Association of cyclic-AMP-dependent protein kinase with neurofilaments

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Neurofilament preparations isolated from bovine spinal cord contain cyclic-AMP-dependent protein kinase (PKA) activity. Treatment of this preparation with cyclic AMP, to dissociate the regulatory subunit of the kinase from the catalytic subunit, resulted in retention of the kinase activity but loss of cyclic AMP regulation. This suggests that PKA is associated via its catalytic subunit with the neurofilament preparation. The association of exogenous PKA from bovine heart with the neurofilament preparation and with neurofilaments reconstituted from purified neurofilament proteins was also investigated. Either the free catalytic subunit or combinations of the catalytic and regulatory subunits of PKA were incubated with the preparations, and the degree of association was determined as the level of kinase activity that co-sediments with neurofilaments. The results indicate that the free catalytic subunit of PKA co-sediments with neurofilaments reconstituted from purified proteins. The regulatory subunit of PKA from bovine heart, when pre-mixed with the catalytic subunit, decreased the level of kinase that co-sediments with the neurofilament fraction in a dose-dependent manner. This effect of the regulatory subunit was reversed by inclusion of cyclic AMP in the incubation medium before centrifugation. The above findings suggest that the regulatory subunit, when attached to the catalytic subunit, has an inhibitory effect on its association with neurofilaments, with the implication that the association may be a cyclic-AMP-regulated event.

INTRODUCTION

Cyclic-AMP-dependent protein kinase (PKA) is a holoenzyme composed of two catalytic and two regulatory subunits. Binding of cyclic AMP to the regulatory subunits causes their dissociation from the catalytic subunits, thus producing two free catalytic subunits that are active. A large proportion of PKA in the nerve cell is associated with various intracellular structures, membranes and organelles [1–4]. The best characterized association of PKA is its interaction with microtubules, a major component of the neuronal cytoskeleton. This association, first described by Vallee and co-workers, is via binding of the type II regulatory subunit to microtubule-associated protein 2 (MAP2) [4–6]. The type II regulatory subunit of PKA also binds to a number of other proteins [7]. Such interactions are thought to direct the kinase to specific sites within the cell. The catalytic subunit of PKA has been reported to bind only two proteins, the regulatory subunit and Walsh inhibitor [8], both of which inhibit its activity.

In the present study, we investigated the association of PKA with neurofilaments (NFs), another major component of the neuronal cytoskeleton. These are 10 nm filaments, composed of three subunits, NF-H, NF-M and NF-L, in the mammal. NF-enriched preparations from different species contain associated PKA activity [9,10]. Previous work in our laboratory indicated that an NF-enriched preparation from squid axoplasm contains protein kinase activity that phosphorylates kemptide, a peptide substrate for PKA, and which is inhibited by wiptide, a specific inhibitor for PKA [11]. This activity, however, was not affected by cyclic AMP [11]. These observations suggest that the catalytic subunit of PKA and not the holoenzyme is associated with the fraction. Similarly, Caputo *et al.* [12] demonstrated that a NF-enriched preparation from rat contains kinase activity that phosphorylates PKA-specific substrates, although no activation by cyclic AMP was detected.

The results described here indicate that the endogenous PKA

in a NF preparation from bovine spinal cord is associated via the catalytic subunit. In addition, we show that the free catalytic subunit isolated from bovine heart can associate with NFs reconstituted from purified proteins. The regulatory subunit of PKA from bovine heart, when attached to the catalytic subunit, inhibits the association.

EXPERIMENTAL

[γ - 32 P]ATP was purchased from Amersham Corp. and [14 C]methylated ovalbumin was from NEN. Kemptide and wiptide were purchased from Peninsula Laboratories. ATP, cyclic AMP and the isolated catalytic and regulatory subunits of PKA from bovine heart were purchased from Sigma.

The activity of the catalytic subunit of PKA was 40–56 enzyme units/ μ g of protein (1 enzyme unit transfers 1 pmol of phosphate/min from ATP to casein at pH 6.5, at 30 °C). The activity of the regulatory subunit of PKA was 83 units/ μ g of protein (1 unit of regulatory subunit inhibits 1 enzyme unit of catalytic-subunit phosphorylating activity). The regulatory and catalytic subunits, which were obtained as freeze-dried powder, were reconstituted by adding a solution containing 1 mg of BSA/ml and 6 mg of dithiothreitol/ml, and shaking for a few minutes at room temperature. When combinations of catalytic and regulatory subunits were to be used, catalytic-subunit solutions prepared as above were added directly to the freeze-dried regulatory subunit.

