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Inhibition of a Low K_m GTPase Activity in Rat Striatum by Calmodulin

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Abstract: In rat striatum, the activation of adenylate cyclase by the endogenous Ca^{2+}-binding protein, calmodulin, is additive with that of GTP but is not additive with that of the nonhydrolyzable GTP analog, guanosine-5'-(β, γ-imido)triphosphate (GppNHp). One possible mechanism for this difference could be an effect of calmodulin on GTPase activity which has been demonstrated to "turn-off" adenylate cyclase activity. We examined the effects of Ca^{2+} and calmodulin on GTPase activity in EGTA-washed rat striatal particulate fractions depleted of Ca^{2+} and calmodulin. Calmodulin inhibited GTP hydrolysis at concentrations of 10^{-9}–10^{-6} *M* but had no effect on the hydrolysis of 10^{-5} and 10^{-6} *M* GTP, suggesting that calmodulin inhibited a low K_m GTPase activity. The inhibition of GTPase activity by calmodulin was Ca^{2+}-dependent and was maximal at 0.12 μ*M* free Ca^{2+}. Maximal inhibition by calmodulin was 40% in the presence of 10^{-7} *M* GTP. The IC_{50} for calmodulin was 100 n*M*. In five tissues tested, calmodulin inhibited GTP hydrolysis only in those tissues where it could also activate adenylate cyclase. Calmodulin could affect the activation of adenylate cyclase by GTP in the presence of 3,4-dihydroxyphenylethylamine (DA, dopamine). Calmodulin decreased by nearly 10-fold the concentration of GTP required to provide maximal stimulation of adenylate cyclase activity by DA in the striatal membranes. The characteristics of the effect of calmodulin on GTPase activity with respect to Ca^{2+} and calmodulin dependence and tissue specificity parallel those of the activation of adenylate cyclase by calmodulin, suggesting that the two activities are closely related. Inhibition of a low K_m GTPase activity by calmodulin could represent an action of calmodulin in increasing the association of a GTP-binding protein with the catalytic subunit activity resulting in a reduction of the "turn-off" GTPase activity.
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Adenylate cyclase (EC 4.6.1.1) activity in mammalian brain is regulated by several effectors including neurotransmitters, guanyl nucleotides, and calcium (Kebabian, 1977). Binding of GTP to a guanyl nucleotide-binding protein (N) transduces the signal from the neurotransmitter receptor to the adenylate cyclase catalytic subunit (Rodbell, 1980; Limbird, 1981). Activation or attenuation of adenylate cyclase activity through receptor occupation is mediated by distinct guanyl nucleotide-binding proteins (Hildebrandt et al., 1983). Ca^{2+} activates adenylate cyclase activity by binding to the endogenous Ca^{2+}-binding protein, calmodulin (CaM) (Brostrom et al., 1975; Cheung et al., 1975). In rat striatum, in addition to stimulating basal adenylate cyclase activity, CaM increased the sensitivity of adenylate cyclase to 3,4-dihydroxyphenylethylamine (DA, dopamine) three- to fourfold (Gnegy and Treisman, 1981). *In vivo* studies have demonstrated a relationship between CaM content in rat striatal membranes and the sensitivity of DA receptors (Gnegy et al., 1980; Gnegy, 1982; Memo et al., 1982).

Several studies have shown that adenylate cyclase in brain consists of at least two components, one of which is stimulated by CaM and submicromolar concentrations of Ca^{2+} (Brostrom et al., 1977; Heideman et al., 1982; Treisman et al., 1983). The two components have different sensitivities to activation by guanyl nucleotides (Brostrom et al., 1978; Treisman et al., 1983). The molecular mechanism by which CaM affects either basal or DA-stimulated adenylate cyclase activity in the striatum is not known. Several studies have suggested that

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Abbreviations used: AppNHp, Adenosine-5'-(β, γ-imido)triphosphate; CaM, Calmodulin; DA, 3,4-Dihydroxyphenylethylamine (dopamine); GppNHp, Guanosine-5'-(β, γ-imido)triphosphate; NTPase, Nucleotide triphosphatase.

CaM acts directly at the catalytic subunit of adenylate cyclase (Salter et al., 1981; Heideman et al., 1982). Synergistic interactions between CaM and guanyl nucleotides, however, have been reported (Brostrom et al., 1978; Heideman et al., 1982). Brostrom et al. (1978) found that CaM decreased the concentration of GTP required for maximal activation of the CaM-dependent adenylate cyclase component. The effect of CaM on the sensitivity of adenylate cyclase for the nonhydrolyzable GTP analog, guanosine-5′-(β, γ-imido)triphosphate (GppNHp), was less pronounced than that for GTP. In rat striatal membranes, we found that the activation of adenylate cyclase by CaM in the presence of GTP differed from that in presence of GppNHp (Treisman et al., 1983). Activation of striatal adenylate cyclase by CaM was additive with activation by GTP. In contrast, kinetic analysis suggested that CaM and GppNHp were competitive activators at the CaM-sensitive component of adenylate cyclase (Treisman et al., 1983). One explanation for the difference in the interaction of CaM with GppNHp as compared to GTP could be that CaM is affecting GTP hydrolysis.

