ACTH₁₋₂₄ Releases a Protein from Synaptosomal Plasma Membranes

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Abstract: Brain membranes contain several protein kinases, all of which appear to play a role in the regulation of neuronal functioning. These membranes also contain numerous (phospho) proteins. It has been proposed that the degree of phosphorylation of some of these proteins may affect neuronal membrane properties. In a series of previous reports we showed that ACTH₁₋₂₄ inhibits the endogenous phosphorylation of several synaptosomal plasma membrane (SPM) proteins including the B-50 protein. Although we have speculated that the degree of phosphorylation of B-50 may be important in regulating the turnover of membrane (poly)-phosphoinositides, the exact nature of the interaction between ACTH₁₋₂₄ and B-50/B-50 protein kinase is unknown. The purpose of the present study was to determine whether treatment of SPM with ACTH₁₋₂₄ will lead to a specific release of proteins from SPM. We found that ACTH₁₋₂₄ specifically releases a 41,000 M_r protein from rat brain SPM. Although we are not certain about the biological significance of the release of this polypeptide, it is of sufficient interest for further research in view of the lack of success of finding binding of labeled ACTH to brain membranes. **Key Words:** Synaptosomal plasma membrane—ACTH₁₋₂₄—B-50 protein kinase. Aloyo V. J. et al. ACTH₁₋₂₄ releases a protein from synaptosomal plasma membranes. *J. Neurochem.* 38, 871–875 (1982).

Brain membranes contain several protein kinases, all of which appear to play a role in the regulation of neuronal functioning. These kinases include cyclic AMP-dependent kinases type I and II (Walter et al., 1978), calcium-dependent kinase (Schulman and Greengard, 1978), nucleotide-independent kinase (Miyamoto et al., 1978), and the ACTH-sensitive B-50 kinase (Zwiers et al., 1980). In addition, these membranes contain numerous (phospho)proteins. It has been proposed that the degree of phosphorylation of some of these proteins may affect neuronal membrane properties (Greengard, 1979; Rodnight, 1979).

In a series of previous studies we have shown that ACTH₁₋₂₄ inhibits the endogenous phosphorylation of several synaptosomal plasma membrane (SPM) proteins including the B-50 protein (M_r 48,000; IEP 4.5) (Zwiers et al., 1978). Although we have speculated that the degree of phosphorylation of B-50 may be important in regulating the turnover of

membrane (poly)-phosphoinositides (Jolles et al., 1980), the exact nature of the interaction between ACTH₁₋₂₄ and B-50/B-50 protein kinase is unknown. Considering that the inhibition of endogenous protein kinases may be brought about by changes in the accessibility of the acceptor protein to the catalytic subunit, we have in the present work studied whether treatment of SPM with ACTH₁₋₂₄ will lead to a specific release of proteins from SPM. Such a release has been reported for the treatment of erythrocyte plasma membranes with either 1 M-NaCl (Rubin, 1979) or 1 μM-cyclic AMP (Owens and Haley, 1978). In this communication we report that ACTH₁₋₂₄ specifically releases a protein (M_r 41,000) from rat brain SPM.

MATERIALS AND METHODS

Cerebral cortex SPM was prepared from 150 g female rats according to the method of Terenius (1973). The rats

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Abbreviations used: M_r, Relative molecular weight; PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium dodecyl sulfate; SPM, synaptosomal plasma membranes.

were sacrificed by decapitation, their cerebral cortices were quickly dissected free of other brain parts, and then homogenized in 7.5 ml of ice-cold 0.32 M-sucrose per cortex. Unless otherwise specified, all subsequent procedures were carried out at 0–4°C. The homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was carefully separated from the pellet before being centrifuged at 10,000 *g* for 20 min. The resulting pellet (P2) was shocked by resuspension in 3.5 ml water per cortex. After stirring for 10 min this suspension was recentrifuged at 10,000 *g* for 20 min. The supernatant containing the light membrane fragments were carefully layered over a discontinuous sucrose gradient consisting of a bottom layer of 1 M-sucrose and an upper layer of 0.4 M-sucrose and centrifuged at 100,000 *g*_{max} for 80 min in a Beckman SW 27.1 rotor. The resulting SPM was obtained by collecting the material at the 0.4 M–1.0 M-sucrose interface. These membranes were washed by resuspension in buffer A (10 mM-sodium acetate, 10 mM-magnesium acetate, 1 mM-calcium acetate, pH 6.5) and then by centrifugation at 100,000 *g*_{max} for 20 min. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Extraction was performed by resuspending the SPM at a final concentration of 5 mg protein per milliliter of buffer A with or without various extractants. The suspension was placed in a 30°C water bath for 5 min and then centrifuged at 100,000 *g*_{max} for 30 min. The supernatant (ex-

tract) was dialyzed against two changes of buffer A and then used without further treatment. The pellet was washed by resuspending at 5 mg protein per milliliter of buffer A and recentrifuging. Finally, the pellet was resuspended in the original volume of buffer A and dialyzed against two changes of buffer A before use.

