NH)p and calmodulin.

[e] Striatal membranes were preincubated with (+)-butaclamol, (−)-butaclamol or propranolol for 5 min at 37° before the addition of remaining assay components.

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have yielded equivocal results. Calcium has generally been considered to be inhibitory to DA-sensitive adenylate cyclase activity because stimulation in homogenates can be detected only in the presence of EGTA, a calcium-chelating agent. This effect occurred because EGTA decreased basal adenylate cyclase activity (as shown in Table 1) and permitted expression of DA sensitivity (21, 22). The inhibitory effect of EGTA on basal and guanine nucleotide-dependent adenylate cyclase activity in homogenates of rat caudate nucleus could be partially reversed by the addition of calcium (23), but high concentrations of calcium are inhibitory to basal and DA-stimulated adenylate cyclase activity in homogenates (22). However, in other systems, calcium has been shown to enhance catecholamine stimulation of cyclic AMP production. Schwabe *et al.* (24) have shown that, in rat cerebral cortical slices, the addition of EGTA will selectively decrease the stimulation of cyclic AMP production by *alpha*-adrenergic agonists and to some extent that of *beta*-adrenergic agonists. However, these results could be interpreted as a selective requirement for extracellular calcium. A stronger role for a stimulatory effect of intracellular calcium in NE stimulation of cyclic AMP was shown by Brostrom *et al.* (25), using C6 glioma cells. The ability of C6 glioma cells to accumulate cyclic AMP in response to NE was reduced 60–70% following nearly total calcium depletion of the cells in culture by EGTA.

The effects of calcium on adenylate cyclase activity in brain are mediated at least in part through an endogenous calcium-binding protein, calmodulin. Since calmodulin is so involved with the effects of calcium on cyclic nucleotide metabolism in the brain, one cannot properly determine the effects of calcium on adenylate cyclase activity without consideration of calmodulin. Calmodulin is enriched in synaptic membranes (2), and its subcellular distribution is similar to that of adenylate cyclase and PDE (26). Calmodulin has been shown to stimulate adenylate cyclase activity in cerebral cortex in a calcium-dependent manner (1, 11, 27). We found that calmodulin stimulated basal adenylate cyclase activity in rat striatum in a biphasic manner that was dependent upon the concentration of calcium. Since calmodulin is in excess of adenylate cyclase in brain membranes, the stimulatory effects of calcium and calmodulin are not apparent unless the particulate fraction has been prepared and washed several times in buffer containing millimolar quantities of EGTA. This procedure causes dissociation and removal of most (but not all) of the calcium and calmodulin from the particulate fraction. Stimulation of adenylate cyclase by calmodulin was maximal at a calculated effective calcium concentration of 0.12 μM, which is within resting levels of calcium in nerve membranes (28). The actual concentration of free calcium may be slightly greater because the particulate fraction may not have been totally depleted of calcium. Calcium and calmodulin inhibited basal adenylate cyclase activity at calculated free calcium concentrations of more than 1 μM. At low resting levels of calcium, or even after a slight increase in calcium, adenylate cyclase activity would be increased by calmodulin, but as calcium levels would continue to rise to micromolar concentrations, perhaps by altered synaptic activity, the system would be inhibited. It has been reported that the inhibitory phase of calcium and calmodulin effects on guinea pig cerebral cortical adenylate cyclase corresponds to the calcium concentrations eliciting an increase in calmodulin-dependent PDE activity (27).

This concept would apply to DA stimulation of adenylate cyclase activity as well. DA sensitivity was abolished in calcium- and calmodulin-depleted membranes and could not be restored by the addition of either agent. A dose-dependent stimulation of adenylate cyclase by DA was found only when 10 μM GTP or Gpp(NH)p was included in the assay. We found 1.4- to 1.5-fold stimulation by maximal concentrations of DA in the presence of 10 μM Gpp(NH)p and 1.7- to 1.9-fold stimulation in the presence of 10 μM GTP. This stimulation is comparable to that found by others in similar preparations (22, 23, 29). The addition of 125 μM calcium (0.12 μM calculated free calcium) strongly inhibited the DA-stimulated adenylate cyclase in the presence of either guanyl nucleotide. However, when calmodulin was present the maximal velocity of the reaction was restored and the sensitivity to DA was even greater than that found in the presence of the guanyl nucleotide alone. Thus calmodulin affected both the K_{act} for DA and V_{max} of the DA-sensitive adenylate cyclase activity. This finding suggests that at physiological, resting concentrations of calcium, DA will not greatly stimulate adenylate cyclase activity unless calmodulin is present.

Our data further suggest that there is an interaction among calcium, calmodulin, and guanyl nucleotides. We found that both GTP and Gpp(NH)p stimulated basal adenylate cyclase activity in our membrane preparation and were required for DA stimulation. The effects of guanyl nucleotides on basal and DA-stimulated adenylate cyclase activity are variable and may depend on the type of membrane or homogenate preparation as well as the temperature of incubation (22, 23, 29). DA-stimulated adenylate cyclase activity in striatum may have the same molecular organization and mechanism of stimulation by guanyl nucleotides as those described for the *beta*-adrenergic receptors in erythrocytes and in other cells (30–32). Guanyl nucleotides regulate both binding to the *beta*-adrenergic receptor and stimulation of adenylate cyclase activity. These effects are mediated by a GTP-binding protein with a molecular weight of 42,000, alternately referred to as G-protein (31), G/F factor (32), or *N* (30). In a schema described by Limbird *et al.* (31), binding of a *beta*-adrenergic agonist to its receptor site promotes the formation of a receptor-G-protein complex. This association causes GDP to unload from a GTP-binding site on the G-protein, allowing GTP subsequently to bind. As GTP binds to the G-protein, the receptor dissociates from the G-protein which then associates with the catalytic subunit of adenylate cyclase and stimulates the formation of cyclic AMP. Guanyl nucleotides, via the G-protein, thus decrease the affinity of the receptor site for agonist but increase the coupling between receptor and adenylate cyclase subunit.

