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ACTH-INDUCED INHIBITION OF ENDOGENOUS RAT BRAIN PROTEIN PHOSPHORYLATION IN VITRO: STRUCTURE ACTIVITY

HENK ZWIERS, VICTOR MARIUS WIEGANT, PETER SCHOTMAN, AND WILLEM HENDRIK GISPEN

Division of Molecular Neurobiology,
Institute of Molecular Biology,
Rudolf Magnus Institute for Pharmacology and Laboratory for Physiological Chemistry,

University of Utrecht,
Padualaan 8, Utrecht,
The Netherlands

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$ACTH_{1-24}$ inhibits the endogenous phosphorylation in vitro of distinct SPM protein bands. Using N-terminal fragments of ACTH, the structure–activity requirements for this effect were studied. A rather complex interaction of the ACTH fragments with endogenous SPM phosphorylation was observed. The effects were not only dependent on the primary structure of the peptide used, but also on the protein band studied and the ATP/SPM ratio used in the incubation system. $ACTH_{1-24}$ did not interfere with the ATP-hydrolyzing activity of the SPM preparation, nor did it influence the endogenous phosphatase activity. Therefore, a direct interaction of ACTH with SPM protein kinase(s) is likely to be responsible for its effect on phosphorylation.

INTRODUCTION

ACTH and peptides derived from its N terminus are known to influence brain cell physiology and metabolism and hence brain function (learned behavior, excessive grooming, morphine-induced analgesia, etc.; see references 1, 2). Neurochemical studies revealed that such peptides may affect RNA and protein metabolism and catecholamine turnover in rat

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brain (3, 4). The correlation of these various effects is poorly understood. Recently it was found that synthetic $ACTH_{1-24}$ when added in vitro reduced the endogenous phosphorylation of a number of rat brain synaptosomal plasma membrane (SPM) proteins (5). The present study deals with structural requirements for this effect and the structure–activity relationship found is compared with that of other ACTH–CNS interactions. The effect of $ACTH_{1-24}$ was studied under a low and a high ATP/SPM ratio (6), and some evidence is presented concerning the nature of this peptide-induced inhibition of endogenous SPM phosphorylation in vitro.

EXPERIMENTAL PROCEDURE

Animals and Tissue Fractionation

Male albino rats of an inbred Wistar strain (TNO, Zeist, The Netherlands) were used, weighing 160–200 g. After decapitation of the animal, the cerebral cortex was excised and used for the isolation of synaptic plasma membranes (SPM) according to Terenius (7). The enriched SPM pellet was suspended in a medium containing 50 mM Na acetate and 10 mM Mg acetate (pH 6.5).

In Vitro Phosphorylation Assay

The phosphorylation assays were carried out at 30°C under two conditions of SPM and ATP concentrations (6).

Condition A. 7.5 μM ATP; 2.2 μg SPM protein/μl; final volume 50 μl.

Condition B. 200 μM ATP; 0.4 SPM protein/μl; final volume 25 μl.

Peptides were dissolved in 10 μl 0.01 N HCl and further diluted with incubation medium to a final concentration of 5×10^{-4} M. 10 μl (condition A) or 5 μl (condition B) of peptide solution or control solution (the same mixture without peptide) was added to the incubation mixture 10 sec prior to the addition of ATP.

Synthetic peptides (over 85% pure peptide) were obtained from Organon International B.V., Oss, The Netherlands.

Separation of Individual Phosphoprotein Bands; Autoradiography

After termination of the phosphorylation reaction as described in the preceding paper (6), 20-μl (condition A) or 35-μl (condition B) aliquots of each incubation mixture were subjected to SDS-polyacrylamide gel electrophoresis on slab gels, as described by Lugtenberg et al. (8). Staining, destaining, autoradiography, and scanning were carried out as reported previously (5, 6). Comparisons were made only between samples of one gel. On each gel reference SPM samples were included, and the exposure time allowed a linear relationship between radioactivity and grain density per band. Peak heights above background were computed and taken as a relative measure of radioactivity in the gel (5, 9, 19).

