Modulation of Adenylate Cyclase by Protein Phosphorylation: Effects of ACTH

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INTRODUCTION

Previous studies from our laboratory have demonstrated that neuropeptides related to ACTH may function as regulators of brain adenylate cyclase activity (Wiegant et al., 1979). In μM and higher concentrations $ACTH_{1-24}$ inhibits the activity of adenylate cyclase in brain synaptic plasma membranes in vitro. The presence of calcium ions is essential for this effect (Wiegant et al., 1979; Wiegant, Reul and Gispen, unpublished), indicating that the inhibition of adenylate cyclase by $ACTH_{1-24}$ involves a calcium-dependent process.

From other investigations it became clear that ACTH and congeners specifically inhibit the endogenous phosphorylation of a number of proteins in brain synaptic plasma membranes in vitro (Zwiers et al., 1976, 1978). The peptide concentration at which this effect occurs, is identical to that needed for the inhibition of adenylate cyclase. Zwiers et al. (1978, 1979, 1980) have shown that the inhibition by ACTH of the phosphorylation of at least one specific membrane protein, B-50, is the result of a direct interaction of the peptide with a calcium-dependent, cyclic AMP (cAMP)-insensitive, protein kinase.

Structure–activity relationships for this effect appeared to be very similar to those found for the inhibition of adenylate cyclase (Wiegant et al., 1981). In general, the determination of adenylate cyclase is performed using ATP as a substrate, under conditions where phosphorylation of proteins can also take place. Interestingly, the inhibitory effect of the peptide on adenylate cyclase and on B-50 protein kinase turned out to be dependent on the concentration of ATP in the incubation mixture (Wiegant et al., 1979; Zwiers et al., 1978). These similarities between the interactions of ACTH with adenylate cyclase and endogenous protein phosphorylation in brain synaptic plasma membranes (dose range, structure–activity relationships, calcium dependence and ATP dependence) led us to consider that ACTH influences adenylate cyclase activity through a mechanism involving peptide-sensitive phosphorylation. This consideration is in line with recent observations by Richards et al. (1981) and Ehrlich et al. (this volume) that membrane phosphoproteins are involved in the regulation of basal and receptor-coupled adenylate cyclase activity.

In the present report we present experimental data supporting this idea. It is suggested that phosphate turnover in the membrane may function as an important pathway for neuromodulators to change the efficacy of transmitter adenylate cyclase interactions, thereby modulating nerve impulse flow.

EXPERIMENTAL DESIGN

Light synaptic plasma membranes (SPM) were prepared according to Terenius et al. (1973) from rat cerebral cortex, septum, basal ganglia and hippocampi. To control the state of phosphorylation of the membrane proteins prior to the determination of adenylate cyclase activity, a two-phase incubation system was used. First, membranes were incubated at 30° C in buffer (20 mM Tris–HCl, pH 7.5), containing 5 mM Mg^{2+} for 30 min, in the presence or absence of 10^{-4} M $ACTH_{1-24}$ and of varying amounts of ATPγS. This analog of ATP is used by protein kinases as a phosphate donor. As the thiophosphate-ester bond is not hydrolysed by phosphatases as a substrate, thiophosphorylation in essence is an irreversible process (Gratecos and Fischer, 1974; Cassidy et al., 1979). Immediately after the incubation, the membranes were rapidly spun down, washed twice with ice-cold buffer and resuspended. Aliquots of the suspension were then reincubated to determine the adenylate cyclase activity. Adenylate cyclase assays were carried out at 30° C for 5 min, using 0.5 mM [α-^{32}P]ATP as substrate in the presence of 5 mM Mg^{2+}, an ATP-regenerating system, and caffeine to inhibit phosphodiesterase activity. Samples were processed and cAMP accumulation was determined as previously described (Wiegant et al., 1979). In parallel reincubations the amount of free phosphorylatable sites in the membrane suspension was estimated. Aliquots were reincubated for 10 sec at 30° C in the presence of 7.5 μM [γ-^{32}P]ATP, 10 mM Mg^{2+} and 1 mM Ca^{2+}. Phosphoproteins were solubilized with SDS, separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography according to Zwiers et al. (1976). Incorporation of [^{32}P]phosphate into specific protein bands was determined by liquid scintillation counting.

