INHIBITION BY Ca^{2+} OF THE ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE-STIMULATED PHOSPHORYLATION OF PROTEINS IN MEMBRANES FROM OX NEUROHYPOPHYSEAL SECRETOSOMES

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1. Introduction

During the last few years, evidence has been presented suggesting a functional role for adenosine 3′:5′-monophosphate (cAMP) in the secretion of neurohypophyseal hormones [1–4]. A role for Ca^{2+} in this organ, as a necessary secretion trigger has been well established (reviewed [5]).

In the nervous system, stimulation by cAMP of the phosphorylation of membrane proteins from synaptic structures has been described [6–8], and an inhibitory effect of Ca^{2+} on this type of system has been reported [9]

In view of these findings, we examined the endogenous phosphorylation activity in membranes from ox neurohypophyses with respect to possible interactions between Ca^{2+} and cAMP. A complex picture of several phosphorylated bands was revealed using an SDS–polyacrylamide electrophoresis system. The cAMP-stimulated phosphorylation of one of the bands, corresponding to a mol. wt 80 000 protein, was inhibited by Ca^{2+} at down to 0.1 mM or less.

2. Materials and methods

[γ-^{32}P]Adenosine 5′-triphosphate, spec. act. > 1000 Ci/mM, was purchased from the Radiochemical Centre, Amersham. It was lyophilized to remove ethanol before use. Adenosine 5′-triphosphate, sodium salt, and adenosine 3′:5′-monophosphoric acid, crystalline, were from Sigma Chemical Company, St Louis, MO. All other chemicals were of analytical grade.

Secretory nerve endings from ox neurohypophyses (neurosecretosomes) were prepared in a homogenization medium containing sucrose, 0.25 M and *N*-Tris-(hydroxymethyl)methyl-2-amino ethane sulfonic acid (TES) 20 mM (pH 7.0) as in [10]. The neurosecretosomes were lysed in 20 vol. TES 5 mM (pH 8.0) for 1 h in the cold. Aliquots of the lysate were sonicated on ice for 5 s, 50 W, the membranes were sedimented at 100 000 × g_{max} for 45 min, and stored in the homogenization medium at −18°C with no loss of phosphorylation activity within 1 week

Membrane protein, 0.3 mg, was used in each phosphorylation assay. In the routine assay, the concentrations in the final reaction vol. 0.125 ml were (mM) TES 20.0 (pH 7.0), sucrose 100, ethylene glycol-bis-(β aminoethyl ether)*N,N*′tetraacetic acid (EGTA) 1.0, Mg_{free}^{2+} 2.0, [γ-^{32}P]ATP 0.01 (spec. act. 2–6 Ci/mM). Cyclic AMP, where used, was 5 μM, and free Ca^{2+} concentration was varied as specified in section 3

Prior to adding [γ-^{32}P]ATP, the membranes were preincubated on ice in the reaction mixture for 5 min, in the next 2 min the mixture was brought to 30°C. ATP was added, and after 10 s with vigorous shaking the reaction was stopped with sodium dodecyl sulphate (SDS) and β-mercaptoethanol, added to final conc 2% for each.

To determine the nature of the incorporated $^{32}PO_4$ activity, the reactions were stopped with 2.0 ml ice-cold trichloroacetic acid (TCA) 10%, and the mix-

tures treated in one of the following ways

(i) The precipitate was placed on a boiling water bath for 3 min, then collected by centrifugation, washed in 2.0 M phosphate buffer, at pH 7.8, and resuspended for electrophoresis in SDS 2%, β-mercaptoethanol 2%

(ii) The cold precipitate was sedimented and resuspended in 0.2 ml ice-cold NaOH, 0.5 M, kept on ice for 5 min, reprecipitated with 10% TCA, again sedimented and washed with phosphate buffer and resuspended for electrophoresis as in (i) above.

(iii) Same as (ii), except that after adding 0.5 M NaOH, 3 min on a boiling bath preceeded the 5 min on ice.

(iv) As (i), but boiling in TCA was omitted and the pellet after the buffer wash was dispersed in 1.0 ml diethyl ether–ethanol mixture, 1:1, and kept at room temperature for 10 min. The protein was then sedimented and resuspended for electrophoresis.

(v) As (iv), except that the pellet after buffer wash was resuspended in 20 mM phosphate buffer (pH 7.8). The material was then incubated with either pronase, (10 μg, for 30 min at 37°C) or ribonuclease (2 μg for 60 min at 37°C). SDS and β-mercaptoethanol were then added to the usual final concentration.