Cassel and Selinger (1976, 1977) demonstrated that the hydrolysis of GTP to GDP through a specific GTPase (EC 3.6.1.-) activity represents a "turn-off" mechanism for catecholamine-stimulated adenylate cyclase systems. Many hormones that either stimulate or inhibit adenylate cyclase activity have been shown to stimulate GTPase activity (Jakobs, 1979; Limbird, 1981). Cholera toxin, on the other hand, strongly activates adenylate cyclase activity but inhibits hormone-stimulated GTPase activity (Cassel and Selinger, 1977). Although there are many GTPase- and nonspecific nucleotide triphosphatase (NTPase)-related activities in membranes the adenylate cyclase-coupled GTPase activity can be assessed by its high affinity for GTP. Its apparent K_a for GTP is between 10^{-7} *M* and 10^{-6} *M* (Cassel and Selinger, 1976; Koski and Klee, 1981). We investigated the capacity of CaM and Ca^{2+} to affect GTPase activity in a rat striatal particulate fraction. We found that CaM inhibited GTP hydrolysis at low concentrations of GTP in a Ca^{2+}-dependent manner but did not affect GTP hydrolysis at concentrations $>10^{-6}$ *M*. The inhibition was dose-dependent with respect to Ca^{2+} and CaM and was specific to tissues where CaM can stimulate adenylate cyclase activity. CaM decreased the concentration of GTP required to provide maximal stimulation of adenylate cyclase activity by DA in rat striatal membranes. Inhibition of a low K_m GTPase activity by CaM could represent an effect of CaM to increase the association of the GTP-binding protein (N) with the catalytic subunit of adenylate cyclase and effectively increase GTP-mediated adenylate cyclase activity.

MATERIALS AND METHODS

Materials.

Male, Sprague-Dawley rats (175–200 g) [strain Crl:CD(SD)BR] were obtained from Charles River, Wilmington, MA. [γ-^{32}P]GTP (26 Ci/mmol) and [α-^{32}P]ATP (54 Ci/mmol) were purchased from Amersham, Searle. Creatine phosphokinase, creatine phosphate, ouabain, and dithiothreitol were purchased from Sigma Chemical. HPLC-purified GTP, ATP, adenosine-5′-(β, γ-imido)triphosphate (AppNHp) and GppNHp were purchased from International Chemical and Nuclear. Phenyl Sepharose resin was obtained from Pharmacia. PEI cellulose thin-layer plates on plastic backing were purchased from E. M. Reagents. All other materials were the highest commerically available grade reagents.

Purification of CaM

CaM was purified by a modification of the method of Gopalakrishna and Anderson (1982) and Dedman et al. (1977) developed in collaboration with Dr. Michael J. Welsh, Department of Anatomy, The University of Michigan. A bovine brain was homogenized in a blender in 10 volumes of 50 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* EGTA. The homogenate fraction was heated to 95°C for 1 min and then centrifuged at 20,000 × *g* for 30 min. The resulting pellet was extracted with the above buffer, pelleted again, and the supernatant fractions were mixed together. The supernatant fraction was fractionated between 40 and 100% saturation using solid ammonium sulfate and centrifuged at 15,000× *g*. The resulting pellet was resuspended in 50 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* $CaCl_2$ with no EGTA (Buffer A). The material was dialyzed for 14 h against Buffer A with three changes and applied to a phenyl Sepharose column (1.5 × 20 cm) equilibrated in Buffer A. After washing the column with 10 volumes of Buffer A, protein was further eluted with Buffer A containing 0.5 *M* NaCl until no ultraviolet absorbance was detectable. CaM was then eluted from the column with 50 m*M* Tris-HCl buffer, pH 7.5, containing 50 m*M* EGTA with no $CaCl_2$ (Buffer B). This fraction was applied to a DEAE column (1.0 × 20 cm) equilibrated with Buffer B. After washing with 10 bed volumes of Buffer B, CaM was eluted with Buffer B containing a gradient of 0–500 m*M* NaCl. CaM eluted at a salt concentration of approximately 300 m*M* NaCl. The CaM was highly purified and gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with an M_r of 16,700. Silver stain of the gels revealed impurities of <0.01%. The CaM was extensively dialyzed against 0.05 *M* ammonium bicarbonate, lyophilized, and stored at −80°C. It remains stable for over a year.