RESULTS

Rat brain SPM were incubated for 30 min in the presence of various concentrations of ATPγS, then washed twice at 4° C and resuspended in buffer. Reincubation of aliquots of this suspension for 10 sec in the presence of [γ-^{32}P]ATP resulted in the incorporation of [^{32}P]phosphate into proteins. Subsequently, these proteins were solubilized and then separated on SDS–PAGE. As is illustrated by the autoradiograph (Fig. 1), the incorporation of phosphate was inversily related to the concentration of ATPγS employed in the incubation. Radiolabelling of all phosphoprotein bands present was decreased by incubation with ATPγS. Maximal inhibition of incorporation of phosphate into proteins was observed at 1 mM ATPγS. The absolute amount of phosphate incorporated during reincubation into the two most prominent phosphoprotein bands (apparent molecular weights 48 K and 56 K) was determined by liquid scintillation counting. Phosphate labelling of these proteins showed an inverse linear relationship with the log concentration of ATPγS present in the incubation (Fig. 2). From the data depicted in Fig. 2, the EC_{50} of ATPγS on phospholabelling of these bands was computed, being 60 and 20 μM, respectively.

As expected, ATPγS did not abolish the protein kinase activity of the membranes. For histone added to the reincubation mixture became highly phosphorylated by endogenous SPM protein kinases, irrespective of the ATPγS concentration during the incubation (data not shown). These data suggest that ATPγS does not affect the process of phosphorylation in SPM, but indeed serves as a (thio)phosphate donor resulting in irreversible thiophosphorylation of membrane proteins.

