Proc. Natl. Acad. Sci. USA
Vol. 73, No. 11, pp. 3887–3890, November 1976
Biochemistry

Regulation of dopamine stimulation of striatal adenylate cyclase by an endogenous Ca^{++}-binding protein

(adenosine 3′:5′-cyclic monophosphate/release of phosphodiesterase activator/protein phosphorylation)

M. E. GNEGY, P. UZUNOV, AND E. COSTA

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeth's Hospital, Washington, D.C. 20032

Communicated by John J. Burns, August 12, 1976

ABSTRACT **Membranes of rat caudate nucleus contain a dopamine-dependent adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] and a Ca^{++}-binding protein that activates phosphodiesterase (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17). This activator can be released from the membranes by a phosphorylation with a 3′:5′-cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37). Under the conditions of membrane phosphorylation and activator release, dopamine fails to activate striatal adenylate cyclase. The basal activity of this enzyme is not decreased by the release of the protein activator but the activation by NaF is reduced. Adenylate cyclase is not phosphorylated when the dopamine activation is blocked after the release of the activator, but other membrane proteins are phosphorylated. It is postulated that the endogenous protein stored in striatal membranes can regulate the intracellular concentration of cAMP by an activation of adenylate cyclase while stored in striatal membrane, and by an activation of phosphodiesterase when released into the cytosol after membrane phosphorylation.**

The molecular mechanism whereby neurotransmitters (1–8) activate adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] is still unclear. Recently Brostom *et al.* (9) reported that a Ca^{++}-binding protein could regulate the activity of a detergent-dispersed adenylate cyclase preparation from porcine cerebral cortex. This protein has properties identical to those of the endogenous Ca^{++}-dependent protein which, according to Cheung, activates cyclic nucleotide phosphodiesterase (3′:5′-cAMP 5′-nucleotidohydrolase, EC 3.1.4.17) (10–12). We reported that this phosphodiesterase-activating protein is located in the synaptic membranes of brain tissue (13) and can be released from these membranes by phosphorylation promoted by a cAMP-dependent ATP:protein phosphotransferase (protein kinase, EC 2.7.1.37) (14). Several lines of evidence (13, 14) indicate that when cAMP content in postsynaptic cells reaches a certain level, this second messenger releases phosphodiesterase activator from its membrane storage site by a cAMP-dependent phosphorylation of synaptic membranes (14). Once released into the cytosol this protein decreases the K_m of high-K_m phosphodiesterase, thereby lowering the concentration of cAMP by promoting its hydrolysis. This information (13, 14) and the report that the endogenous activator can regulate adenylate cyclase activity (9) prompted us to study whether transmitters could still activate adenylate cyclase after this protein was released from its storage site.

Rat caudate nucleus, which contains a dopamine-dependent adenylate cyclase (15–17) and a Ca^{++}-binding protein that is releasable through cAMP-dependent phosphorylation (18), was used in this study. The present experiments demonstrate that the depletion of the endogenous protein activator from striatal membranes impairs the activation of striatal adenylate cyclase by both dopamine and NaF.

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; Gpp(NH)p, guanosine 5′-(β, γ-imino)triphosphate.

METHODS

Uniformly labeled [^{3}H]cAMP (50 Ci/mmol), [8-^{14}C]ATP (51.6 mCi/mmol), and [γ-^{32}P]ATP (22 mCi/mmol) were obtained from New England Nuclear Corp. (Boston, Mass). Unlabeled cAMP, ATP, aminophylline, phospho*enol*pyruvate, and *Ophiophagus hannah* snake venom were purchased from Sigma Chemical Co. (St. Louis, Mo.). Lubrol-PX was obtained from ICIUS, Inc. (Wilmington, Del.) and guanosine 5′-(β, γ-imino)triphosphate [Gpp(NH)p] was purchased from ICN Pharmaceuticals (Cleveland, Ohio). Pyruvate kinase (10 mg/ml) was obtained from Boehringer Mannheim Co., (New York, N.Y.). Dowex 50 resin (AG 500 W-X8, 200–400 mesh) was purchased from Bio-Rad Laboratories (Richmond, Calif.).