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on slab gels containing 11% acrylamide as previously described (Zwiers et al., 1976). After separation, the gel was fixed and stained with 0.1% Fast Green in MeOH:acetic acid:water, 50:10:40 (by vol.) and then destained in the same solvent. Densitometric tracings of the proteins were made by scanning the gel at 650 nm with a Zeiss KM3 chromatogram spectrophotometer. For gels containing radioactive proteins, the gels were dried and subjected to autoradiography as previously described (Wiegant et al., 1978).

Protein kinase activity of the SPM was assayed by the modified method of Zwiers et al. (1976). The endogenous B-50 kinase activity was determined using 30 μ g of SPM protein, 7.5 μ M-ATP, 1 μ Ci [γ -³²P]ATP. In some cases the effects of 100 μ M-ACTH_{1–24} (final concentration) were tested on kinase activity; a 5- μ l aliquot was added 10 s before initiating the reaction. After incubation at 30°C for 20 s the reaction was terminated by the addition of stop mix containing SDS (Zwiers et al., 1976) before being applied to an SDS-PAGE slab gel.

FIG. 1. Autoradiogram of extracted SPM and the protein staining pattern of several SPM extracts. **(A)** Autoradiogram of the endogenous phosphorylation of residual SPM proteins after extraction. SPM was extracted with either buffer A (1) or buffer A containing 1 M-NaCl (2). The residual proteins were collected by centrifugation and then dialyzed overnight before being phosphorylated with (+) or without (–) 100 μ M-ACTH_{1–24}. After phosphorylation the proteins were separated by SDS-PAGE and an autoradiogram prepared. The molecular weights of the indicated phosphoproteins are: A-50, 78,000; B-30, 57,000; B-40, 53,000; and B-50, 48,000. **(B)** The SDS-PAGE protein pattern of SPM (1,9) buffer A extract of SPM (2), 100 μ M cyclic AMP extract (3), 100 μ M cyclic GMP extract (4), 100 μ M ACTH_{1–24} extract (5), 1 M-NaCl extract (6), the catalytic subunit of beef heart cyclic AMP-dependent protein kinase (Sigma P2645) (7), and molecular weight marker proteins (Pharmacia low molecular weight calibration kit (8) are shown.

RESULTS

Figure 1A shows that incubation of buffer A-extracted SPM with [γ -³²P]ATP results in the phosphorylation of several protein bands. The phosphorylation pattern is similar to that found previously in our laboratory for unextracted SPM (data not shown) (see Zwiers et al., 1978). Addition of 100 μ M-ACTH₁₋₂₄ 10 s before initiating the phosphorylation reaction results in the specific inhibition of the phosphorylation of B-50 protein (Fig. 1A). Extraction of SPM with 1 M-NaCl and subsequent removal of the salt by dialysis reduces the endogenous phosphorylation of residual SPM proteins, especially bands A-50 and B-50 (Fig. 1B). However, the capacity to catalyze the phosphorylation of B-50 in the NaCl-extracted membranes is still ACTH-sensitive.

The protein patterns of the several extracts after SDS-PAGE are shown in Fig. 1B. The 1 M-NaCl extraction procedure releases a number of proteins from SPM, whereas 100 μ M-ACTH₁₋₂₄ extracts only one major protein band with an apparent molecular weight of approximately 41,000 (Fig. 1B). The buffer A, 100 μ M cyclic AMP, and 100 μ M cyclic GMP extracts contain only trace amounts of protein including very low amounts of the 41,000 M_r protein.

Figure 2A shows the dose dependence of the extraction of the 41,000 M_r protein by ACTH₁₋₂₄, cyclic AMP, and cyclic GMP. At all concentrations tested cyclic AMP and cyclic GMP extract only trace amounts of the protein. For ACTH₁₋₂₄ a nearly linear dose-response curve exists and at a concentration as low as 5 μ M noticeable extraction occurs. The structure-activity study (Fig. 2B) shows that the activity resides in the C-terminal part of ACTH₁₋₂₄ as the sequences (1-24) and (11-24) are active and the sequences (1-10) and (1-16)-NH₂ are not (Fig. 2B). In the last column the effect of the peptides on the endogenous phosphorylation of B-50 *in vitro* taken from Gispen et al. (1979) is indicated. There is a clear difference in structural requirements for releasing the 41,000 M_r protein and for inhibiting the B-50 protein kinase.