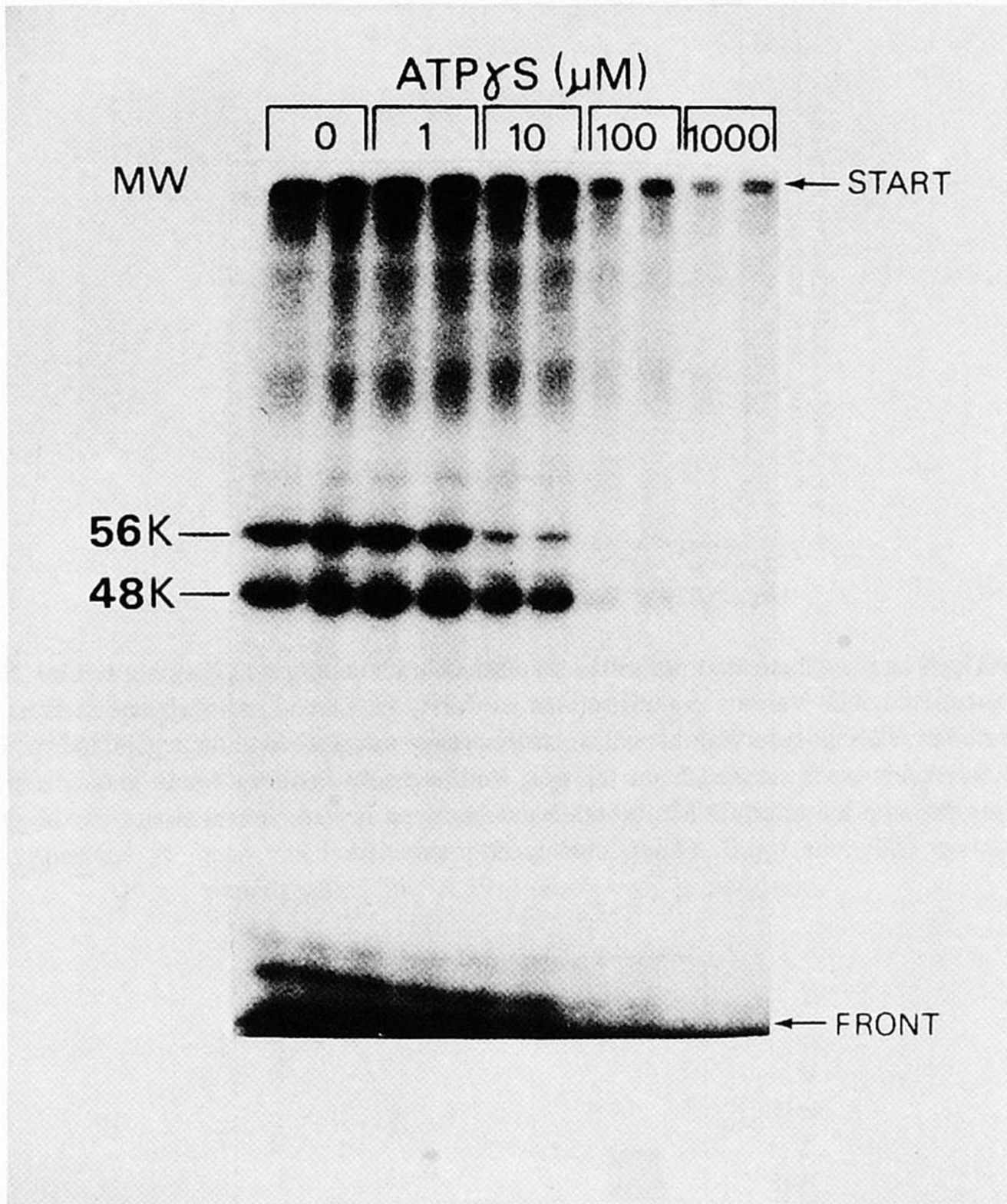

Fig. 1. Effect of ATPγS on the phosphorylation of brain membrane proteins. Synaptic plasma membranes were incubated with 5 mM Mg^{2+} and various concentrations of ATPγS, washed and resuspended in buffer. Aliquots were reincubated with [γ^{32}P]ATP (7.5 μM) in the presence of Mg^{2+} (10 mM) and Ca^{2+} (1 mM). Proteins were solubilized with SDS and separated by SDS–polyacrylamide slab gel electrophoresis. The radiolabelled protein bands were visualized by autoradiography. The autoradiograph depicted here shows the inverse relationship between incorporation of [^{32}P]phosphate and the concentration of ATPγS during the incubation. Incubation in the presence of 1000 μM ATPγS completely prevented incorporation of phosphate into proteins during reincubation. The major phosphoprotein bands in this preparation had apparent molecular weights of 48 K and 56 K.

Also, following incubation with ATPγS, aliquots of the membrane suspension were reincubated for 5 min in a system containing 0.5 mM [α-^{32}P]ATP to assess the effect of thiophosphorylation on the activity of adenylate cyclase. Fig. 3 shows that incubation of SPM in the presence of ATPγS markedly stimulated the basal activity of adenylate cyclase in a concentration-dependent manner. A linear relationship existed between the activity of the enzyme and the log concentration of ATPγS used in the incubation. Half-maximal stimulation was observed at a concentration of 60 μM, whereas maximal stimulation occurred at 0.5 mM ATPγS. By comparing Figs. 1, 2 and 3, a strong correlation between the degree of phosphate incorporation into proteins and the activity of adenylate cyclase in the same membrane preparation becomes apparent.