Particulate preparation

Striatal particulate fractions depleted of Ca^{2+} and CaM were prepared as described (Gnegy and Treisman, 1981). In brief, rats were sacrificed by decapitation; the striata were removed and homogenized in 9 volumes of 10 m*M* Tris-maleate buffer, pH 7.5, containing 1 m*M* $MgSO_4$ and 1.2 m*M* EGTA. The homogenate fraction 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 m*M* Tris-maleate buffer, pH 7.5, containing 1.5 m*M*

EGTA and 1 m*M* $MgSO_4$. Protein content was determined by the method of Lowry et al. (1951). EGTA-washed particulate fractions from bovine retina, and rat liver and testis were prepared in this manner. Rat adipocytes were graciously prepared by Dr. Christin Carter-Su, Department of Physiology, University of Michigan. Fat cells were isolated from the epididymal adipose tissue of 150–200 g male Sprague-Dawley rats by collagenase digestion according to the method of Rodbell (1966). The adipocytes were homogenized and an EGTA-washed particulate fraction was prepared as described above.

GTPase assay

GTPase activity was assayed in the EGTA-washed particulate fractions using[γ-^{32}P]GTP according to the method of Cassel and Selinger (1976). The assay contents were altered to be more similar to those used in the adenylate cyclase assay (Gnegy and Treisman, 1981). Assay contents, in a volume of 100 μl, were: 12.5 m*M* Tris-HCl buffer, pH 7.5; 5 m*M* $MgCl_2$; 1 m*M* ATP; 1 m*M* AppNHp; 1 m*M* creatine phosphate; 5 units creatine phosphokinase; 1 m*M* ouabain; 2 m*M* dithiothreitol; 5 μg membrane protein; 150 μ*M* EGTA; and 50,000 cpm [γ-^{32}P]GTP plus various additions such as nonradioactive GTP, Ca^{2+}, and CaM. Incubations were conducted for 10 min at 37°C. Assays were terminated and free $^{32}P_i$ was determined as described by Cassel and Selinger (1976).

Assay products were analyzed by PEI cellulose TLC (McSwigan et al., 1980) to ensure that the activity we observed was a GTPase activity as opposed to guanylate cyclase activity or pyrophosphate cleavage. Experiments were carried out as described for the GTPase assay except that [α-^{32}P]GTP was used in place of [γ-^{32}P]GTP. Thin layers were developed using 1 *M* formate containing 0.25 *M* LiCl and all samples were run with cold carriers containing 0.5 *M* GTP, GDP, cyclic GMP, GMP, and guanosine. Carrier spots were visualized under ultraviolet light and cut out. The nucleotides were eluted in 1 *M* $MgCl_2$ and counted in a liquid scintillation counter. Under assay conditions (10 min at 37°C), >95% of the counts were found to correspond to GTP or GDP with <2% as cyclic GMP or GMP. With assay times >1 h, production of GMP and guanosine became significant.

Adenylate cyclase activity

Adenylate cyclase activity was determined in the EGTA-washed particulate fractions as described by Gnegy and Treisman (1981). Incubations were conducted for 10 min at 37°C.

Determination of free Ca^{2+} concentration

Free Ca^{2+} was calculated using a dissociation constant for Ca-EGTA of 4.08×10^{-8} *M* according to the method of Nanninga and Kempen (1971).

RESULTS

Effect of CaM on GTPase activity in rat striatal membranes

Figure 1 illustrates an isotopic dilution curve for GTPase activity. Increasing amounts of nonradioactive GTP decreased the formation of $^{32}P_i$ from [γ-^{32}P]GTP. This represents a concentration-dependent increase in GTPase activity. The GTPase activity at concentrations of GTP $>10^{-4}$ *M* represent activity of nonspecific NTPases (Cassel and Selinger, 1976).

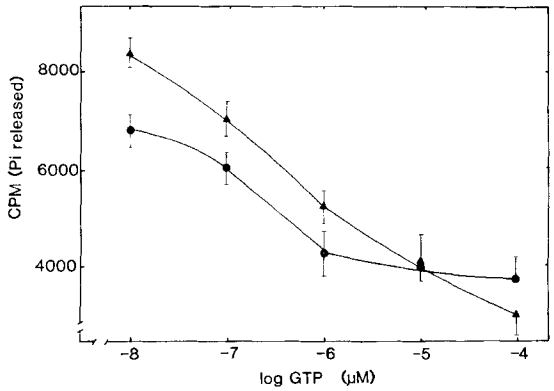

FIG. 1. Effect of CaM on isotope dilution curve for GTPase activity. GTPase activity was determined in a striatal particulate fraction as described in Materials and Methods in the absence (▲) and presence of 0.12 μ*M* free Ca^{2+} (125 μ*M* $CaCl_2$ in the assay) and 560 n*M* CaM (●). Each assay contained 10 n*M* [γ-^{32}P]GTP (50,000 cpm) plus various concentrations of unlabeled GTP. Each point represents the mean ± SEM for three separate experiments performed in triplicate.