Preparation and Phosphorylation of Membrane Fractions from Caudate Nucleus. Caudate nuclei were dissected from male Sprague-Dawley rats (Zivic Miller, Allison Park, Pa.) and homogenized in 9 volumes of 0.32 M sucrose. The pellet, obtained after centrifugation at 100,000 × *g* for 1 hr, was resuspended in an equal volume of sucrose. The membranes (1.6 mg of protein) were phosphorylated in an incubation mixture of 1.6 ml containing 50 mM NaOAc buffer, pH 6.0, 10 mM MgOAc, pH 6.0, 160 μg of bovine heart cAMP-dependent protein kinase, 25 mM ATP, and either 0.5 μM or 1.0 μM cAMP. The reaction, carried out at 30° for 5 min, was stopped by plunging the tubes into ice-cold water. Nonphosphorylated samples contained no ATP. After centrifugation (30,000 × *g* for 10 min at 4°), the phosphorylated membranes were resuspended in 10 mM Tris-maleate buffer, pH 7.5, to assay for adenylate cyclase activity. The supernatant fluid was used to measure the phosphodiesterase protein activator that had been released from the membranes by phosphorylation.

Assay of Adenylate Cyclase. The standard mixture used to assay adenylate cyclase activity at 37° for 3 min contained: 10 mM Tris-maleate buffer, pH 7.4, 5 mM $MgSO_4$, 1 mM ATP (1 μCi of [8-^{14}C]ATP), 2 mM cAMP, 4 mM phospho*enol*pyruvate, 30 μg of pyruvate kinase, 10 mM aminophylline, and approximately 200 μg of membrane protein in a final volume of 0.2 ml. The reaction was terminated by diluting the sample with 0.2 ml of water and heating the tubes at 95° for 1 min. Cyclic AMP was purified from the reaction mixture by separation on a column of cationic exchange resin (19) followed by a $ZnSO_4$-$Ba(OH)_2$ precipitation. Cyclic [^{14}C]AMP was determined on a liquid scintillation spectrometer.

Protein was measured by the method of Lowry *et al.* (20) using bovine serum albumin as a standard.

Assay of Endogenous Protein Activator. The activity of the endogenous protein activator was determined (21) by measuring the degree of activation of a purified activator-deficient preparation of phosphodiesterase (22) elicited by various amounts of heat-inactivated supernatant fluids. Phosphodies-

Table 1. Effect of striatal membrane phosphorylation on the activation of adenylate cyclase by dopamine

Phosphorylation reaction	Adenylate cyclase activity (pmol of cAMP/mg of protein per min)			Activator release (units/mg of protein)
	Basal	1 μM Dopamine	10 μM Dopamine	
None	96 ± 10	153 ± 18	191 ± 24	88 ± 11
Protein kinase, no ATP, 0.5 μM cAMP	88 ± 9	149 ± 16	202 ± 27	93 ± 13
Protein kinase, ATP, 0.5 μM cAMP	87 ± 11	111* ± 17	146* ± 13	604† ± 38
Protein kinase, ATP, 1 μM cAMP	91 ± 14	102† ± 10	117† ± 9	953† ± 80

Rat caudate nucleus was homogenized in 9 volumes of 0.32 M sucrose, spun at 100,000 × *g* for 1 hr, and resuspended in an equal volume of sucrose. The membranes were phosphorylated in the standard incubation mixture described in *Methods*. Nonphosphorylated samples contained no ATP. The reaction, carried out at 30° for 5 min, was stopped by plunging the tubes into ice water. After centrifugation, the membranes were resuspended in 10 mM Tris-maleate buffer, pH 7.5, and adenylate cyclase activity was determined as described in *Methods*. The endogenous protein activator was measured in the heat-treated supernatant fluid, obtained by centrifugation of the phosphorylation reaction mixture, according to the procedure of Uzunov *et al.* (21). Each figure is the mean of 12 enzyme determinations (±SEM). Protein kinase, 160 μg, was purified from beef heart.