Determination of Endogenous ATP-Hydrolyzing Activity in SPM

As an indication of precursor hydrolysis, the production of free phosphate during the incubation was studied under condition A and B with or without the presence of 10^{-4} M $ACTH_{1-24}$. This experiment was carried out as described above, but the reaction was terminated by adding TCA, resulting in a final concentration of 5%. After centrifugation at 1000 *g* the supernatant was used for analysis of the free phosphate content. Since it was found that $ACTH_{1-24}$ severely interfered with the phosphate determination, the peptide was removed by application of the supernatant to a small Dowex 50 WX4 column (H^+ form, 1.0 × 0.5 cm) and samples constituting the standard curve were treated likewise. Subsequently, after elution with 1 N HCl, the phosphate content of the eluate was measured (11).

Discrimination between Endogenous SPM Protein Kinase and Phosphatase Activity

SPM (550 μg/225 μl) was preincubated for 5 min. Then the phosphorylation reaction was initiated at $t = 0$ sec by addition of [γ-^{32}P]ATP, resulting in a final concentration of 7.5 μM ATP and a final volume of 250 μl. At 5 and 20 sec thereafter two aliquots of 25 μl per sampling point were taken. In such aliquots the reaction was stopped with SDS as described for condition B (6).

At $t = 22$ sec $ACTH_{1-24}$ (10 μl, final concentration 10^{-4} M) or 10 μl medium was added and again 25-μl aliquots were taken at $t = 40$ sec, 1, 5, 10, 20, and 30 min. In addition, at $t = 30$ min, 40 μl of the incubation mixture was taken and added to the preincubated fresh sample of [γ-^{32}P]ATP resulting in a concentration of 7.5 μM ATP and a final volume of 50 μl. 20 sec thereafter from this new incubation mixture a last 25-μl aliquot was taken. After the stop procedure all samples were subjected to polyacrylamide gel electrophoresis followed by densitometric scanning of the autoradiographs as described above.

RESULTS

Effect of $ACTH_{1-24}$ on Endogenous SPM Phosphorylation: Structure–Activity Relation

In Figure 1 the relative phosphorylation of a number of SPM protein bands is presented, measured under two different conditions of ATP/SPM ratios (A and B). As already shown (5), $ACTH_{1-24}$, added in vitro, reduced the incorporation of radioactive ^{32}P into SPM proteins of low molecular weight (bands B-50, D-10, D-20, D-25, and D-30) when the incubation was performed under condition A. In addition, under condition B bands D-15 and D-26 were also affected (for SPM phosphorylation profiles and band numbering, see reference 6).

The structure-activity relationship was studied using 10^{-4} M concentration of the ACTH fragments: $ACTH_{1-10}$, $ACTH_{11-24}$, $ACTH_{1-10}$ + $ACTH_{11-24}$, $ACTH_{1-16}$-NH_2, $ACTH_{1-13}$-NH_2, (Lys^{17},Lys^{18}) $ACTH_{5-18}$, $ACTH_{5-16}$-NH_2, $ACTH_{7-16}$-NH_2, and $ACTH_{4-10}$. None of these peptides affected the phosphorylation of phosphoprotein bands