As shown in Fig. 1, 560 n*M* CaM, in the presence of 0.12 μ*M* free Ca^{2+}, decreased the hydrolysis of low concentrations of GTP. CaM significantly inhibited GTPase activity at concentrations of GTP from 10 n*M* to 1 μ*M*. CaM appeared to have no effect on the activity of nonspecific NTPases, as it did not alter GTPase activity at 10 μ*M* or 100 μ*M* GTP. The activity of the low K_m activity can be closely approximated by correcting for the hydrolysis of GTP by high K_m NTPase activity (Cassel and Selinger, 1976; Koski and Klee, 1981). In the following experiments, low K_m GTPase activity was calculated as the difference between the number of cpm of $^{32}P_i$ released in the absence and presence of 100 μ*M* GTP (Cassel and Selinger, 1977; Koski and Klee, 1981).

Dose-dependent effect of Ca^{2+} and CaM on GTP hydrolysis

The inhibition of GTPase activity by CaM required the presence of Ca^{2+}. As shown on the ordinate in Fig. 2, 560 n*M* CaM, in the absence of Ca^{2+}, did not affect the hydrolysis of 10^{-7} *M* GTP. Similarly, Ca^{2+}, in the absence of CaM, had no significant effect on GTP hydrolysis. In the presence of 560 n*M* CaM, however, there was a dose-dependent decrease in GTP hydrolysis as a function of the free Ca^{2+} concentration (Fig. 2). Inhibition was maximal at 0.12 μ*M* free Ca^{2+} (corresponding to 125 μ*M* $CaCl_2$ in the assay). This is the maximally effective Ca^{2+} concentration for activation of adenylate cyclase by CaM in the same striatal membrane preparation (Gnegy and Treisman, 1981). The

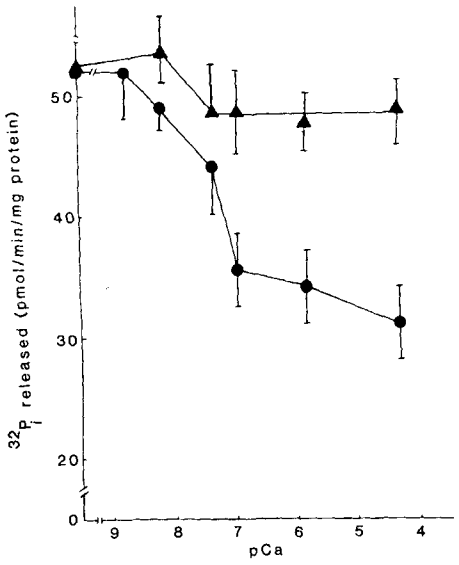

FIG. 2. Ca^{2+} dependence of the inhibition of GTP hydrolysis by CaM. The hydrolysis of 10^{-7} *M* GTP was measured as described in Materials and Methods in the presence of various concentrations of Ca^{2+} alone (▲) or Ca^{2+} plus 560 n*M* CaM (●). The pCa was calculated as described in Materials and Methods. Each point represents the mean ± SEM for three separate experiments performed in triplicate.

inhibitory effects of CaM did not change at concentrations of Ca^{2+} >0.12μ*M*.

Inhibition of GTP hydrolysis was increased as a function of the CaM concentration. The dose-dependent effect of CaM in inhibiting GTPase activity measured at 10^{-7} *M* GTP demonstrated that maximal inhibition of GTPase activity was achieved at 560 n*M* CaM (Fig. 3). The IC_{50} for CaM in inhibiting GTP hydrolysis was approximately 100 n*M*. This is not an exact measure of the inhibition constant because we cannot deplete all of the CaM from the striatal membranes (Gnegy and Treisman, 1981). The maximum inhibition of the low K_m GTPase activity by CaM was 40%.

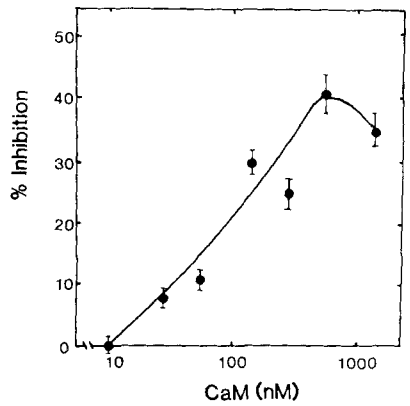

FIG. 3. Dose-dependent inhibition of the GTPase activity by Ca^{2+} and CaM. The hydrolysis of 10^{-7} *M* GTP was measured in a striatal particulate fraction as described in Materials and Methods. Assays contained 100 μ*M* $CaCl_2$ (0.12 μ*M* free Ca^{2+}). Basal GTPase activity was 25 pmol/min/mg protein. Each point represents the value ± SEM for three determinations.