* $P < 0.05$ when compared with values obtained in absence of phosphorylation.

† $P < 0.01$ when compared with values obtained in absence of phosphorylation.

terase activity was measured by the method of Filburn and Karn (23). The incubation mixture, in a final volume of 100 μl, contained 20 μM cAMP (150,000–180,000 cpm ^3H), 32 mM Tris·HCl buffer, pH 8.0, 1 mM $MgSO_4$, 20 μM $CaCl_2$, 0.6 mM dithiothreitol, activator-deficient phosphodiesterase (0.23 μg/μl), and different quantities of various heated preparations containing the activator.

RESULTS

Effect of the release of the endogenous protein activator by phosphorylation of striatal membranes on the basal and dopamine-stimulated adenylate cyclase activity

Dopamine stimulated by about 2-fold the adenylate cyclase activity of a membrane preparation of striatum which was previously incubated in the phosphorylating medium lacking ATP (Table 1). Under these conditions, the amounts of endogenous protein activator released from these membranes into the supernatant were minimal (Table 1).

When striatal membranes were incubated in a phosphorylating medium, including ATP, the membrane content of the endogenous protein activator was reduced by less than 50% and its release into the supernatant fraction was increased by 10-fold (Table 1). As shown in the same table, the basal adenylate cyclase activity remained unchanged in the phosphorylated striatal membranes. However, phosphorylation and the release of the endogenous protein activator from striatal membranes inhibited the stimulation of adenylate cyclase by dopamine (1 μM and 10 μM). The reduction in the stimulation of adenylate cyclase activity by dopamine which occurs after phosphorylation can be correlated with the amount of activator released from the membranes. As shown in Table 1, the inhibition of the dopamine-elicited increase of adenylate cyclase activity was in reverse correlation with the endogenous activator released from the membrane. However, the readdition in the presence of 20 μM Ca^{++} of purified endogenous activator to the membrane preparations listed in Table 1 neither activated the membrane-bound adenylate cyclase nor facilitated the activation of the enzyme by dopamine (data now shown).

Effect of the release of the endogenous protein activator by phosphorylation of striatal membranes on the NaF stimulation of adenylate cyclase activity

Since NaF is believed to activate adenylate cyclase by a mechanism different from those described for peptide hormones and neurotransmitters (8), we studied whether the phosphorylation of striatal membranes also inhibited the NaF stimulation of adenylate cyclase activity. As shown in Table 2, the phosphorylation and associated depletion of the endogenous protein activator from the striatal membrane was followed by a decrease in the responsiveness of adenylate cyclase to NaF activation. The adenylate cyclase activity of normal striatal membranes was stimulated nearly 3-fold by 10 mM NaF. However, striatal membrane phosphorylation reduced the NaF stimulation without changing the basal adenylate cyclase activity. Again, as shown in Table 2, there was a clear correlation between the release of the endogenous protein activator and the inhibition of adenylate cyclase activation caused by NaF.

Adenylate cyclase is not a substrate for phosphorylation during the release of endogenous protein activator

