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ACTH, CYCLIC NUCLEOTIDES, AND BRAIN PROTEIN PHOSPHORYLATION IN VITRO

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Endogenous phosphorylation of proteins from rat brain synaptosomal plasma membranes was studied in vitro. Cyclic AMP (cAMP) markedly stimulated ^{32}P incorporation in three protein bands with molecular weights of 75,000, 57,000, and 54,000, respectively. The effect of the behaviorally active peptide $ACTH_{1-24}$ on this endogenous phosphorylation in vitro was studied using peptide concentrations from 10^{-10} to 10^{-4} M. In a number of protein bands, a biphasic effect of $ACTH_{1-24}$ was observed: in concentrations of 10^{-4}–10^{-5} M, a reduced amount of ^{32}P was found; in concentrations of 10^{-6}–10^{-7} M, hardly any effect could be detected, whereas consistently at concentrations around 10^{-8} M, a significant decrease was again observed. The phosphoprotein bands affected by in vitro addition of $ACTH_{1-24}$ were of a smaller molecular weight than those affected by in vitro addition of cAMP.

INTRODUCTION

Phosphorylation of proteins may affect their tertiary and quarternary structure, and can modify their functional properties. With respect to phosphorylation of membrane proteins, changes in membrane permeability both in nerve and peripheral tissue have been reported (1,2). For nervous tissue, such alterations may influence neurotransmission (2). Phosphorylation of proteins in the cell appears to be regulated by

numerous factors such as the activity of protein kinases and phosphatases, the levels of ATP and divalent cations, and for some proteins, the level of cyclic AMP (cAMP). There seems to be some correlation between phosphorylation of plasma membranes of brain and the acquisition and extinction of learned behavior (3–5). ACTH and ACTH-like peptides are involved in learning processes (6). They exert their influence by a direct action on central nervous structures that at the neurochemical level results in an enhanced synthesis of brain proteins (for a review, see reference 7). It was deemed of interest, therefore, to study whether phosphorylation processes underlie the neurotropic action of ACTH. The present report deals with the effects of $ACTH_{1-24}$ and cyclic nucleotides on the phosphorylation of synaptic plasma membrane proteins in vitro.

EXPERIMENTAL PROCEDURE

Membrane Preparation. Male albino rats of an inbred Wistar strain (TNO Zeist, The Netherlands) weighing 160–200 g, were used. The animals were killed by decapitation, and the cortex cerebrum was excised and used for the isolation of synaptic plasma membranes (SPM) according to Terenius (8). The enriched pellet was suspended (10 mg protein/ml) in 50 mM Na-acetate, pH 6.5, containing 10 mM Mg-acetate, and stored in small aliquots at −80°C.

Phosphorylation Assay. The procedure was in essence that described by Routtenberg and Ehrlich (9). The assays were carried out in small glass tubes in a total volume of 50 μl, containing 150 μg SPM protein as determined by the method of Lowry (10). The incubation mixture consisted of 50 mM Na-acetate, pH 6.5, and 10 mM Mg-acetate. Samples were preincubated for 5 min at 30°C, and the assay was started by addition of 3–6 μCi [γ-^{32}P]ATP (about 15 Ci/mmol). The final concentration of ATP was 7.5 μM. The reaction was stopped by adding a solution resulting in (final concentration): Tris-HCl, 62.5 mM, pH 6.8; SDS, 2%; glycerol, 10%; bromphenol blue, 0.001%; 2-mercaptoethanol, 5%. Subsequently, the reaction mixture was placed in boiling water for 5 min to ensure complete solubilization of membrane proteins and reaction with SDS. $ACTH_{1-24}$ or the cyclic nucleotides were added 5 sec prior to the introduction of [γ-^{32}P]ATP.