Tissue specificity of the CaM inhibition of GTPase activity

CaM-stimulated adenylate cyclase activity is found predominantly in nervous tissue such as brain, spinal cord (Wang and Waisman, 1979), retina (Gnegy et al., 1984), and some secretory tissues (Valverde et al., 1979; Brostrom et al., 1982). On the other hand, Ca^{2+} and CaM do not activate adenylate cyclase activity in many peripheral tissues such as liver (Leoni et al., 1978). To assess whether the inhibition of GTP hydrolysis by CaM was selective for tissues in which CaM stimulated adenylate cyclase activity, we examined the capacity of CaM to inhibit GTPase activity in EGTA-washed particulate fractions from five tissues: rat striatum, liver, testis, adipocytes, and bovine retina. Under our assay conditions, CaM stimulated adenylate cyclase activity in striatum and retina but not in liver, testis, or adipocytes (Fig. 4, top). Although basal adenylate cyclase activity was low in liver and testis, 100 μ*M* GppNHp readily stimulated the activity (Fig. 4, top), as did forskolin (data not shown). The effect of CaM on GTP hydrolysis paralleled the capacity of CaM to activate adenylate cyclase. As shown in Fig. 4, bottom, CaM inhibited the hydrolysis of 10^{-7} *M* GTP in striatum and retina but did not have an inhibitory effect on GTPase activity in liver, testis, or adipocytes. Interestingly, CaM and 150 μ*M* Ca^{2+} inhibited adenylate cyclase activity and slightly increased GTPase activity in liver and testis particulate fractions.

Effect of CaM on the capacity of GTP to activate DA-sensitive adenylate cyclase activity

One might expect that CaM would increase the activation of adenylate cyclase by GTP if CaM directly inhibited GTPase activity. In previous studies, however, we had not been able to demonstrate a potentiating effect of CaM on the activation of adenylate cyclase by GTP in rat striatal membranes (Treisman et al., 1983). Since CaM could increase the sensitivity of adenylate cyclase for DA, it is possible that Ca^{2+} and CaM could potentiate the capacity of GTP to support stimulation by DA. We examined whether CaM would affect the concentration of GTP required to attain maximal stimulation of adenylate cyclase activity by DA. Figure 5A illustrates the dose-dependent stimulation of adenylate cyclase activity by GTP in the presence of 50 μ*M* DA, 0.12 μ*M* free Ca^{2+}, and 560 n*M* CaM. Ca^{2+} and CaM did not significantly alter the capacity of GTP to activate adenylate cyclase activity in the absence of DA. Consideration of the circular symbols in Fig. 5A demonstrates that GTP is re-

