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GTP-PREFERRING PROTEIN PHOSPHORYLATION SYSTEMS IN BRAIN MEMBRANES: POSSIBLE ROLE IN ADENYLATE CYCLASE REGULATION

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Preincubation of brain membranes with GTP under phosphorylating conditions resulted in activation of adenylate cyclase which withstood sedimentation and washing. Investigation into the possible mechanism(s) underlying this activation revealed that these membranes contain endogenous systems which prefer to utilize GTP, rather than ATP, in the phosphorylation of specific protein substrates with apparent M.W. of 54K and 33K. This activity is highly stimulated by Mn++ ions, inhibited by cyclic AMP and independent of Ca++. Triton-X-100 extracts of brain membranes, which contain the catalytic and regulatory subunits of adenylate cyclase, were found to be enriched in endogenous activity which phosphorylated the 54K protein with GTP, but not ATP. These findings provide a means for direct testing of the hypothesis that protein phosphorylation plays a role in adenylate cyclase regulation.

INTRODUCTION

Several lines of investigation have implicated the phosphorylation of membrane-bound proteins in the regulation of adenylate cyclase activity and its hormonal sensitivity (1-5). Guanosine triphosphate (GTP), a potent modulator of adenylate cyclase, is thought to exert its main effects by binding to a specific regulatory component of the enzyme (6). In addition, it has been suggested that some effects of guanine-nucleotides on adenylate cyclase may involve a covalent reaction (7), possibly catalyzed by phosphorylative activity (8,9). In search of evidence for such a mechanism, we have identified endogenous enzymatic system(s) in brain membranes which prefer to utilize GTP rather than ATP in the phosphorylation of specific protein substrates. This membrane-bound phosphorylation system can now serve as a specific target in studies attempting to demonstrate a direct role for protein phosphorylation in the regulation of adenylate cyclase.

METHODS

Membrane fractions were prepared from the neostriatum or cerebral cortex of decapitated male rats by lysis of P_2 fractions in a hypotonic buffer containing 50 μM $CaCl_2$ as described (10). For preparing EGTA-washed membranes, the lysis buffer contained 2 mM Ethylenebis (oxyethylenenitrilo) tetracetate (pH 7.4). [γ-^{32}P]ATP and GTP were synthesized by the method of Walseth and

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Johnson (11). Conditions of endogenous phosphorylation assays were as detailed previously (12), except that protein concentration was 0.5 mg/ml in a final reaction volume of 50 µl that always contained 10 µCi of [γ-^{32}P] ATP or GTP (see figure legends). Sodium dodecyl sulfate (SDS)solubilized reaction products were separated in slab-polyacrylamide gel gradients that were then stained, dried, and autoradiographed as described (12). Adenylate cyclase activity in EGTA-washed cortical membranes after preincubation with GTP was carried out using [α-^{32}P] ATP (ICN, California) as described previously (5), except that Ca++ was not included in the buffers. Cyclic AMP was isolated by the method of Salomon (13) and percent recovery was routinely greater than 60%. Each of the autoradiographs shown in this report is representative of results obtained with 4-12 separate membrane preparations.

RESULTS

Inclusion of GTP (1-10 µM) in the reaction medium of assays measuring adenylate cyclase activity in rat cerebral-cortical membranes did not exert significant effects on basal enzyme activity, confirming previous reports utilizing cortical membranes (14, 15). When such membrane preparations were preincubated for 5 minutes at 30°C with GTP (10 µM) and $MgCl_2$ (10 mM), chilled, sedimented, washed, resuspended, and then assayed for adenylate cyclase, a 34 $\pm$ 9.3% increase in activity was observed compared to control membranes, preincubated without GTP (control: 46.78 $\pm$ 6.89 pmoles cAMP/mg protein/minute, n=6, $p < 0.01$). This effect was dependent on preincubation time, with peak activation obtained after 4-6 minutes preincubation and reversal of the effect following longer preincubations (Fig. 1). When $MgCl_2$ was omitted from the preincubation medium, there was no increase in adenylate cyclase activity measured in the reincubation phase of the assay (closed triangle in Fig. 1). This effect of preincubation with GTP could be mimicked by ATP, but a 100-fold greater concentration (1 mM) was required (data not shown). Thus, the preincubation modified the membranes in a manner which withstood sedimentation and washing, and resulted in increased adenylate cyclase activity. Subsequent experiments of the present study have examined whether such modification(s) might involve phosphorylation of membrane-bound proteins by an endogenous activity utilizing GTP as a phosphate donor.