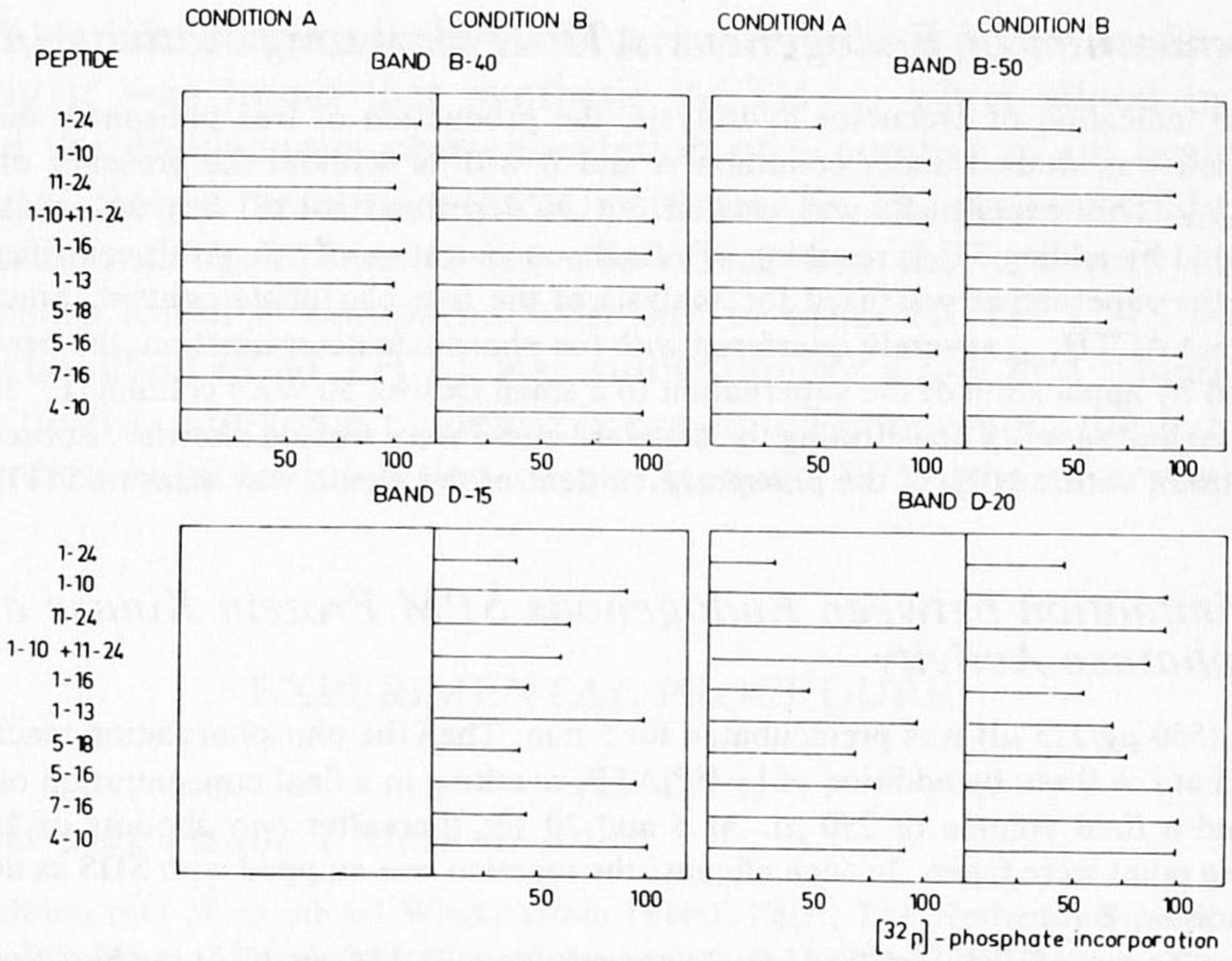

FIG. 1. Effect of N-terminal fragments of ACTH (10^{-4} M) on endogenous phosphorylation of four SPM protein bands. The incubations were carried out in triplicate under different conditions: (A) 7.5 μM ATP, 2.2 μg SPM/μl; (B) 200 μM ATP, 0.4 μg SPM μl. The results were obtained using the densitometric method, are expressed as percentage of control, and are representative of three separate experiments. Band D-15 is not detectable under condition A.

other than those influenced by $ACTH_{1-24}$. Representative for this absence of effect are the data obtained for band B-40, shown in Figure 1. Yet, the effect of $ACTH_{1-24}$ on the low-molecular-weight bands appeared to be dependent on certain structural requirements. As representative for the structure–activity relationship in Figure 1 data are shown for bands B-50, D-15, and D-20.

Under both kinetic conditions (ATP/SPM ratios) the sequence (1–16) was as effective as (1–24) and the short sequences (1–10) and (4–10) were completely inactive. The effectiveness of the other sequences depended on the ATP/SPM ratio in the incubation mixture and differed for the various low-molecular-weight SPM phosphoprotein bands. Under condition A, band B-50 was only affected by (1–24) and (1–16) and band D-20 by (1–24), (1–16), (5–18), and (5–16). Under condition B these bands were both affected by (1–24), (1–16), (1–13), and (5–18). This latter observation was also true for band D-30 (data not shown). The phosphorylation of two other bands, D-15 and D-26, was affected differently, as is shown for band D-15 (Figure 1); (1–24), (11–24), the combination of

(1–10)+(11–24), (1–16), (5–18), and (7–16) reduced the amount of phosphorylation.