(vi) The control sample was resuspended for electrophoresis directly after washing the TCA precipitate with phosphate buffer. Prior to the electrophoresis, all samples were boiled for 3 min. Protein, 70 μg, was applied to the gel in each sample. The electrophoresis was performed in a discontinuous SDS–gel system as in [11], using a Biorad vertical slab gel apparatus. The stacking gel contained 5% polyacrylamide, 0.125 M Tris-(hydroxymethyl)aminoethane (Tris), (pH 7.8) and 0.1% SDS. The separating gel contained a linear gradient of 7.5–17.5% polyacrylamide and 0.5–2.5% glycerol, 0.360 M Tris (pH 8.7) and 0.1% SDS. The electrode buffer contained 0.025 M Tris, at pH 8.3, 0.192 M glycin and 0.1% SDS. The electrophoresis was run at a constant 80 V and 200 V, for the passage of proteins through the stacking and the separating gel, respectively. The running time was about 4 h. After staining with Coomassie brillant blue the wet gels were cut into 1 mm slices, which were digested for 3 h at 65°C in 200 μl Nuclear Chicago Solubilizer-dioxane (2.1) and counted after cooling, adding of 3 ml. Instaflour scintillation liquid and dark adaptation in the cold for at least 2 h. Counting efficiency for ^{32}P was 100%.

Protein was determined as in [12].

Calculations: Peak area was used to express the amount of label incorporated into the protein on the gel. To obtain the desired free Mg^{2+} and Ca^{2+} concentrations in the reaction mixtures containing EGTA and ATP, the total amounts of ions to be added were calculated from the appropriate acid and complex dissociation constants by means of a Univac 1100 digital computer, as in [13].

3. Results and discussion

Figure 1 shows the effect of cAMP in the absence of added Ca^{2+}, on the net level of phosphorylation of several components in the neurosecretosomal membranes. Without cAMP, about 10 radioactive bands could be discerned. Addition of 5 μM cAMP caused a marked increase in the incorporation of $^{32}PO_4$ into bands A, B and C The profiles in fig.1 represent a typical label distribution under the conditions employed. We were mainly concerned with the behaviour of band A, since this component showed the greatest dependence on Ca^{2+} under our experimental conditions.

The nature of the radioactivity associated with band A was examined as in section 2. Extraction with lipid solvents and ribonuclease treatment did not affect the protein staining or the radioactivity distribution on the gel in band A or in its vicinity. Nor was any change seen after cold incubation of the labelled preparation with 0.5 NaOH, or boiling in 10% TCA. Conversely, both pronase and boiling with 0.5 NaOH removed the radioactivity from the position of band A, pronase treatment resulting also in removal of the stained bands from most of the gel, including the band A region. These results suggested that the labelling of the band A involves an ester bond linkage between ^{32}P-phosphate and a hydroxyamino acid residue of the protein present in this position. Both Coomassie blue staining and a typical appearance of the radioactive band as a double or shoulder-carrying

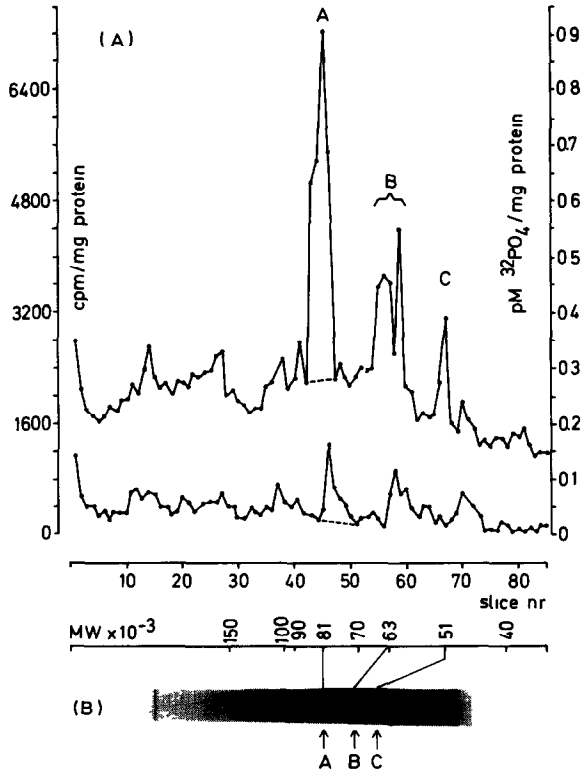

Fig.1A The distribution of the $^{32}PO_4$ activity on the gel after the SDS–polyacrylamide electrophoresis of the phosphorylated membranes from neurosectretosomes, in the absence of added Ca^{2+} cAMP (5 μM) was present (upper profile) or absent (lower profile) in the reaction mixture The general conditions for phosphorylation and for counting of the radioactivity are in section 2. Peak area above the dashed line was used for quantitation The figures are given as cpm and pM $^{32}PO_4$ (the left- and right-hand ordinates, respectively) in a gel slice/mg total protein applied to the gel The zero point for the upper profile has been displaced upwards by 1200 and 0 150 units along the left- and right-hand ordinate, respectively. Fig 1B The Coomassie blue staining pattern of the neurosecretosomal membrane proteins following SDS–polyacrylamide gel electrophoresis

peak indicated the presence of 2 incompletely separated components. When proteins of known molecular weight were run on the gel parallel to the samples, these components could be estimated to lie within mol. wt 81 000–82 000. Because of the incomplete separation, the designation 'protein A' was maintained and a total area, including the second peak where present, was used for quantification.