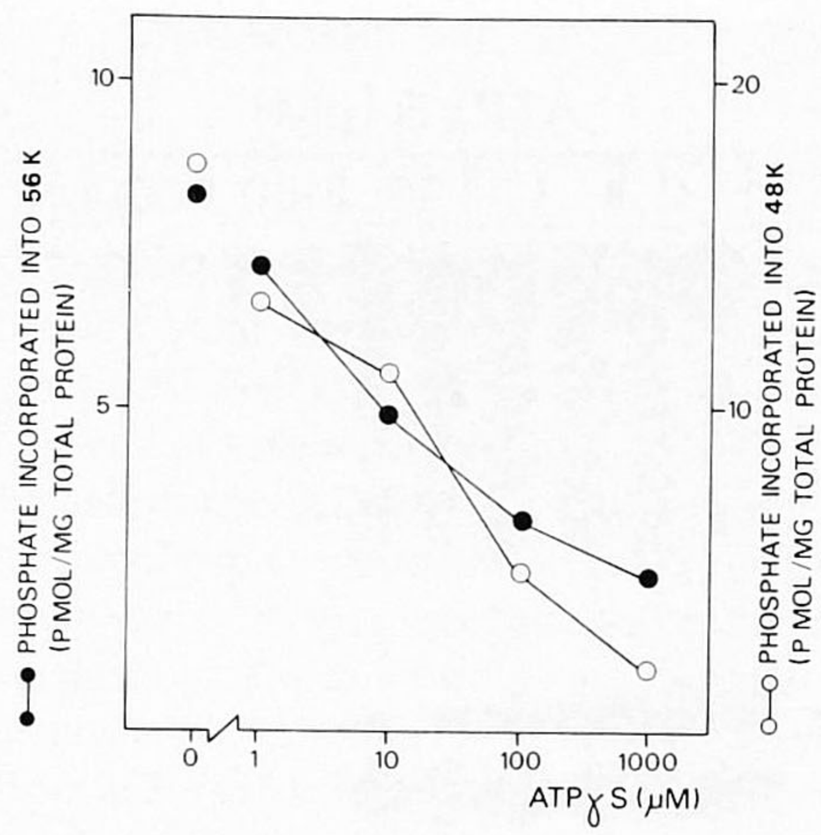

Fig. 2. Effect of ATPγS on phosphate incorporation into distinct brain membrane phosphoproteins. Synaptic plasma membranes were incubated with various concentrations of ATPγS, washed, reincubated in the presence of [γ-^{32}P]ATP and separated by SDS–polyacrylamide gel electrophoresis as described in the legend to Fig. 1. The 48 K and 56 K phosphorprotein bands were excised from the gel. Radioactivity in these bands was determined by liquid scintillation counting and the incorporation into both bands showed inverse correlation with the concentration of ATPγS during the incubation.

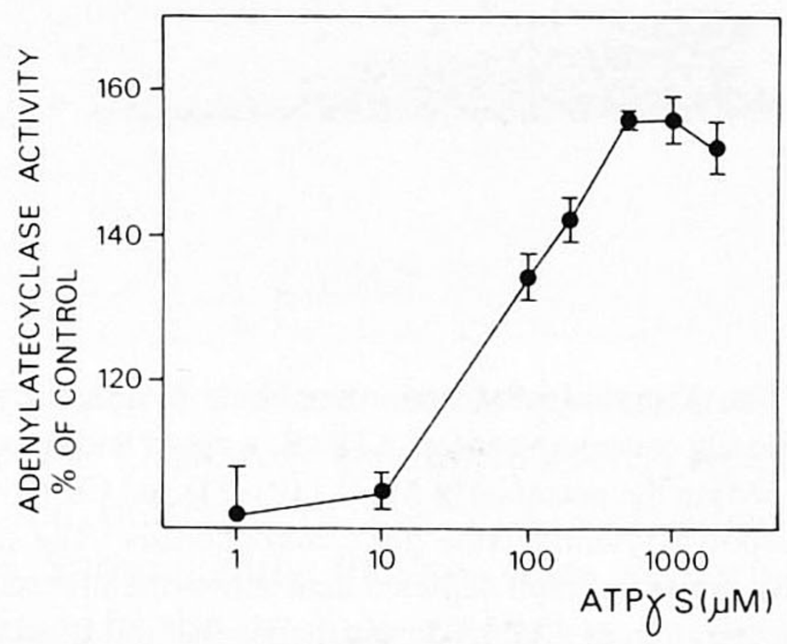

Fig. 3. Activation of brain membrane adenylate cyclase by ATPγS. Synaptic plasma membranes were incubated with various concentrations of ATPγS under phosphorylation conditions (5 mM Mg^{2+}), washed, resuspended and reincubated to assay adenylate cyclase activity. ATPγS dose dependently activated adenylate cyclase. Values are expressed as mean $\pm$ S.E.M. of triplicate determinations. Basal adenylate cyclase activity in this experiment: 720.3 $\pm$ 2.3 pmoles/mg protein/min.