Effect of $ACTH_{1-24}$ on Endogenous ATP-Hydrolyzing Activity of SPM

The ATP-hydrolyzing activity of SPM was studied under both conditions A and B. After an initial linear relationship with time, the free phosphate production leveled off between 20 and 40 sec (see reference 6). The presence of 10^{-4} M $ACTH_{1-24}$ did not influence the appearance of inorganic phosphate into the incubation medium.

Does $ACTH_{1-24}$ Affect Phosphorylation or Dephosphorylation?

To study a possible effect of $ACTH_{1-24}$ on phosphatase activity, SPM material was incubated under the low ATP/SPM ratio condition (A) and the peptide was added to the incubation mixture at $t = 22$ sec, when only net dephosphorylation is measured (6) $ACTH_{1-24}$ (10^{-4} M) did not alter the amount of ^{32}P recovered in separated protein bands, measured over a long incubation time (30 min). In Figure 2 this is shown for band B-50, taken as a representative of the ACTH-sensitive protein bands.

If, however, at the end of this period, a new aliquot of [γ-^{32}P]ATP was added (final concentration 7.5 μM) and again protein phosphorylation

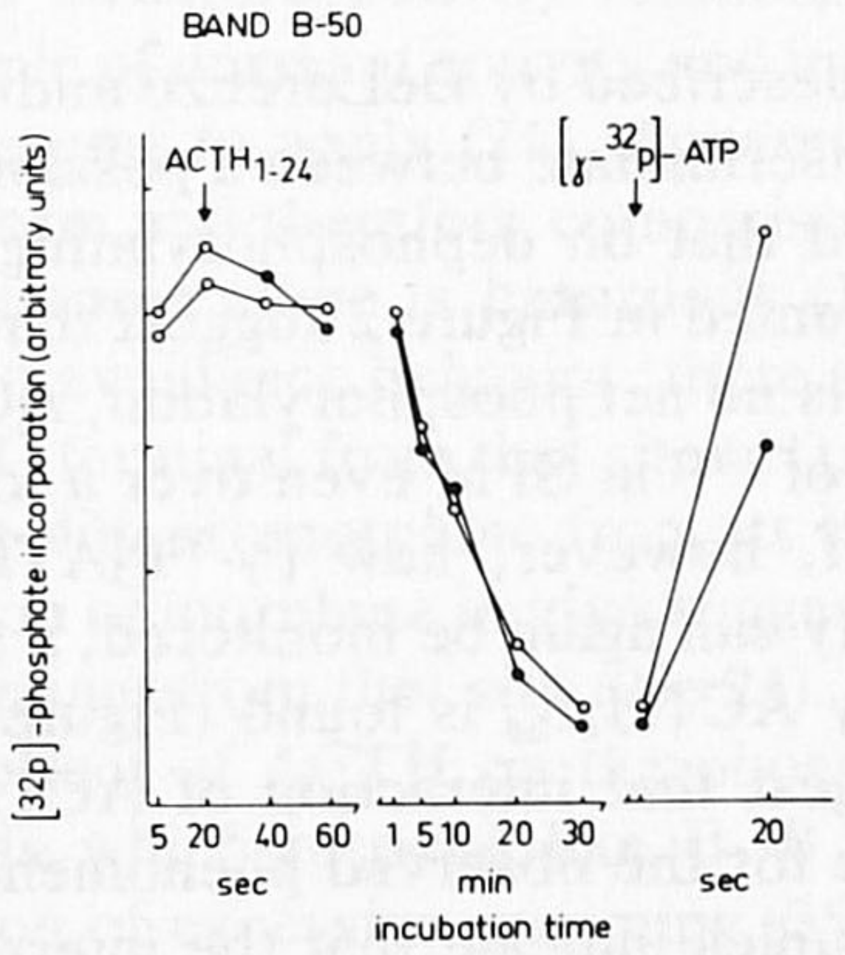

FIG. 2. Endogenous phosphorylation of SPM protein band B-50 as a function of incubation time, measured by the densitometric method, and expressed in arbitrary units. The incubations were performed under condition A (7.5 μM ATP, 2.2 μg SPM/μl). At 22 sec (arrow) either buffer (○——○) or $ACTH_{1-24}$, resulting in a concentration of 10^{-4}M (●——●), was added. At 30 min. (arrow) [γ-^{32}P]ATP was added, to increase the ATP concentration with 7.5 μM. Results are representative of three separate experiments.