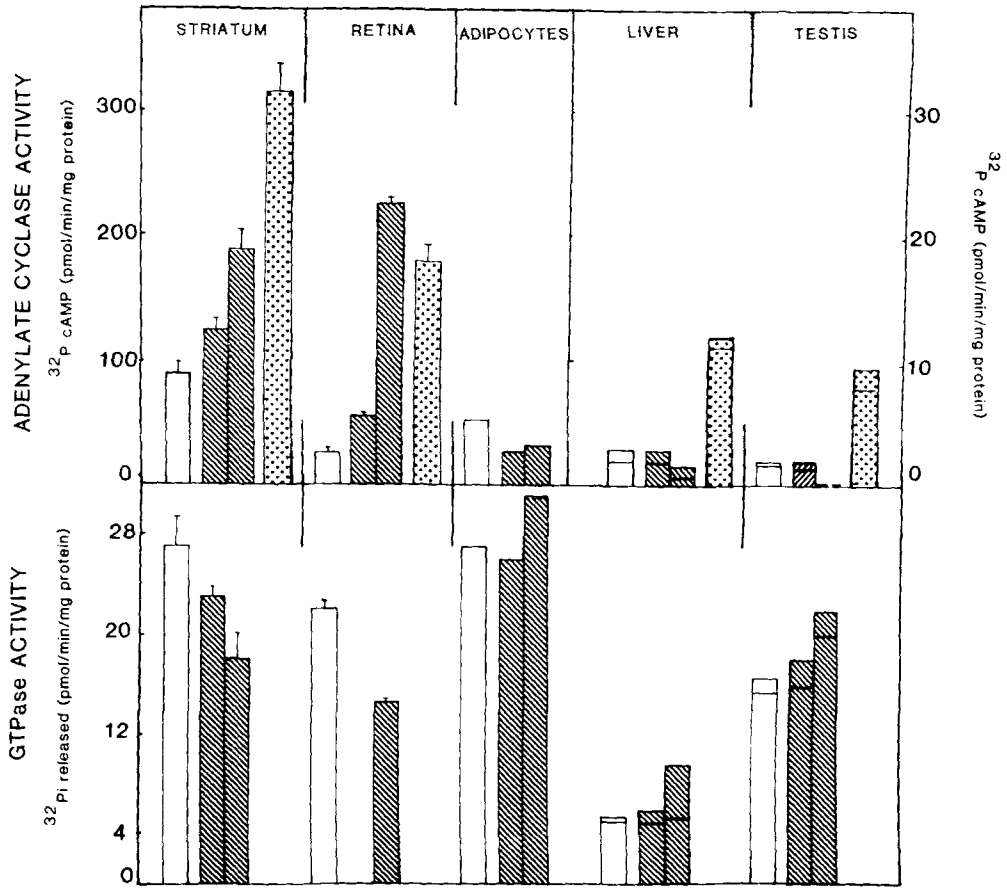

FIG. 4. Top: Effects of CaM and GppNHp on adenylate cyclase activity from bovine retina and rat striatum, liver, testis, and adipocytes. EGTA-washed particulate fractions were prepared as described in Materials and Methods. Adenylate cyclase activity was measured at a tissue protein concentration of 0.6 mg/ml in the presence of the following additions: none (open bars); 560 n*M* CaM and 50 μ*M* $CaCl_2$ (6.6 n*M* free Ca^{2+}) (first cross-hatched bars); 280 n*M* CaM and 125 μ*M* $CaCl_2$ (120 n*M* free Ca^{2+}) (second cross-hatched bars); and 100 μ*M* GppNHp (stippled bars). Results are given ± SEM for five determinations in striatum and three determinations in retina. Two experiments were performed using liver and testis; for these results, the horizontal bars represent the results from the two trials. For adipocytes, the value is the average of triplicate determinations. **Bottom:** Effect of CaM on GTPase activity in bovine retina and rat striatum, liver, testis, and adipocytes. The hydrolysis of 10^{-7} *M* GTP was measured in EGTA-washed particulate fractions of the various tissues prepared as described in Materials and Methods with the following additions: none (open bars); 560 n*M* CaM and 125 μ*M* $CaCl_2$ (120 n*M* free Ca^{2+}) (first cross-hatched bars); 560 n*M* CaM and 150 μ*M* $CaCl_2$ (1.6 μ*M* free Ca^{2+}) (second cross-hatched bars). The number of determinations are the same as described for the top figure.

quired for activation of adenylate cyclase by DA in the rat striatal particulate fraction. Activation of adenylate cyclase by 50 μ*M* DA, a maximally activating concentration (Gnegy and Treisman, 1981), becomes significant at 0.5 μ*M* and is maximal at 5 μ*M* (Fig. 5A). On the other hand, consideration of the triangular symbols in Fig. 5A reveals that in the presence of Ca^{2+} and CaM, 50 μ*M* DA significantly stimulated adenylate cyclase activity at 0.1 μ*M* GTP and that activation by DA was maximal at 0.5–1 μ*M* GTP. The effect of CaM on the activation of adenylate cyclase by 50 μ*M* DA as a function of the GTP concentration is shown more clearly in Fig. 5B. The apparent K_a for GTP in supporting maximal stimulation of adenylate cyclase by 50 μ*M* DA is 0.6 μ*M* in the absence of Ca^{2+} and CaM and 0.07 μ*M* in the presence of Ca^{2+} and CaM. Thus Ca^{2+} and CaM increase the effectiveness of GTP in supporting DA activation of adenylate cyclase activity by nearly 10-fold.

DISCUSSION

Cassel and Selinger (1976) have proposed that the hydrolysis of GTP to GDP is a "turn-off" mechanism that returns activated adenylate cyclase to a basal inactivated state (see Fig. 6). The GTP hydrolysis related to adenylate cyclase activity occurs at low concentrations of GTP and is affected by several hormones and toxins, such as cholera toxin, which modulate adenylate cyclase activity (Limbird, 1981).

We have demonstrated that the endogenous Ca^{2+}-binding protein, CaM, inhibits the hydrolysis of concentrations of GTP <1 μ*M* in a rat striatal particulate fraction but has no effect on hydrolysis