Incubation of cortical or striatal membranes from rat brain with 1 µM [γ-^{32}P] ATP and 10 mM $MgCl_2$, followed by sodium dodecyl sulfate (SDS)-gel electrophoresis and autoradiography, revealed the usual pattern of endogenously phosphorylated proteins that we have designated bands A through H (Fig. 2, lane 1, see references 3, 10, 12 for M.W. and details). When 1 µM [γ-^{32}P]GTP was substituted for ATP, phosphorylation of only one band with an apparent M.W. of 54,000 daltons was prominent (Fig. 2, lane 2). Substituting $MnCl_2$ for $MgCl_2$ markedly increased the phosphorylation of most of the endogenous protein substrates by [γ-^{32}P]ATP (Fig. 2, lane 4). Quantitation by densitometric scanning revealed that phosphorylation of the 54K band with AT^{32}P was not stimulated by Mn++ ions. On the other hand, when [γ-^{32}P]GTP was the phosphate donor, phosphorylation of the 54K protein band was greatly stimulated by Mn++

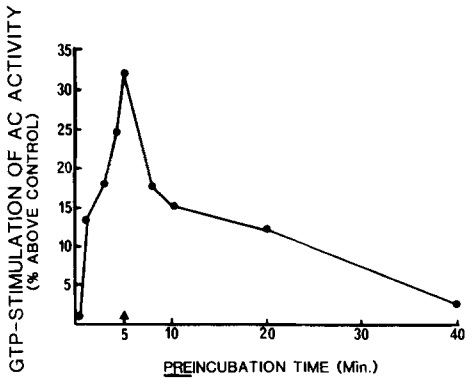
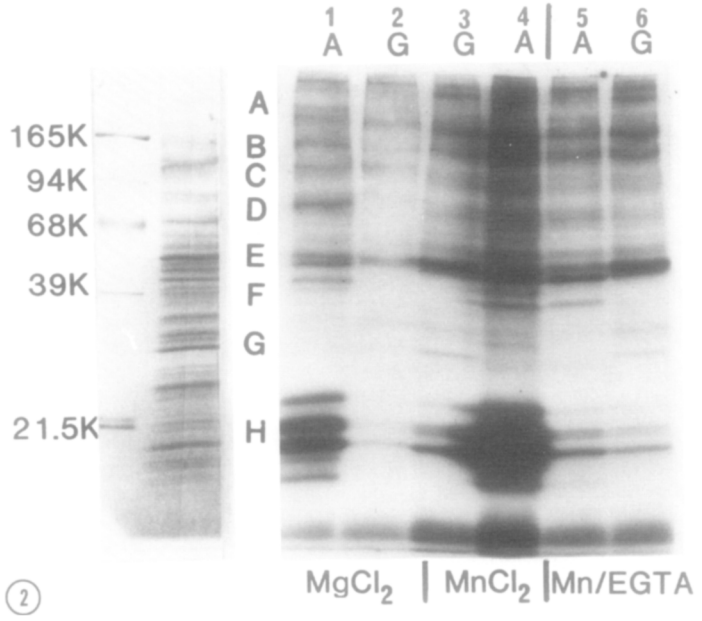


Figure 1. Effect of Preincubation with GTP on Adenylate Cyclase (AC) Activity in Resuspended Membranes.

Aliquots of membranes prepared from P_2 of rat cerebral cortex lysed in EGTA-containing buffer were preincubated with 10 mM $MgCl_2$ and 10 μM GTP for the times indicated and then sedimented, washed twice and assayed for adenylate cyclase activity as described in "Methods". Control activity (preincubation for 5 min without GTP) was 49.57 ± 1.86 pmoles cyclic AMP/mg protein/min. Activity in membranes preincubated with GTP but without $MgCl_2$ (▲) was identical to that of control. Data shown are the means of two experiments in each of which each point was run in triplicate. The SEM for all data points did not exceed 10% of the mean.