DA-sensitive adenylate cyclase in the striatum may follow a similar activation mechanism. Guanyl nucleotides decrease the affinity for DA and its agonist but do not affect antagonist binding at DA receptors (33). We

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and others (23, 29) have shown that guanyl nucleotides stimulate basal adenylate cyclase activity and are required for DA stimulation. There is a lag period in stimulation of adenylate cyclase activity by both GTP and Gpp(NH)p that could indicate the time required for binding of GTP to the G-protein and association with the catalytic subunit. Stimulation of adenylate cyclase by the nonhydrolyzable analogue Gpp(NH)p is considered to represent a persistently activated state of the enzyme, as opposed to stimulation by GTP. GTP can be hydrolyzed by GTPase, an enzyme that is proposed to turn off the cyclase reaction (30). We found that stimulation by Gpp(NH)p was inhibited by calcium even at very low concentrations. GTP stimulation of adenylate cyclase activity, on the other hand, was much less sensitive to calcium and was inhibited only at calculated free concentrations of calcium greater than 2 μM. DA-sensitive adenylate cyclase activity was inhibited by calcium in the presence of either Gpp(NH)p or GTP. As with Gpp(NH)p alone, the inhibition was largely prevented by calmodulin. The molecular association or activated state induced by Gpp(NH)p in striatal membranes may be similar to that achieved in the presence of GTP and hormone. Our data suggest that it is this association or "activation state" that can be inhibited by calcium and with which calmodulin can interact.

The mechanism by which calmodulin can reverse this calcium inhibition is not yet known. Calmodulin could be simply binding to the calcium, preventing it from interacting with the DA-sensitive adenylate cyclase and causing inhibition. However, there are several factors which suggest that this is not the case. First, calmodulin actually stimulated adenylate cyclase activity in the presence of calcium. It did not simply block inhibition by calcium, since calcium alone had little effect on basal adenylate cyclase activity. Second, troponin C, a calcium-binding protein with some sequence homology to calmodulin, could not mimic the action of calmodulin on basal or DA-stimulated adenylate cyclase activity, even at concentrations 10 times that of calmodulin. Troponin C binds calcium with an affinity similar to that of calmodulin. Furthermore, the calcium concentration range in which troponin C activates PDE is similar to that of calmodulin (34). Third, we have shown that, under the conditions used in this assay, ^{125}I-labeled calmodulin will bind to the striatal membranes in a calcium-dependent manner and the calcium concentration dependence of ^{125}I-labeled calmodulin binding is the same as that for stimulation of adenylate cyclase activity (35).

It is also highly unlikely that calcium is complexing with the guanyl nucleotides. The calculated free calcium concentration is quite low because of the EGTA·calcium buffer system. Furthermore, since the assays contained 5 mM $MgSO_4$ the guanyl nucleotides are most likely present as Mg^{2+} complexes. The presence of a regenerating system would ensure the maintenance of the GTP concentration (11). Our data do not rule out the possibility that there are two separate adenylate cyclase enzymes, one stimulated by guanyl nucleotides and inhibited by calcium and another stimulated by calcium and calmodulin. Brostrom *et al.* (11) and Toscano *et al.* (36) have found evidence for a Ca^{2+}-sensitive and Ca^{2+}-insensitive form of adenylate cyclase activity in cerebral cortex. It is possible that in the presence of guanyl nucleotide, calcium, and calmodulin the resulting adenylate cyclase activity is due to both calcium inhibition of the guanyl nucleotide-sensitive form and calcium and calmodulin stimulation of a separate cyclase. However, Toscano *et al.* (36) found that calmodulin stimulation of a calmodulin-dependent adenylate cyclase required the presence of a GTP-dependent protein fraction. Another possible explanation is that the adenylate cyclase subunit has two calcium sites. One site would have a high affinity for calcium that would cause inhibition of adenylate cyclase activity. The second site would be a calmodulin-binding site through which calmodulin and calcium would stimulate adenylate cyclase activity. High concentrations of calcium would tend to inhibit adenylate cyclase activity at both sites.

Our findings correlate with previous work concerning the relationship between calmodulin and dopaminergic supersensitivity in rat striatum (7–9, 37). Animals chronically treated with antipsychotic drugs exhibit behavioral supersensitivity to apomorphine and have increased calmodulin content in their striatal membranes. The adenylate cyclase in the striatal membranes of the drug-treated animals showed an increased sensitivity to DA as evinced by a 3- to 4-fold decrease in the apparent K_{act} for DA (7–9). Similarly, the membrane concentration of calmodulin is increased in the striata of rats after unilateral brain hemitransection (37) lesioning the nigrostriatal pathway. This further supports a role for calmodulin in dopaminergic activity and even supersensitivity to DA in the rat striatum. During normal synaptic activity or under conditions of greatly altered synaptic input, calmodulin could be vital in attempting to maintain normal dopaminergic function through both stimulatory and inhibitory effects.

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