occurs (6), then $ACTH_{1-24}$ appeared to inhibit the renewed incorporation of phosphate into proteins (Figure 2).

DISCUSSION

It has been suggested that phosphorylation of cerebral membrane proteins is important in neural functioning (for review see reference 12). Indeed correlation of protein phosphorylation and behavioral performance (13–16) or neuronal electrical activity (12) has been reported. The present paper elaborates on the previously reported effect of the behaviorally active peptide $ACTH_{1-24}$ on the endogenous phosphorylation of SPM proteins in vitro (5).

As discussed elsewhere the incorporation of ^{32}P into proteins, as measured in vitro, is the result of phosphorylation by protein kinases, dephosphorylation by protein phosphatases, and exhaustion of the radioactive ATP by ATPases (6, 17, 18). ACTH appeared to inhibit the phosphorylation of low-molecular-weight SPM protein bands in vitro (5; Figure 1). The mechanism by which ACTH exerts this effect is not known. It seems highly unlikely that the effect is caused by a stimulation of endogenous ATP-hydrolyzing activity present in the SPM preparation used. This would have resulted in only a general rather than a more specific decrease of labeling due to limitation of the ^{32}P donor. Furthermore, no effect of $ACTH_{1-24}$ on the breakdown of ATP by SPM could be detected.

Using the approach described by DeLorenzo and Greengard (19) a first attempt was made to discriminate between a possible effect of $ACTH_{1-24}$ on phosphorylating and that on dephosphorylating activity in the SPM fraction. The data presented in Figure 2 suggest that after the exhaustion of ATP (6) when there is no net phosphorylation, $ACTH_{1-24}$ is ineffective in altering the amount of ^{32}P in SPM even over a long incubation period (dephosphorylation). If, however, new [γ-^{32}P]ATP is added and thus phosphorylation activity can again be monitored, a subsequent inhibition of ^{32}P incorporation by $ACTH_{1-24}$ is found (Figure 2). Therefore, these data are taken to suggest that interaction of ACTH with SPM protein kinase(s) is responsible for the observed phenomenon.

Structure–activity studies indicate that the interaction of ACTH with endogenous SPM phosphorylation is complex. There is clearly a differential effect depending on protein bands, ATP/SPM ratio, and peptide used. In evaluating the data in terms of structure–activity we will confine ourselves to those obtained under the high ATP/SPM ratio condition, for reasons discussed in the previous paper (6).

It would appear that the capability of $ACTH_{1-24}$ to inhibit phosphorylation of the low-molecular-weight SPM bands (represented by B-50 and D-20, Figure 1) is confined to the N-terminal part of the molecule. The shortest active sequence with the N terminus intact is (1–13). The sequence (5–18) seems as active as (1–13), and it may therefore be concluded that the active site is in the region (5–13). Possibly, C-terminal elongation is necessary for expression of the activity since (5–16) is inactive but (5–18) is active. (Figure 1).

Clearly the structure–activity relation for band D-15 (and D-26) is different from that of bands B-50, D-10, D-20, D-25, and D-30. Again this suggests that the phosphorylation of these bands has other characteristics, as was concluded from their differential behavior under the two ATP/SPM conditions (6). Interestingly bands D-15 and D-26 are the only bands that are affected by $ACTH_{11-24}$. This inhibitory effect by the sequence (11–24) is apparently not influenced by the presence of an equimolar amount of $ACTH_{1-10}$. It should be noted that the sequence (11–24) has affinity for the ACTH receptor in the adrenal membranes but antagonizes the effects of both $ACTH_{1-39}$ and $ACTH_{1-10}$ on steroidogenesis (20). From the inactivity of (1–13) and the activity of (5–18) and (7–16) on band D-15 and D-26, one might conclude that the phosphorylation of these bands is affected by an amino acid sequence located C-terminal from that involved in the effect on the other bands. The inactivity of sequence (5–16) is incompatible with such reasoning or inexplicable at present.