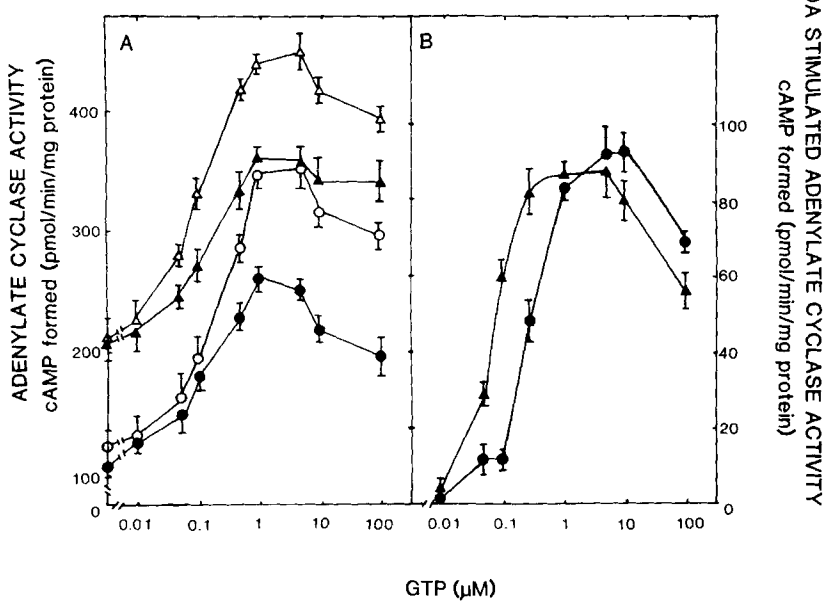

FIG. 5. A: GTP-dependent activation of adenylate cyclase activity in rat striatal particulate fractions in the presence of CaM and DA. Adenylate cyclase activity was measured in EGTA-washed striatal particulate fractions as described in Materials and Methods at various concentrations of GTP in the absence (●) and presence of 50 μ*M* DA (○); 560 n*M* CaM and 0.12 μ*M* free Ca^{2+} (▲); and 50 μ*M* DA, 560 n*M* CaM, and 0.12 μ*M* free Ca^{2+} (△). Each point represents the mean ± SEM for four separate experiments. **B:** Effect of CaM on the GTP concentration dependence of DA-stimulated adenylate cyclase activity. The DA-stimulated adenylate cyclase activity was calculated from the data presented in (A). DA-stimulated adenylate cyclase activity was calculated as the pmol/min/mg protein of cyclic AMP formed in the presence of 50 μ*M* DA above that formed in the presence of GTP alone (●) or GTP, 560 n*M* CaM, and 0.12 μ*M* Ca^{2+} (125 μ*M* $CaCl_2$ in the assay) (▲). Values for DA-dependent adenylate cyclase activity were determined from four separate experiments and expressed as the mean ± SEM.

of higher concentrations of GTP. Several lines of evidence suggest that the inhibition of GTPase activity by CaM is related to the activation of adenylate cyclase by CaM. First, Ca^{2+} was required for the inhibition of GTPase activity by CaM and is similarly required for CaM to activate adenylate cyclase in striatal membranes (Gnegy and Treisman, 1981). The maximally effective concentration of free Ca^{2+} required for CaM to both inhibit GTPase and stimulate adenylate cyclase activities is 0.12 μ*M*.

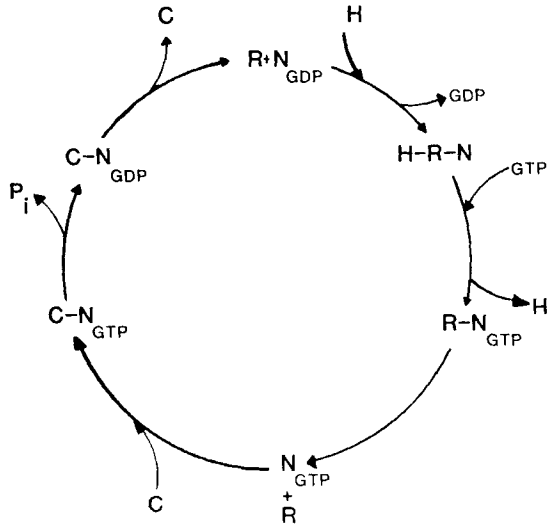

FIG. 6. Model depicting the hormone-mediated increase in adenylate cyclase activity. For a review see Limbird (1981). The receptor (R) can associate with a GTP-binding protein (N) containing GDP at the active site. Activation of R by a hormone or neurotransmitter (H) such as DA dislodges GDP and permits occupation of N by active GTP. After dissociation of R, N-GTP can associate with the catalytic subunit (C) and activate adenylate cyclase. The reaction can be turned off by hydrolysis of GTP to GDP, returning the system to a basal state.

Second, the concentration dependence for CaM is comparable for both enzyme activities. The IC_{50} for CaM in the inhibition of GTPase activity was 100 n*M* whereas the apparent K_a for CaM in activation of adenylate cyclase was 70 n*M* at the maximally effective free Ca^{2+} concentration of 0.12 μ*M* (Gnegy and Treisman, 1981). Third, the effect of CaM on GTPase activity appears to be selective for tissues where CaM activates adenylate cyclase activity. CaM both activated adenylate cyclase activity and inhibited GTP hydrolysis in tissues such as rat striatum and bovine retina but had no effect on these enzymes under similar conditions in rat liver, testis, and adipocytes.