Separation of Membrane Proteins by Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis on slab gels was performed according to Lugtenberg et al. (11). The slab was 1.3 mm thick and consisted of a running gel (11% acrylamide, 9 cm) with a stacking gel (3% acrylamide, 2 cm). Routinely, 25 μl of the reaction mixture containing 25 μg protein was applied directly to the gel. On each slab, 25 samples were run simultaneously. Electrophoresis was carried out at room temperature using 30 mA (100–200 V) per gel. When the tracking dye (bromophenol blue, added to the sample) had run as far as 1 cm from the bottom of the gel ($2\frac{3}{4}$ h), electrophoresis was terminated, and the gels were stained overnight in a solution of 0.1% Fast Green FCF in 50% methanol–10% acetic acid (vol/vol). Quantitative autoradiography of the gels was performed to visualize radioactive material in the gels. To this end, the wet slabs were mounted on a glass-plate holder and sealed in a thin polyethylene bag, preventing desiccation of the gel. Kodak Royal X-Omat X-ray film was used for exposure (2–7 days). Densitometric scanning of the

autoradiogram was performed using a linear gel-scanner (slit width 0.2 mm) and a Zeiss-PMQII spectrophotometer. Quantitation of the ^{32}P radioactivity was obtained by measuring peak heights above background (9,12). Good agreement was found comparing such data with those obtained by liquid scintillation counting of ^{32}P radioactivity in 1-mm gel slices. Estimation of molecular weight was done by comparing the electrophoretic mobilities of the various membrane proteins with those of the following marker proteins: phosphorylase b (92,500), bovine serum albumine (67,000), catalase (60,000), ovalbumin (45,000), lactate dehydrogenase (36,000), chymotrypsinogen (25,000), and hen lysozyme (14,000).

Chemicals. Acrylamide and methylenebisacrylamide were obtained from Serva, Heidelberg, G.F.R.; Fast Green FCF, from Sigma Chemical Company, St. Louis, Missouri; sodium dodecylsulfate, from BDH, Poole, United Kingdom; glycin, from Merck, Darmstadt, G.F.R.; and hen egg lysozyme from Fluka, Buch, Switzerland. Other standard proteins were obtained from Boehringer Mannheim, Amsterdam, The Netherlands. The cAMP and cGMP were obtained from Sigma; the peptides, from Organon, Oss, The Netherlands; and the [γ-^{32}P]ATP from the Radiochemical Centre, Amersham, United Kingdom.

RESULTS

SPM Protein Phosphorylation In Vitro and Its Dependence on Cyclic Nucleotides

Separation of proteins of synaptic plasma membranes (SPM) from cortex cerebrum revealed up to 50 bands when colored with Fast Green (Figure 1). In the autoradiogram, at least 10 different radioactive bands were distinguished and numbered 1–10. The estimated molecular weights of these peptide bands are 78,000 (1), 75,000 (2), 57,000 (3), 54,000 (4), 53,000 (5), 48,000 (6), 20,000 (7), 18,000 (8), 17,000 (9), and 15,000 (10). The phosphorylation of three of the protein bands appears to be cAMP-dependent (Figure 2). When 5×10^{-6} M cAMP was present in the incubation medium, phosphorylation of bands 2, 3, and 4—which are hardly visible in the absence of cAMP—increased dramatically. Quantitative analysis of the autoradiogram revealed about twice as much radioactivity in bands 2, 3, and 4 after 5 sec to 5 min of incubation with [γ-^{32}P]ATP in the presence of cAMP as in its absence. No significant changes in phosphorylation of bands 1 and 5–10 were detected. Using 20 sec as incubation time and increasing doses of cAMP ranging from 10^{-7} to 10^{-4} M, it was found that the phosphorylation of protein bands 2, 3, and 4 was significantly increased at a cAMP concentration of 5×10^{-7} M, whereas the concentration of 5×10^{-6} M had the maximal effect. Under the same experimental conditions, no effects of cGMP on the phosphorylation pattern could be detected at concentrations between 10^{-10} M and 10^{-4} M.