Other ACTH–CNS structure–activity relationships are not identical, but the general principle of dormant activity and induction of such activity by chain elongation seems to apply (21). Apparently, information is encoded in a multiple form and therefore comparison between peptides on basis of primary structures alone is hazardous (1, 22). With respect to the effect of ACTH on avoidance behavior, there seems to exist an active site in (4–7) and one C terminal from that site (21). Also the requirements necessary to displace dihydromorphine from its binding site in rat brain SPM and counteraction of morphine in vivo suggest a site in the sequence (4–10) and one C terminal from that site (23, 24).

Interestingly, the effect of ACTH on the phosphorylation under condition B of the bands which respond like B-50 is very similar to that found for the induction of excessive grooming (25). The peptides (1–24), (1–16), (1–13), (5–18) and to some extent (5–16) induce the display of excessive grooming in the rat after intraventricular administration, whereas (1–10), (4–10), (11–24), (7–16), and the combination of (1–10)+(11–24) are ineffective. Work is in progress to further investigate a possible correlation between this neurochemical and behavioral activity

of ACTH and preliminary observations point to alterations in the phosphorylation of band B-50, D-10, D-20, D-25, and D-30 after in vivo administration of ACTH (26, 27).

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REFERENCES

1. DE WIED, D., and GISPEN, W. H. 1977. Behavioral effects of peptides. Pages 391–442, *in* Gainer, H., and Barker, J. L. (eds.), Peptides in Neurobiology, Plenum Press, New York.
2. GISPEN, W. H., VAN REE, J. M., and DE WIED, D. 1977. Lipotropin and the central nervous system. Int. Rev. Neurobiol. 20:209–249.
3. SCHOTMAN, P., REITH, M. E. A., VAN WIMERSMA GREIDANUS, TJ. B., GISPEN, W. H., and DE WIED, D. 1976. Hypothalamic and pituitary peptide hormones and the central nervous system: With special reference to the neurochemical effects of ACTH. Pages 309–344, *in* Gispen, W. H. (ed.), Molecular and Functional Neurobiology, Elsevier, Amsterdam.
4. DUNN, A. J., and GISPEN, W. H. 1977. How ACTH acts on the brain. Biobehav. Rev. 1:15–23.
5. ZWIERS, H., VELDHUIS, D., SCHOTMAN, P., and GISPEN, W. H. 1976. ACTH, cyclic nucleotides and brain protein phosphorylation in vitro. Neurochem. Res. 1:669–677.
6. WIEGANT, V. M., ZWIERS, H., SCHOTMAN, P., and GISPEN, W. H. 1977. Endogenous phosphorylation of rat brain synaptosomal plasma membranes in vitro: Methodological aspects. Neurochem. Res. (this volume).
7. TERENIUS, L. 1973. Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. Acta Pharmacol. Toxicol. 32:317–320.
8. LUGTENBERG, B., MEIJERS, J., PETERS, R., VAN DER HOEK, P., and VAN ALPHEN, L. 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. FEBS Lett. 58:254–258.
9. UEDA, T., MAENO, H., and GREENGARD, P. 1973. Regulation of endogenous phosphorylation of specific proteins in synaptic membrane fractions from rat brain by adenosine 3′,5′-monophosphate. J. Biol. Chem. 248:8295–8305.
10. EHRLICH, Y. H., and ROUTTENBERG, A. 1974. Cyclic AMP regulates phosphorylation of three protein components of rat cerebral cortex membranes for thirty minutes. FEBS Lett. 45:237–243.
11. HURST, R. O. 1964. The determination of nucleotide phosphorus with a stannous chloride-hydrazine sulphate reagent. Can. J. Biochem. 42:287–292.
12. GREENGARD, P. 1976. Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters. Nature 260:101–108.
13. PERUMAL, R., GISPEN, W. H., WILSON, J. E., and GLASSMAN, E. 1975. Phosphoryl-

ation of proteins from the brains of mice subjected to short-term behavioral experiences. Pages 201–207, *in* Gispen, W. H., van Wimersma Greidanus, Tj. B., Bohus, B., and de Wied, D. (eds.), Progress in Brain Research, Vol. 42, Elsevier, Amsterdam.