(Fig. 2, lane 3). Acid treatment to eliminate P-N linkages did not decrease the [^{32}P] content in the 54K band phosphorylated with GTP, whereas base treatment did, indicating a phospho-ester linkage which is a product of protein kinase activity. The predominance of the 54K band among proteins phosphorylated by GT^{32}P was particularly apparent in reactions carried out with membranes that had been prepared in EGTA-containing buffers (Fig. 2, lane 6). As reported previously (12, 16), chelation of Ca++-ions greatly reduces endogenous phosphorylation in synaptic membranes incubated with Mg·AT^{32}P. It can be seen that this was also the effect with Mn·AT^{32}P (compare lanes 4 and 5, Fig. 2). Endogenous phosphorylation of the 54K protein band by Mn·GT^{32}P, however, was not reduced in EGTA-washed membranes (compare lanes 3 and 6, Fig. 2). In accordance with these results, we have found that addition of $CaCl_2$ and calmodulin (10 units) caused a 3- to 4-fold increase in phosphorylation of proteins with M.W. of 50-52K, 60-63K, 80-90K, and greater than 100K, as previously reported with AT^{32}P (16), but did not stimulate phosphorylation of the 54K band by Mn·GT^{32}P (not shown). In addition to the 54K substrate, bands with apparent M.W. of 30K and 33K (see gel region "G" in Fig. 2) were also phosphorylated preferentially by GTP (compare lanes 5 to 6, and 3 to 4). In contrast, phosphorylation of bands in the M.W. range of 40K to 47K (gel region "F" in Fig. 2) was consistently seen with AT^{32}P but not with GT^{32}P.

Electrophoretic separation under conditions that improve resolution in the M.W. range of 40K-60K revealed that the GTP-prefering protein substrates in this gel region constitute a group of three bands with apparent M.W. of 52K-



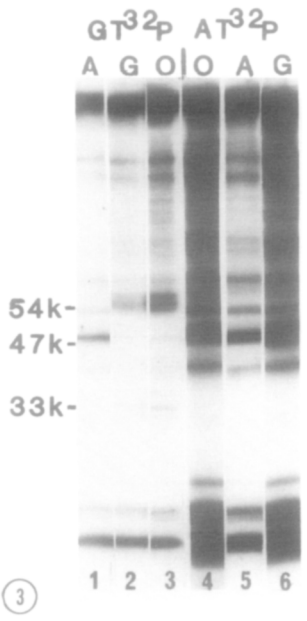


Figure 2. Identification of Specific Substrates for GTP-Preferring Protein Phosphorylation Systems.
Membranes from rat neostriatum were prepared in Ca++ (lanes 1-4) or EGTA (lanes 5,6) containing buffers as described in Methods. Phosphorylation reactions were carried out by incubating the membranes (40 μg protein) with 1 μM [γ-^{32}P]ATP (A) or 1 μM [γ-^{32}P]GTP (G), each at 10 μCi/assay tube, in the presence of 10 mM $MgCl_2$ (lanes 1, 2) or $MnCl_2$ (lanes 3-6). SDS-solubilized reaction products were separated in 7-14% linear polyacrylamide gel gradient which was stained and autoradiographed for 22 hours as described (12). The pattern of protein staining (which was the same for all lanes) and position of M.W. marker proteins is shown on the left. Reactions carried out with EGTA membranes in $MgCl_2$ gave patterns essentially as seen in lanes 5 and 6, but required a minimum of 4-6 days autoradiography for visualization. Results essentially as seen here with striatal membranes were also obtained with preparations from cerebral cortex.

Figure 3. Effects of ATP on Phosphorylation of Proteins by GTP.
Reactions were carried out with 1 μM [γ-^{32}P] GTP ($GT^{32}P$) or ATP ($AT^{32}P$) using Ca++-prepared neostriatal membranes and 10 mM $MnCl_2$ as described in the legend to Fig. 2. O = no additions; G = 100 μM nonlabeled GTP added with the radioactive nucleotide triphosphate; A = 100 μM nonlabeled ATP added. SDS-solubilized membranes were separated in 10-16 % linear polyacrylamide gel gradients.