CaM did not inhibit completely the hydrolysis of low concentrations of GTP. The level of inhibition of GTPase activity by CaM is comparable to the degree to which hormones and certain toxins have been demonstrated to affect GTPase activity in crude membranes. Neurotransmitters, such as DA (Onali et al., 1983) and opiates (Koski and Klee, 1981; Koski et al., 1982), have been shown to increase GTPase activity. On the other hand, toxins such as cholera toxin (Cassel and Pfeuffer, 1978) or the *Bordetella pertussis* toxin (Katada et al., 1982) inhibit hydrolysis of low concentrations of GTP from 30 to 50%. The GTPase activity measured at low concentrations of GTP in the striatal mem-

branes most likely represents more that one GTPase activity (Aktories et al., 1982). It has been demonstrated that cholera toxin can more completely inhibit a specific catecholamine-stimulated GTPase activity in turkey erythrocyte membranes that is associated with the 45,000 dalton stimulatory N protein (Cassel and Selinger, 1977; Hildebrandt et al., 1983). We attempted to assess the effect of CaM on DA-stimulated GTPase activity. We were unable to reliably measure activation of GTPase by DA in our striatal particulate preparation. The activation of GTPase activity at 10^{-8}–10^{-6} *M* GTP by 100 μM DA was only 10–20%, and because of variability was not always statistically significant. Improved assay conditions or membrane preparations are being sought to assess this activity.

CaM may be selectively inhibiting a particular GTPase activity at one of the components of adenylate cyclase in striatum (Brostrom et al., 1978; Treisman et al., 1983). The inhibition of GTP hydrolysis by CaM might be more complete at the CaM-sensitive component of adenylate cyclase activity. This could explain the fact that CaM did not potentiate the activation of adenylate cyclase by GTP in the striatal particulate fraction. Our previous studies showed that activation of adenylate cyclase by CaM and GTP in striatum appears to represent two independent components of adenylate cyclase activity (Treisman et al., 1983). GppNHp, on the other hand, could activate both components. GDP may be tightly bound at a guanyl nucleotide-binding protein (N) that affects the CaM-sensitive component of adenylate cyclase activity and may become dissociated only when DA binds to the receptor. Our study showed that CaM did increase a GTP-mediated activation of adenylate cyclase but the effect was apparent only in the presence of DA.

We do not know the mechanism by which CaM inhibits GTPase activity. Cholera toxin and *Bordetella pertussis* toxin, for instance, inhibit GTP hydrolysis through ADP ribosylation of the stimulatory and the inhibitory GTP-binding proteins, Ns and Ni, respectively (Cassel and Pfeuffer, 1978; Katada and Ui, 1982). Evidence suggests that CaM acts at the catalytic subunit (C) of adenylate cyclase (Salter et al., 1981) and does not require GTP to stimulate adenylate cyclase activity (Seamon and Daly, 1982; Heideman et al., 1982). A schema depicting our present knowledge of hormone and neurotransmitter activation of adenylate cyclase activity is shown in Fig. 6 (Limbird, 1981). Receptor occupation by the neurotransmitter leads to a displacement of GDP at the N protein by GTP. A subunit of N then binds in some manner to the catalytic subunit and activates adenylate cyclase activity. A specific GTPase activity can then reset the cycle by hydrolyzing GTP to GDP, leading to the dissociation of N and the catalytic subunit. Since purified Ns did not demonstrate GTPase activity (Sternweiss et al., 1981) it has been proposed that the GTPase activity resides at the association of GTP-occupied N and C. Recently, however, Brandt et al. (1983) have demonstrated catecholamine-stimulated GTPase activity in phospholipid vesicles containing purified β-adrenergic receptor and Ns purified from liver. Assuming that the GTPase inhibited by CaM is related to adenylate cyclase, CaM may act at C to increase the association between GTP-N and C. In this manner CaM could potentiate the DA receptor-mediated stimulation of adenylate cyclase activity by GTP and inhibit GTPase activity. In the absence of GTP or hormone the association of CaM with C itself will activate adenylate cyclase activity. GppNHp, the nonhydrolyzable analog of GTP, causes an "irreversible" activation of adenylate cyclase activity (Limbird, 1981) and strongly inhibits GTPase activity (Cassel and Selinger, 1977). We have shown that activation of adenylate cyclase by GppNHp is competitive with that of CaM in rat striatal membranes. GppNHp-occupied N may bind to C in the striatum in such a manner as to prohibit or mask the action of CaM.

We cannot discount the possibility, however, that CaM may be acting at an inhibitory GTP-binding protein, Ni, preventing it from attenuating adenylate cyclase activity. This could lead to a decrease in a GTPase activity that may occur at Ni (Aktories et al., 1982). Purification of the coupling components and reconstitution with a CaM-sensitive adenylate cyclase activity will be required to determine the molecular events involved in the inhibition of GTPase activity by CaM and Ca^{2+}.

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