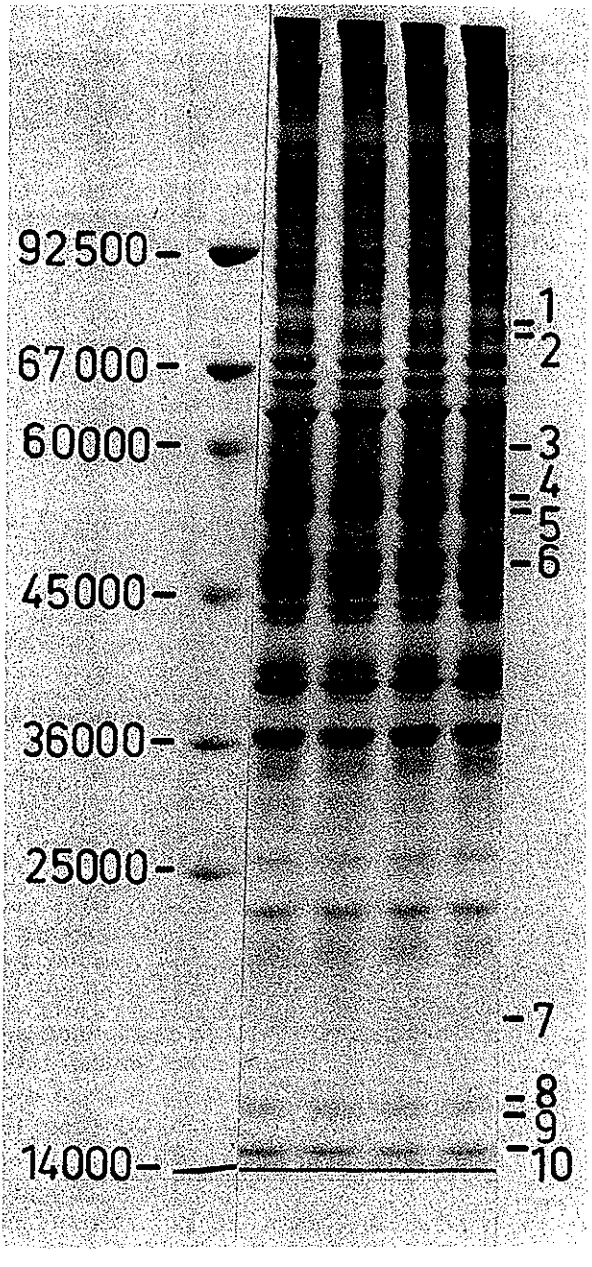

FIG. 1. Electrophoretic separation and Fast Green staining of SPM proteins (see the Experimental Procedure section). The electrophoretic mobility of 7 molecular weight standards (see the Experimental Procedure section) is shown on the left side; on the right, the positions of the 10 phosphoprotein bands under study are indicated.