14. PERUMAL, R., GISPEN, W. H., GLASSMAN, E., and WILSON, J. E. 1977. Phosphorylation of proteins of synaptosome-enriched fractions of brain during short term training experience: the effects of various behavioral treatment. Behav. Biol. 21:341–357.
15. ROUTTENBERG, A., EHRLICH, Y. H., and R. R. RABJOHNS. 1975. Effect of a training experience of phosphorylation of a specific protein in neocortical and subcortical membrane preparations. Fed. Proc. 34:17.
16. EHRLICH, Y. H., RABJOHNS, R. R., and ROUTTENBERG, A. 1977. Experiental input alters the phosphorylation of specific proteins in brain membranes. Pharmacol. Biochem. Behav. 6:169–175.
17. RODNIGHT, R., REDDINGTON, M., and GORDON, M. 1975. Methods for studying protein phosphorylation in cerebral tissues. Pages 324–367, *in* Marks, N., and Rodnight, R. (eds.), Research Methods in Neurochemistry, Vol. 3, Plenum Press, New York.
18. WELLER, M., and MORGAN, I. 1976. Distribution of protein kinase activities in subcellular fractions of rat brain. Biochim. Biophys. Acta 436:675.
19. DELORENZO, R. J., and GREENGARD, P. 1973. Activation by adenosine 3′:5′-monophosphate of a membrane-bound phosphoprotein phosphatase from toad bladder. Proc. Natl. Acad. Sci. U.S.A. 70:1831–1835.
20. SEELIG, S., SAYERS, R., SCHWYZER, R., and SCHILLER, P. 1971. Isolated adrenal cells: $ACTH_{11-24}$, a competitive antagonist of $ACTH_{1-30}$ and $ACTH_{1-10}$. FEBS Lett. 19:233.
21. GREVEN, H. M., and DE WIED, D. 1977. The influence of peptides structurally related to ACTH and MSH on active avoidance behaviour in rats. A structure-activity relationship study. Pages 140–152, *in* Tilders, F., Swaab, D., and van Wimersma Greidanus, Tj. B. (eds.), Control, Chemistry and Effects of MSH, Karger, Basel.
22. WIEGANT, V. M., and GISPEN, W. H. 1977. ACTH-induced excessive grooming in the rat: Latent activity of $ACTH_{4-10}$. Behav. Biol. 19:554–558.
23. TERENIUS, L., GISPEN, W. H., and DE WIED, D. 1975. ACTH like peptides and opiate receptors in the rat brain: structure activity studies. Eur. J. Pharmacol. 33:395–399.
24. GISPEN, W. H., BUITELAAR, J., WIEGANT, V. M., TERENIUS, L., and DE WIED, D. 1976. Interaction between ACTH fragments, brain opiate receptors and morphine induced analgesia. Eur. J. Pharmacol. 39:393–397.
25. GISPEN, W. H., WIEGANT, V. M., GREVEN, H. M., and DE WIED, D. 1975. The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: Structure activity studies. Life Sci. 17:645–652.
26. ZWIERS, H., WIEGANT, V. M., SCHOTMAN, P., and GISPEN, W. H. 1977. Neuropeptides and rat brain phosphoproteins. Abstract 6th Int. Meeting of the Int. Soc. for Neurochemistry, Copenhagen, August 1977, Abstract No. 461.
27. ZWIERS, H., WIEGANT, V. M., SCHOTMAN, P., and GISPEN, W. H. 1977. Intraventricular administered ACTH and changes in rat brain protein phosphorylation: A preliminary report. Pages 267–273, *in* Roberts, S., Lajtha, A., and Gispen, W. H. (eds.), Mechanism Regulation and Special Functions of Protein Synthesis in the Brain, Elsevier, Amsterdam.