A number of mechanisms can be considered to be a basis for the decrease in adenylate cyclase stimulation caused by phosphorylation of striatal membranes. We entertained the possibility that the change in the properties of striatal adenylate cyclase could be due to a phosphorylation of this enzyme. A membrane preparation from rat caudate nucleus was phosphorylated as described in the *Methods* using [γ-^{32}P]ATP. The supernatant, which contained the activator released by phosphorylation, was discarded. After incubation at 30° for 5 min the membranes were centrifuged at 30,000 × *g* for 20 min and resuspended in a buffer containing 20 mM Tris·HCl, pH 7.5, 250 mM sucrose, 1 mM $MgSO_4$, 0.5 mM dithiothreitol, and 1% Lubrol-PX. The solubilized membranes were applied to a Sephadex G-200 column equilibrated with the Tris buffer described above as reported by Lynch *et al.* (12). Fig. 1 shows the elution profile from a Sephadex G-200 column of the various proteins present in the membrane preparation after phosphorylation. About 95% of the proteins were eluted between fractions 25 and 50. The activities of the adenylate cyclase and that of the endogenous protein activator were eluted from the column simultaneously (fractions 25 to 38), suggesting that under our experimental conditions, without the addition of ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), adenylate cyclase and the endogenous protein activator may form a complex. However, most of the phosphorylated protein was eluted between fractions 66 and 76, which contain only about 2% of the total protein (Fig. 1). Little or no phosphorylation was present

Table 2. Effect of striatal membrane phosphorylation on the activation of adenylate cyclase by NaF

Phosphorylation reaction	Adenylate cyclase activity (pmol of cAMP/mg of protein per min)		Activator release (units/mg of protein)
	Basal	10 mM NaF	
None	99 ± 11	261 ± 38	74 ± 10
Protein kinase, no ATP, 0.5 μM cAMP	81 ± 9	205 ± 33	91 ± 13
Protein kinase, ATP, 0.5 μM cAMP	87 ± 11	166* ± 31	648† ± 55
Protein kinase, ATP, 1 μM cAMP	87 ± 10	136† ± 29	887† ± 93

A membrane preparation of rat caudate nucleus was phosphorylated, and the activities of both adenylate cyclase and protein activator were measured as described under Table 1, except that the adenylate cyclase assay was conducted at 37° for 10 min. Each figure is the mean of 10 enzyme determinations (±SEM). Protein kinase, 160 μg, was purified from beef heart.

* $P < 0.05$ when compared with values in absence of phosphorylation.

† $P < 0.01$ when compared with values in absence of phosphorylation.

in the fraction containing the activator or the adenylate cyclase (Fig. 1). Previous studies have shown that the activator could not be phosphorylated either *in vitro* or *in vivo* (12, 24). The possibility remains that the protein containing the ^{32}P could represent the binding site of the activator.

DISCUSSION

The experiments reported demonstrate that an endogenous Ca^{++}-binding protein stored in synaptic membranes of striatum regulates the stimulation of the adenylate cyclase activity by the putative neurotransmitter dopamine. Previous studies showed that in the presence of Ca^{++} this endogenous protein activates adenylate cyclase in detergent-dispersed preparations of this enzyme (9, 11, 12). Presumably, in the membrane preparation used in the present experiments to measure adenylate cyclase activity, the supramolecular structure that regulates the relationship existing in striatum between the dopamine recognition site and the adenylate cyclase is not disrupted. Under these conditions, the addition of purified endogenous protein activator to nonphosphorylated membranes did not facilitate the activation of adenylate cyclase by the neurotransmitter dopamine. This gives an indication that a certain functional interaction may exist between the enzyme and the endogenous activator protein in nonsolubilized preparations. Since our previous studies have shown that the endogenous protein which activates phosphodiesterase is stored in synaptic membranes and is released from these membranes by a cAMP-dependent phosphorylation (13, 14), we studied the effect of the release of the activator from its storage site in synaptic membranes on the activation of adenylate cyclase. We compared the adenylate cyclase activity of striatal membranes that had been phosphorylated *in vitro* to that of the intact nonphosphorylated membranes. We found that when the endogenous protein activator is released from the striatal membranes by previous phosphorylation the basal adenylate cyclase activity remains intact. However, the adenylate cyclase has virtually lost the capability to be stimulated by the transmitter dopamine as well as by NaF. The correlation between the release of the endogenous protein activator from synaptic membranes and the degree of impairment of adenylate cyclase activation by the transmitter supports the view that this protein might mediate the adenylate cyclase activation triggered physiologically by the occupancy of the postsynaptic receptor by the natural agonist. The fact that adenylate cyclase is not a substrate for phosphorylation excludes the possibility that this enzyme is regulated by phosphorylation and favors a number of other mechanisms. It is possible that the endogenous protein activator can control the cAMP concentration by a dual