Incubation of SPM in the presence of 10^{-4} M $ACTH_{1-24}$ under non-phosphorylating conditions (i.e., in absence of ATP or ATPγS), resulted in a strong inhibition (32 %) of adenylate cyclase activity detected by reincubation in the presence of [α-^{32}P]ATP (Fig. 4). In a parallel reincubation with [γ-^{32}P]ATP a large decrease in the incorporation of phosphate was observed in the 48 K protein band, but also the labelling of the 56 K band was affected (Fig. 5).

When membrane phosphoproteins are saturated with phosphate, as is the case after incubation with 1000 μM ATPγS (Fig. 2), a change in the activity of protein kinase cannot be expressed in an altered degree of phosphate incorporation in the reincubation. Indeed, 10^{-4} M

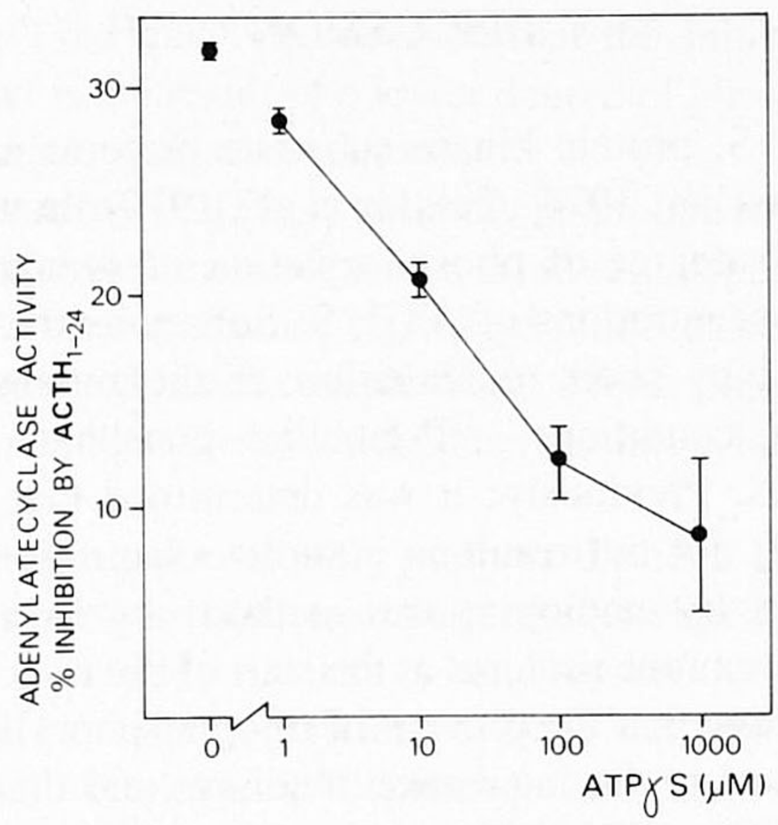

Fig. 4. Effect of ATPγS on $ACTH_{1-24}$-induced inhibition of adenylate cyclase. Synaptic plasma membranes were incubated with various concentrations of ATPγS in the presence or absence of 10^{-4} M $ACTH_{1-24}$ under phosphorylating conditions (5 mM Mg^{2+}). Membranes were washed, resuspended and adenylated cyclase activity was determined. The expression of the inhibitory effect of the peptide on adenylate cyclase activity depended on the concentration of ATPγS during the incubation. Incubation with 1000 μM ATPγS abolished the effect of the peptide. The values are expressed as mean $\pm$ S.E.M. of triplicate incubations. Basal adenylate cyclase activity in this experiment: 836.6 $\pm$ 19.4 pmoles/mg protein/min.