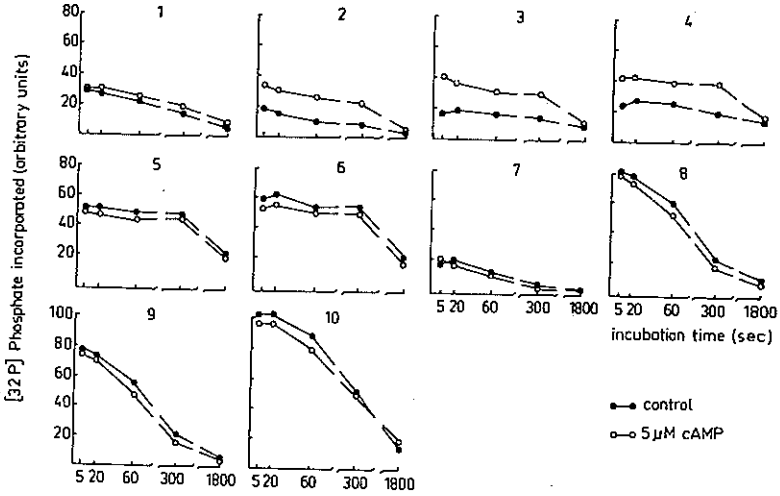

FIG. 2. Pattern of semiquantitative scanning of the ^{32}P radioactivity in the 10 SPM phosphoprotein bands of Figure 1 as a function of incubation time and presence of 5 μM cAMP. Data are expressed in arbitrary units (see the Experimental Procedure section); the number at the top of each graph refers to the appropriate SPM phosphoprotein band.

$ACTH_{1-24}$ and SPM Protein Phosphorylation In Vitro

Incubation of SPM in the presence of $ACTH_{1-24}$ resulted in a significant decrease in the phosphorylation of bands 6–10, and this reduction remained detectable up to 5 min of incubation with [γ-^{32}P]ATP (Figure 3). The effect of $ACTH_{1-24}$ appeared to be dose-dependent and showed biphasic dose–response curves, with the largest decrease at 10^{-4} M, small or no effects at 10^{-6}–10^{-7} M, and a decrease again at 10^{-8} M $ACTH_{1-24}$. Such a dose–response relationship was confirmed in six experiments using four different batches of SPM and three different batches of [γ-^{32}P]ATP; the level of incorporated ^{32}P at 10^{-8} M $ACTH_{1-24}$ was -22% for band 8, -28% for band 9, and -14% for band 10 in comparison with the respective control levels, and differed significantly both from the values for 10^{-6} M and the control values for bands 8–10 ($P<0.05$, two tailed Wilcoxon–matched pairs–signed-ranks test). The band that migrates in front of the tracking dye is lipid-extractable, and is also affected by $ACTH_{1-24}$.

DISCUSSION

It has been suggested that phosphorylation of cerebral membrane proteins is important in neural functioning (2). The present paper deals

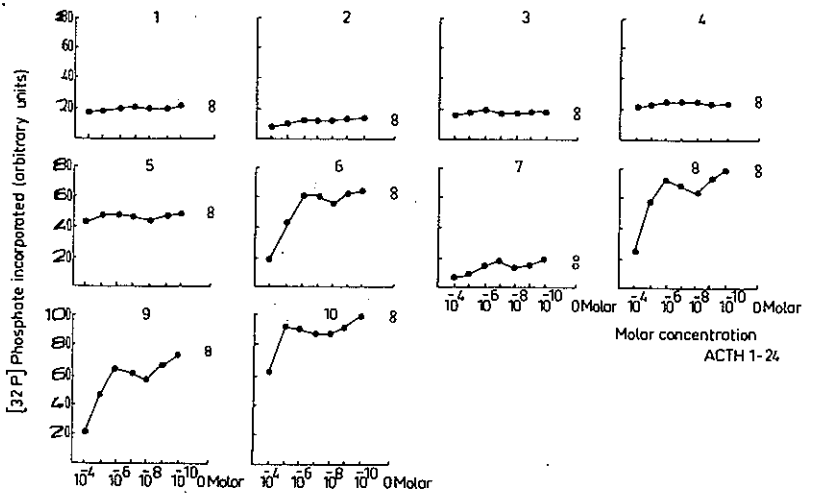

FIG. 3. ^{32}P radioactivity in SPM phosphoprotein bands as a function of $ACTH_{1-24}$ concentration in the incubation mixture, after scanning of the autoradiograph. Data are expressed in arbitrary units (see the Experimental Procedure section); the number at the top of each graph refers to the appropriate SPM phosphoprotein band. ●——●: Incubation at various concentrations of $ACTH_{1-24}$; O: control incubation in duplicate, without $ACTH_{1-24}$ (0 molar).

with the phosphorylation of synaptosomal plasma membranes in vitro in the presence of $ACTH_{1-24}$. An enriched synaptosomal plasma membrane fraction was used, isolated from rat cerebrum (8). [γ-^{32}P]ATP served as substrate in the assay of the phosphorylation of the membrane proteins in vitro (compare reference 9). The incorporation of ^{32}P into proteins, as measured under these conditions, is the resultant of phosphorylation by protein kinases, dephosphorylation by protein phosphatases, and exhaustion of the radioactive ATP by ATPases. Therefore, we do not speculate on the actual mechanism, but merely refer to phosphorylation in keeping with the literature (4,9,12,13).