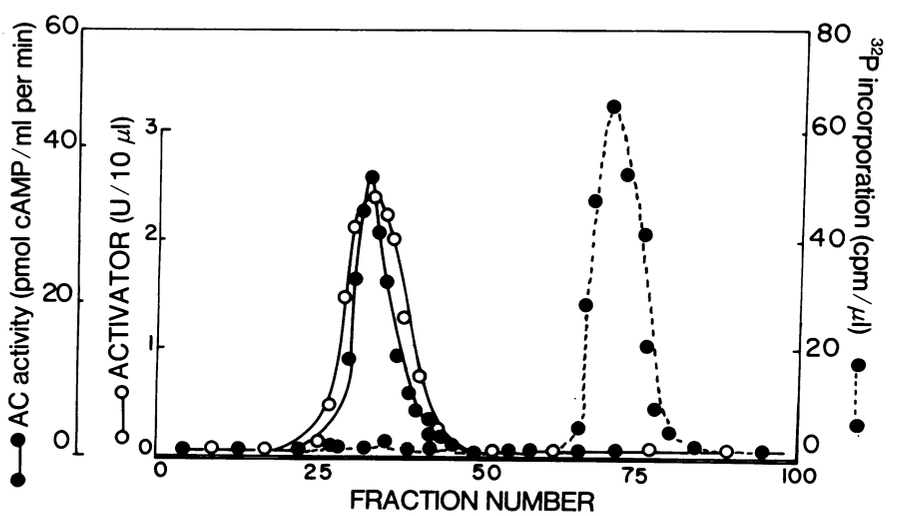

FIG. 1. One milliliter (5 mg) of a striatal membrane preparation solubilized with Lubrol-PX following its phosphorylation with [^{32}P]ATP was applied to a Sephadex G-200 column (1 × 50 cm) equilibrated with 20 mM Tris·HCl buffer, pH 7.5, 250 mM sucrose, 1 mM $MgSO_4$, 0.5 mM dithiothreitol, and 1% Lubrol-PX. The flow rate was adjusted to 15 ml/hr and 0.5 ml fractions were collected. Aliquots were assayed for adenylate cyclase (AC) activity, phosphodiesterase activator activity, and ^{32}P content. Activities per volume of eluate are given. One unit (U) of activator is defined as the amount of this protein which will increase the activity of an activator-deficient phosphodiesterase by 50% under the standard conditions of the assay as defined in *Methods*. The void volume was 10 ml.

mechanism: first, by mediating transmitter-dependent activation of adenylate cyclase while the protein activator is stored in the synaptic membrane, and second, following its release into the cytosol, by activating phosphodiesterase and increasing the metabolism of cAMP (25). The availability of the protein activator in the membrane and the translocation of this protein to the cytosol when the intracellular concentration of cAMP reaches certain levels most probably provides an efficient mechanism of controlling the cAMP response. This regulation of both adenylate cyclase and phosphodiesterase by the endogenous protein activator could be a basis for explaining why, in many tissues, the onset of cAMP increase is coupled to the stimuli, but its duration is not (26). Furthermore, it seems attractive to postulate that changes in the rate of synthesis, release, and metabolism of the endogenous protein activator might explain at least in part the phenomena of sub- and supersensitivity to neurotransmitters. Finally, the interaction of drugs with this protein may explain their capability of inhibiting second messenger responses.