56K (Fig. 3, lane 3). Labelling of these bands (and of the 33K substrate) was greatly decreased when 100 μM unlabeled GTP was added with the 1 μM [γ-^{32}P]GTP (Fig. 3, lane 2) and completely abolished by addition of 1 mM unlabeled GTP (not shown). Addition of GTP to reactions carried out with 1 μM [γ-^{32}P]ATP had very little effect on the phosphorylation pattern (Fig. 3, lane 6). Inclusion of 100 μM unlabeled ATP in reactions carried out with 1 μM [γ-^{32}P]GTP had unexpected effects: Phosphorylation of the 54K and 33K bands was abolished whereas ^{32}P-incorporation into a band with apparent M.W. of 47K was greatly increased (Fig. 3, lane 1). The inhibition of phosphorylation of the 54K and

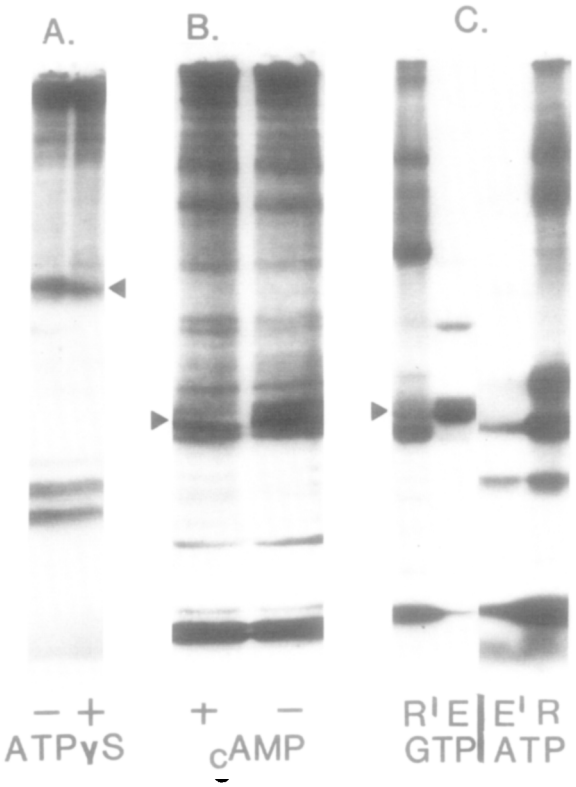


Figure 4. Properties of the GTP-Utilizing Phosphorylation Systems.

4A. Neostriatal Ca++-membranes were preincubated with (+) or without (-) 1 mM nonlabeled ATP-γ-S for 20 min and then sedimented, resuspended and assayed with Mn·GT^{32}P as detailed in Fig. 2. Separation was in a 7-14% linear gel gradient. Arrow indicates position of the 54K band.

4B. Reactions as described in Fig. 1-lane 3 were carried out with (+) and without (-) 10 μM cyclic AMP added to the reaction mixture. Separation was in a 7-14% exponential gel gradient.

4C. Total membrane preparation from rat forebrain was treated with 1.5% Triton X-100 exactly as described in (18) and separated by centrifugation to give the membrane residue (R) and Triton extract (E) which was further treated with Biobeads (19). Each was then assayed for endogenous phosphorylative activity with 10 mM $MnCl_2$ and 1 μM [γ-^{32}P]GTP or ATP as in Fig. 2. Electrophoretic separation was carried out in a 7% polyacrylamide gel slab.

33K bands with GT^{32}P by 100 μM ATP could not be attributed to their acceptance of phosphate from the unlabeled ATP, since these bands were not phosphorylated when membranes were incubated with either 1 μM or 100 μM [γ-^{32}P]ATP (Fig. 3, lanes 4 and 5). The increased incorporation of ^{32}P into the 47K band (Fig. 2, lane 1) may have been caused by [γ-^{32}P]ATP, possibly produced by a phosphotransferase action. This was suggested by the finding that the 47K band is a preferential substrate for [γ-^{32}P]ATP at high (100 μM) concentration (compare lanes 4 to 5 in Fig. 3). In support of this suggestion, we have found that preincubation with ATP-γ-S (under conditions which thiophosphorylated the 47K band irreversibly) (9), decreased the subsequent phosphorylation of the 54K band by Mn·GT^{32}P (Fig. 4A).