After the incorporation period, synaptosomal proteins of 20 incubations were electrophoretically separated into about 50 different bands per aliquot (see Figure 1), and the phosphorylation of these protein bands was studied under various in vitro conditions. Possible adhering free ^{32}Pi is removed during the electrophoretic separation, as was concluded from the absence of radioactivity in gels having aliquots that were stopped at zero seconds (data not shown here). The present paper presents detailed information on the phosphorylation of only 10 bands, since the phosphorylation of these bands could be quantified reproduci-

bly, and addition of cAMP or $ACTH_{1-24}$ to the incubation mixture affected the phosphorylation of only these 10, although phosphorylation in the presence of cAMP seems to result in an overall increase in phosphorylation [and is also visible according to other publications (9,13)].

Quantitation by measuring peak height above background reveals that phosphorylation of 3 of the 10 bands, numbered 2, 3, and 4—with mol. wt. 75,000, 57,000, and 54,000, respectively—was specifically stimulated to a greater extent by cAMP (see Figure 2). Cyclic GMP, on the other hand, did not affect protein phosphorylation, a finding similar to that reported elsewhere (9). Incubation of SPM in the presence of $ACTH_{1-24}$ resulted in a decrease in phosphorylation of the proteins of bands 6–10. This effect seems rather specific, because neither fragment $ACTH_{1-10}$ nor fragment $ACTH_{11-24}$, nor their combination, affected the phosphorylation of these bands (Zwiers, unpublished data). The decrease in phosphorylation in the presence of $ACTH_{1-24}$ shows a biphasic dose–response relationship (see Figure 3). A component of the SPM fraction that is involved in the effect of $ACTH_{1-24}$ appeared to be sensitive to freezing, since the effectiveness of $ACTH_{1-24}$ diminished by a factor of 10 on storage of the SPM at −80°C for 1 month, whereas phosphorylation as such was not affected.

Preliminary data by Wiegant and Zwiers showed that the endogenous ATPase activity of the SPM preparation is not influenced by $ACTH_{1-24}$. Thus, the effect of $ACTH_{1-24}$ on the incorporation of the [γ-^{32}P] of ATP into 5 SPM protein bands seems to result from an alteration in the balance between phosphorylation and dephosphorylation during at least 5 min.

Under the present in vitro conditions, no evidence for a relationship between the effects of cAMP and $ACTH_{1-24}$ was obtained, because different protein bands were involved. Although, from a behavioral point of view, numerous studies suggest a role of ACTH-like peptides in motivational and learning processes through a direct effect on the brain (6), neurochemical data are sketchy and have only established that ACTH-like peptides influence nerve cell protein synthesis at the translational level (7,14–19). Some indication also exists as to their role in noradrenaline turnover (20). Recently, evidence was obtained suggesting that these peptides may influence nerve cell metabolism through an increase in intracellular cAMP content (21,22). However, in vitro $ACTH_{1-24}$ and cAMP affect the phosphorylation of different membrane proteins, and the effects are in opposite directions.

Further information on the interaction of $ACTH_{1-24}$ with intact nervous tissue (e.g., slices or in vivo treatment) is therefore needed to

reach a definite conclusion as to the mechanism of ACTH-induced changes in SPM protein phosphorylation and the relationship of these phenomena to the acquisition and extinction of learned behavior.

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