In the absence of Ca^{2+} and cAMP, 0.088 ± 0.019 (SE, n = 5) pM PO_4 were incorporated into protein A (expressed per mg total protein applied to the gel). With 5 μM cAMP, the corresponding value was 1.446 ± 0.430 (SE, n = 5). These results were not altered when the EGTA concentration was raised from 1.0 to 4.0 mM.

As shown in fig.2, addition of Ca^{2+} to the system caused a decrease in the net level of the cAMP-stimulated component of phosphorylation of band A. This

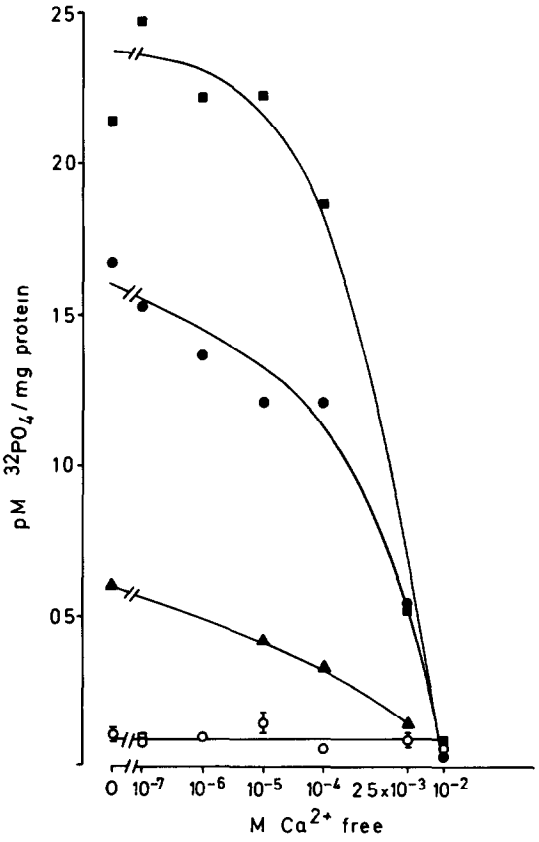

Fig 2 The effect of Ca^{2+} on the net level of phosphorylation of the protein A Closed symbols Activity in the presence of 5 μM cAMP Data for 3 independent membrane preparations are shown (▲, ●, ■). Open symbols Activity in the absence of cAMP. Except for the 10^{-7} M point, where data from 2 experiments are shown, the values are means of at least 3 experiments. The error range is indicated where it was greater than symbols used. The values of the pM $^{32}PO_4$ were derived from the peak area (see fig.1 and section 2) and were expressed per mg total protein applied to the gel The general phosphorylation conditions are in section 2

was in contrast to the lack of effect on the basal component. The effect of Ca^{2+} was constantly apparent at 0.1 mM Ca^{2+}, and > 50% at 2.5 mM Ca^{2+}. Using 2 5 mM Ca^{2+}, this result was seen to be independent of the ATP at 10–100 μM.

Three independent experiments were chosen in fig.2 to demonstrate that the membrane preparations obtained at different occasions showed some variation in the net level of phosphorylation. No firm reason can presently be suggested to explain this observation. It may be mentioned that significant variation in the absolute levels of activity between different animals was earlier found [14] in a study of the subcellular distribution of protein kinase in rat brain

Using a technique permitting recognition of the changes in the total amount of protein-incorporated label rather than following a single posphorylated component, an inhibition of endogenous protein kinase activity in neurosecretory granules from guinea pig neurohypophyses by Ca^{2+} at 1–10 mM was reported [15], both in the presence and the absence of 5 μM cAMP We here report that Ca^{2+} may exert the inhibitory effect on the phosphorylation of the individual protein in the membranes from the neurohypophyseal nerve endings, acting in the concentrations below the range mentioned above, and only affecting the cAMP-stimulated component of the phosphorylation of this protein. An action of Ca^{2+} also in the low concentration range ($10^{-7} - 10^{-5}$ M) is suggested in the present results, but further work on this aspect is needed

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