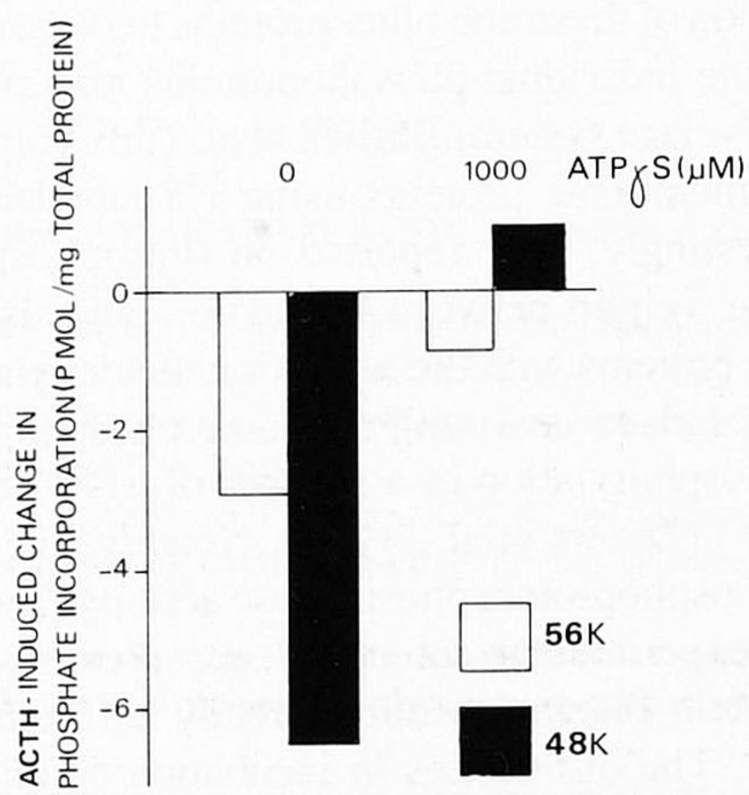

Fig. 5. Effect of ATPγS on the $ACTH_{1-24}$-induced inhibition of phosphate incorporation into SPM proteins. Synaptic plasma membranes were incubated with or without 1000 μM ATPγS in the presence or absence of 10^{-4} M $ACTH_{1-24}$. The membranes were washed, resuspended, reincubated with 7.5 μM [γ-^{32}P]ATP and separated by SDS–polyacrylamide gel electrophoresis. The 48 K and 56 K protein bands were excised from the gel. Radioactivity in these bands was determined by liquid scintillation counting. $ACTH_{1-24}$ inhibited phosphate incorporation into both protein bands. Incubation with 1000 μM ATPγS abolished this effect of the peptide.

$ACTH_{1-24}$ did not affect phospholabelling of the 48 K and 56 K proteins in membranes incubated with 1000 μM ATPγS (Fig. 5). Interestingly, in the same membranes thiophosphorylation also antagonized the expression of the effect of $ACTH_{1-24}$ on adenylate cyclase activity. No inhibition by the peptide was found after incubation with 1000 μM ATPγS (Fig. 4).

DISCUSSION

In the presence of ATPγS, protein kinase substrate proteins are irreversibly (thio)phosphorylated (Gratecos and Fischer, 1974; Cassidy et al., 1979). In this study we have used this feature to predetermine the degree of phosphorylation of synaptic plasma membranes by incubation with varying concentrations of ATPγS. Subsequently, the degree of (thio)phosphorylation was monitored by short reincubation of the membranes in the presence of [γ-^{32}P]ATP. Under these conditions, ^{32}P-labelled phosphate is only bound to free un(thio)phosphorylated sites. Previously, it was determined that the phosphorylation conditions used (7.5 μM ATP) did not result in complete saturation of the acceptor proteins (Wiegant et al., 1978). Yet, by employing this method, a good reflection of the degree of (thio)phosphorylation of membrane proteins at the start of the reincubation could be obtained (Figs. 1 and 2). Our data show that the process of thiophosphorylation is proportional to the concentration of ATPγS used in the incubation medium, and that thiophosphorylation was maximal at 1 mM ATPγS (Figs. 1 and 2).