The effects of cyclic AMP on endogenous phosphorylation of specific proteins in membranes incubated with Mn·GT^{32}P are depicted in Fig. 4B. It can be seen that 10 μM cyclic AMP inhibited the phosphorylation of the 54K band (marked with an arrow). At the same time, the phosphorylation of a duplex band migrating with apparent M.W. of 80K was stimulated by cyclic AMP, as reported previously for assays carried out with [γ-^{32}P]ATP (3, 17). Under these conditions, cyclic GMP had no discernable effects on endogenous phosphorylative activity.

A Triton-X-100 extract was prepared from rat forebrain membranes as described by Neer et al. (18). Assays of adenylate cyclase activity, carried out using the reaction conditions described by these investigators, have confirmed that these extracts contain the catalytic and regulatory subunits of the enzyme. The same preparations were also tested for endogenous phosphorylative activity. As can be seen in Fig. 4C, this extract contained a very prominent endogenous phosphorylative activity that can utilize [γ-^{32}P]GTP, but not [γ-^{32}P]ATP, to phosphorylate protein substrates in the M.W. range of 54K-56K. The extraction also unmasked endogenous activity which phosphorylated a 68K substrate with GT^{32}P. The incorporation of ^{32}P into the 54K and 68K bands of the Triton-extract (lane GTP/E in Fig. 4C) was 3-6 times higher than in the original membranes (before extraction) or in the membrane residue (after extraction; lane GTP/R in Fig. 4C). Preliminary results using gel filtration chromatography revealed that these endogenous phosphorylation systems are present within macromolecular complexes, some of which contained both adenylate cyclase and GTP-utilizing protein kinase activities.

DISCUSSION

This report demonstrates that brain membranes are capable of utilizing [γ-^{32}P]GTP in the phosphorylation of endogenous protein substrates. The inclusion of Mn++ ions in the reaction medium revealed that a specific protein moiety of apparent M.W. 54K is the principal substrate for endogenous phosphorylative activity which prefers to utilize GTP over ATP as the phosphate donor. A minor substrate for this activity is a band of 33K, similar to the one described in rat ovarian plasma membranes (8). In addition, a 68K substrate of a GTP-prefering activity was unmasked with Triton-X-100. These extracts were also found to be enriched in endogenous activity capable of phosphorylating the 54K band by Mn·GTP but not by ATP. Recently, we have detected this GTP-preferring activity in synaptic plasma membrane fractions purified on sucrose density gradients. A most interesting property of the endogenous system that phosphorylates the 54K band by GTP is its inhibition by cyclic AMP. A puzzling property of this system is its inhibition by 100 μM ATP. The mechanism(s) underlying this inhibition may involve phosphorylation of the 47K protein by a kinase with high Km for ATP, or production of cyclic

AMP from the added ATP. Another attractive possibility raised by these findings is that inhibition by cyclic AMP of GTP-supported protein phosphorylation may serve as a feedback mechanism in the control of basal and hormone-sensitive adenylate cyclase.

The properties of the GTP utilizing phosphorylation system described here are consistent with a role in the regulation of adenylate cyclase. Because Mn++ is a most potent activator of adenylate cyclase (20,21), we have tested its effects on endogenous phosphorylative activity. Indeed, it was the use of $MnCl_2$ that enabled us to demonstrate unequivocally the preferential phosphorylation of specific protein substrates by GTP. The known difficulty in demonstrating activation of brain adenylate cyclase by GTP (14,15) may be related to our finding that GTP-supported phosphorylation of proteins in these preparations is inhibited by the simultaneous presence of ATP, an obligatory constituent of adenylate cyclase assays. On the other hand, when GTP was included in a preincubation phase of the assay (before addition of ATP), activation of cyclase could be demonstrated, and was not reversed by extensive washes of the membranes between the pre- and re-incubation phases of the assay.

Much progress has been made in elucidating the mechanism of adenylate cyclase regulation by studying its activity in solubilized preparations from brain membranes (18,21). Our finding that such preparations contain GTP-preferring endogenous phosphorylative activity, in addition to catalytic and regulatory subunits of adenylate cyclase, provides a powerful tool with which to investigate the interactions between these two enzymatic systems.

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