DISCUSSION

Rubin (1979) has demonstrated that treatment of membranes with 1 M-NaCl releases the membrane-bound catalytic subunit of cyclic AMP-dependent protein kinase. This implies that such treatment reduces the phosphorylation of this enzyme's natural substrate proteins in the residual membrane. In this study we have shown that treatment of SPM with 1 M-NaCl specifically reduces the

FIG. 2. Dose-response and structure-activity studies on the extraction of 41,000 M_r protein from SPM. The extracts from equal quantities of SPM were separated by SDS-PAGE and the relative amount of the 41,000 M_r protein was determined by scanning the gel. **(A)** The dose-response curve for the extraction of 41,000 M_r protein by ACTH₁₋₂₄ (○), cyclic AMP (□), or cyclic GMP (×). **(B)** Structure-activity relationship for the extraction of the 41,000 M_r protein by the indicated ACTH fragments. This is compared with the inhibition of the endogenous B-50 phosphorylation in SPM (according to Gispen et al., 1979). All peptides were used at a final concentration of 100 μ M.

phosphorylation of two substrate proteins (B-50 and A-50). This result could be obtained if the NaCl treatment removes either (1) the substrate proteins, (2) the kinase for these substrates, or (3) a modulator of the kinase activity. The removal of the substrate is unlikely to be responsible for the observed NaCl effect since the SDS-PAGE gel protein staining pattern of the residual SPM proteins shows that, at least for B-50, there is no reduction of staining. Since the protein A-50 cannot be distinguished in Fast Green-stained SDS gels of SPM it will be eliminated from further discussion. Further, an examination of the protein staining pattern of the NaCl extract after SDS-PAGE revealed no B-50 protein (Fig. 1B). This observation was confirmed by examination of the autoradiogram obtained after SDS-PAGE of the 1 M-NaCl extract of prephosphorylated SPM (data not shown). These results rule out the removal of the substrate (B-50) from the membranes by the NaCl treatment.

Figure 1 shows, in agreement with our previous results (Zwiers et al., 1976), that ACTH₁₋₂₄ specifically inhibits the phosphorylation of the B-50 protein in SPM. Since both NaCl and ACTH₁₋₂₄ inhibit the phosphorylation of membrane-bound B-50, this suggests a similar mechanism of action. Examination of the protein staining pattern of the ACTH₁₋₂₄ extract of SPM after SDS-PAGE also reveals no B-50 protein (Fig. 1B). However, one major protein band was found corresponding to a major protein of the NaCl extract (Fig. 1B). Rubin's (1979) work suggests that the NaCl extract should contain the catalytic subunit of cyclic AMP-dependent protein kinase. Figure 1B shows that when the ACTH and NaCl extracts are compared by SDS-PAGE with the catalytic subunit of cyclic AMP dependent protein kinase the 41,000 M_r band is similar but not identical to this catalytic subunit.

It is generally accepted, for both the type I and the type II cyclic AMP-dependent protein kinases, that cyclic AMP causes the dissociation of the holoenzyme into the regulatory and catalytic subunits (Krebs and Beavo, 1979). For erythrocytes, 1 μ M cyclic AMP causes release of the catalytic subunit from the plasma membrane. In our experiments, cyclic AMP (Fig. 2A) did not release the 41,000 M_r protein from SPM. In addition, these concentrations of cyclic AMP do not inhibit B-50 phosphorylation in SPM (Zwiers et al., 1976).

From these results it appears unlikely that the 41,000 M_r band is related to the previously identified cyclic AMP-dependent protein kinases. Further evidence that the 41,000 M_r protein is not the B-50 kinase comes from our recent work. We have purified the B-50 protein kinase from rat brain membranes and have shown that this kinase has a molecular weight by SDS-PAGE of 70,000 (Zwiers et al., 1980).

The reported levels of ACTH in various brain re-

gions are 100–500 times lower than the lowest active concentration used in this study (Krieger et al., 1980). However, the possible release of ACTH from peptidergic neurons may allow for locally higher concentrations. Irrespective of the actual physiological concentration of ACTH the dose-response relationship for the release of 41,000 M_r protein (Fig. 2A) and the inhibition of the B-50 protein kinase by ACTH₁₋₂₄ (Zwiers et al., 1976) are of the same order of magnitude. However, the discrepancy between the structure-activity relationships for extraction of 41,000 M_r protein and inhibition of B-50 phosphorylation (Fig. 2B) suggests that the two peptide effects are only remotely related. Thus, although removal of the 41,000 M_r band results in the reduction of endogenous B-50 phosphorylation (Fig. 1), there are sequences such as (1-16)-NH₂ that do not release the 41,000 M_r band but do inhibit the phosphorylation of B-50 (Fig. 2B). Even more disconcerting is the lack of effect of the sequence (11–24) on the B-50 phosphorylation, although it strongly releases the 41,000 M_r band.

Preliminary evidence shows that the ACTH₁₋₂₄ cannot be removed from the 41,000 M_r protein indicating an interaction between the two polypeptides. Although we are not certain about the biological significance of the release of 41,000 M_r, it is of sufficient interest for further research in view of the lack of success of finding binding of labeled ACTH to brain membranes (Witter, 1980). Thus, although in both the NaCl and ACTH₁₋₂₄ extract the release of the 41,000 M_r protein was observed, at present it would appear that the release of this band is not the sole explanation for the inhibition of B-50 protein kinase. It is entirely possible that the interaction with the 41,000 M_r relates to other known chemical effects of ACTH in the brain.

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