Although the exact manner in which the endogenous protein activator interacts with adenylate cyclase is not known, the inhibition of the adenylate cyclase stimulation by dopamine and NaF following the release of the protein activator suggests that this protein may act beyond the dopaminergic receptor. With different brain structures, preliminary results have indicated that membrane phosphorylation and activator release also reduce the stimulation of adenylate cyclase by Gpp(NH)p. This could indicate that the endogenous protein activator participates in the chain of events that converts the adenylate cyclase from a ground state to a highly activated state (6, 8).

1. Klainer, L. M., Chi, Y.-M., Freidberg, S. L., Rall, T. W. & Sutherland, E. W. (1962) *J. Biol. Chem.* **237,** 1239–1243.
2. Birnbaumer, L., Pohl, S. L. & Rodbell, M. (1971) *J. Biol. Chem.* **246,** 1857–1860.
3. DeHaen, C. (1974) *J. Biol. Chem.* **249,** 2756–2762.
4. Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J. & Rodbell, M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3087–3090.
5. Lin, M. C., Salomon, Y., Rendell, M. & Rodbell, M. (1975) *J. Biol. Chem.* **250,** 4246–4252.
6. Cuatrecasas, P., Jacobs, S. & Bennett, V. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1739–1743.
7. Rendell, M., Salomon, Y., Lin, M. C., Rodbell, M. & Berman, M. (1975) *J. Biol. Chem.* **250,** 4253–4260.
8. Rodbell, M. (1975) *J. Biol. Chem.* **250,** 5826–5834.
9. Brostrom, C. O., Huang, Y.-C., McL. Breckenridge, B. & Wolff, D. H. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 64–68.
10. Cheung, W. Y. (1971) *J. Biol. Chem.* **246,** 2859–2869.
11. Cheung, W. Y., Bradham, L. S., Lynch, T. J., Lin, Y. M. & Tallant, E. A. (1975) *Biochem. Biophys. Res. Commun.* **66,** 1055–1062.
12. Lynch, J. J., Tallant, E. A. & Cheung, W. Y. (1976) *Biochem. Biophys. Res. Commun.* **68,** 616–625.
13. Gnegy, M. E., Uzunov, P. & Costa, E. (1976) *Fed. Proc.* **53,** 609.
14. Gnegy, M. E., Costa, E. & Uzunov, P. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 352–355.
15. Kebabian, J. W., Petzold, G. L. & Greengard, P. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 2145–2149.
16. Mishra, R. K., Gardner, E. L., Katzman, R. & Makman, M. H. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3883–3887.
17. Clement-Cormier, Y. C., Parrish, R. G., Petzold, G. L., Kebabian, J. W. & Greengard, P. (1975) *J. Neurochem.* **25,** 143–149.
18. Revuelta, A., Uzunov, P. & Costa, E. (1976) *Neurochem. Res.* **1,** 212–217.
19. Krishna, G., Weiss, B. & Brodie, B. B. (1968) *J. Pharmacol. Exp. Ther.* **163,** 379–385.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
21. Uzunov, P., Revuelta, A. V. & Costa, E. (1975) *Mol. Pharmacol.* **11,** 506–510.
22. Uzunov, P. & Weiss, B. (1972) *Biochim. Biophys. Acta* **284,** 220–226.
23. Filburn, C. R. & Karn, J. (1973) *Anal. Biochem.* **52,** 505–516.
24. Uzunov, P., Gnegy, M. E., Lehne, R., Revuelta, A. & Costa, E. (1976) *Adv. Biochem. Psychopharmacol.* **15,** 283–301.
25. Uzunov, P., Lehne, R., Revuelta, A., Gnegy, M. E. & Costa, E. (1976) *Biochim. Biophys. Acta* **422,** 326–334.
26. Guidotti, A., Zivkovic, B., Pfeiffer, R. & Costa, E. (1973) *Naunyn-Schmiedebergs Arch. Pharmakol.* **278,** 195–206.