The basal activity of adenylate cyclase was directly related to the concentration of ATPγS used in the incubation (Fig. 3) and therefore to the state of (thio)phosphorylation of the membranes. These results corroborate those of Ehrlich et al. (this volume), and they indicate that brain basal adenylate cyclase activity is stimulated by phosphorylation of membrane proteins. Quantitative analysis of the two major phosphoprotein bands with apparent molecular weights of 56 K and 48 K (Fig. 2) revealed a close correlation between thiophosphorylation of these bands and the activity of adenylate cyclase. However, as we did not analyse the kinetics of thiophosphorylation of these and other proteins in more detail, no conclusion can be drawn with respect to the role individual phosphoproteins may play in the regulation of the multicomponent adenylate cyclase system, Ehrlich et al. (this volume) studied the incorporation of thiophosphate into membrane proteins using ^{35}S-labelled ATPγS in a comparable experimental system. Interestingly, they reported on (thio)phosphorylation of two protein bands in SPM with molecular weights between 40 and 60 K and also described a correlation of the phosphorylation of these proteins with the activation of adenylate cyclase. It remains to be shown whether both studies indeed deal with the same phosphoproteins.

$ACTH_{1-24}$ inhibits the phosphorylation of a number of SPM proteins via interaction with endogenous protein kinase(s) (Zwiers et al., 1976). Notably, the peptide was shown not to influence brain membrane phosphoprotein phosphatase activity (Zwiers et al., 1978). It should be kept in mind that the experimental set up of our previous studies on the effect of neuropeptides on brain protein phosphorylation greatly differ from those employed here (Zwiers et al., 1976, 1978). The differences in incubation conditions (time of exposure to $ACTH_{1-24}$, incubation/reincubation, etc.) may be cause of the different phosphorylation pattern and sensitivity of the kinases to ACTH. For instance, recently we reported that exposure of membranes to a number of fragments of ACTH resulted in the release of a protein (molecular weight 41 K) from these membranes (Aloyo et al., 1982). Albeit that the meaning of this observation is not yet totally understood, it certainly may have resulted in differences between previous and present endogenous membrane phosphorylation. Although no proper immunochemical or two-dimensional identification of the 48 K protein band was performed, it is almost certain that this protein is identical to the B-50 protein first described by Zwiers et al. (this volume).

The inhibitory effect of $ACTH_{1-24}$ on SPM protein kinase(s) could be detected by reincubation in the presence of [γ^{32}P]ATP, even after the membranes had been washed extensively (cf. Fig. 5). The adenylate cyclase assay was performed under phosphorylating conditions

(0.5 mM ATP, 5 mM Mg^{2+}). Hence, it could be that the inhibition of adenylate cyclase activity by $ACTH_{1-24}$ in fact is the result of a lower degree of phosphorylation of a number of proteins essential in adenylate cyclase regulation.

Inhibition of protein kinase by $ACTH_{1-24}$ will only result in a lower degree of phosphorylation of substrate proteins, if these are present in a relatively dephosphorylated from as is the case after incubation of SPM in the absence of ATPγS. Indeed, no inhibition of phosphorylation by the peptide was observed when complete and irreversible (thio)phosphorylation had taken place (Fig. 5). Also, under these conditions, the adenylate cyclase activity was not inhibited by the peptide.

In conclusion, the present results strongly support the idea that phosphoproteins in the membrane are intimately involved in the regulation of adenylate cyclase activity (Richards et al., 1981; Whittemore et al., 1981; Ehrlich et al., this volume). Moreover, they indicate neuropeptides like ACTH can alter adenylate cyclase activity by changing the state of phosphorylation of one or more relevant phosphoproteins. At present, no information on the exact nature and role of the phosphoprotein(s) is available. However, there seems to be a strong parallel to the observed regulation of membrane DPI kinase by the degree of phosphorylation of B-50 (Jolles et al., 1980, 1981). With respect to adenylate cyclase, it has been suggested (Schleifer et al., 1980) that phosphorylation of the guanine nucleotide regulatory protein may be involved in the regulation of the activity of the receptor adenylate cyclase complex. Such a mechanism would allow putative neuromodulators like ACTH and other neuropeptides to change the efficacy of neurotransmission by affecting membrane processes involved in the generation of second messengers (cAMP, calcium).

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