Procedures to obtain an NF preparation from bovine spinal cord (see Fig. 1a, lane 2, for protein composition and Fig. 1b for electron micrograph) and for purification of NF subunits were as described previously [10]. The NF preparation was depleted of kinase activity by treatment with 0.8 M-KCl, centrifugation, and incubation for 10 min in boiling water (heat-inactivated NF preparation). Protein was measured by the method of Bradford [13], and SDS/PAGE (7.5% acrylamide in resolving gel) was carried out by the method of Laemmli [14].

Table 1. Endogenous PKA activity in control and cyclic-AMP-washed NF preparations

NF preparation was resuspended in medium containing 1 mg of BSA/ml, 0.3 mM-EGTA, 0.2 mM-EDTA, 3% (v/v) ethylene glycol, 17% (v/v) glycerol, 0.7 mM-dithiothreitol, 10 μ g of leupeptin/ml and 20 mM-Hepes/Tris, pH 7.0, in the absence (Control) or presence (cAMP-washed) of 5 μ M-cyclic AMP, at a final concentration of 3.12 mg of protein/ml. The samples were left in ice for 20 min and were then centrifuged in an Airfuge (130000 *g* for 30 min). The pellets were resuspended in medium A containing 100 μ g of leupeptin/ml, to yield a final protein concentration of 4.7 mg/ml. Regulatory subunit from bovine heart (15.6 μ g/ml) was also added at this stage to one group (cAMP-washed + RS). Portions (10 μ l) from the resuspended samples were assayed for kinase activity, with kemptide (200 μ M) as substrate. Where indicated, cyclic AMP (5 μ M) and wiptide (5 μ M) were included in assay media. The reaction was carried out for 10 min at 30 °C. Values represent kinase activities per mg of protein of NF preparation, as pmol of P_i transferred in 10 min, and are means $\pm$ S.E.M. of (*n*) estimations, each corresponding to samples centrifuged in separate tubes. For every treatment (rows), the significance of the difference between 'basal' and '+cAMP' groups was evaluated by *t* tests. The results show that the difference between 'basal' and '+cAMP' is significant in 'Control' and 'cAMP-washed + RS' samples ($P < 0.005$ and $P < 0.05$ respectively) and non-significant in 'cAMP-washed' samples.

Treatment	Kinase activity (pmol of P_i transferred)			
	Basal	+cAMP	+Wiptide	+cAMP/wiptide
Control	745 $\pm$ 67 (6)	1383 $\pm$ 147 (6)	677 $\pm$ 45 (6)	672 $\pm$ 69 (6)
cAMP-washed	1319 $\pm$ 156 (6)	1515 $\pm$ 126 (6)	617 $\pm$ 39 (6)	596 $\pm$ 27 (5)
cAMP-washed + RS	681 $\pm$ 15 (4)	1111 $\pm$ 166 (4)	715 $\pm$ 69 (3)	638 $\pm$ 56 (3)

Fig. 1. Morphology and protein composition of NF preparation and NFs reconstituted from purified subunits

(a) SDS/PAGE, protein staining patterns: lane 1, molecular-mass markers (rabbit muscle phosphorylase *b*, BSA and hen egg-white ovalbumin); lane 2, NF preparation (4.5 μ g of protein); lane 3, reconstituted NFs (1.8 μ g of protein). (b) and (c) Electron micrographs. Suspensions of NF preparation (b) and of reconstituted NFs (c) were applied on carbon-coated grids, stained with 1% uranyl acetate and viewed at $\times 50000$ magnification. Scale bar: 0.1 μ m. Arrowheads point to typical filaments.

Reconstitution of NFs from purified subunits

Purified NF subunits in urea were combined in the proportions 2:1:1 (by wt.) of NF-L, NF-M and NF-H respectively. The samples were dialysed against 170 mM-NaCl/0.5 mM-dithiothreitol/0.1 mM-EGTA/10 mM-Hepes/Tris, pH 7.0, with three changes of the buffer, for 15–20 h. They were concentrated to about 0.7 mg of protein/ml by placing the dialysis bags on poly(ethylene glycol) 20000. Alternatively, dialysis was carried out in low-salt medium (0.1 mM-EGTA/0.5 mM-dithiothreitol/10 mM-sodium phosphate, pH 7.0) and, after concentration, reconstitution was achieved by adding NaCl to a final concentration of 170 mM and incubation for about 15 min at

30 °C. Immediately before use, reconstituted NFs were recovered by centrifugation in a Microfuge (16000 *g* for 20 min). Fig. 1(a), lane 3, shows the protein composition and Fig. 1(c) is an electron micrograph of the reconstituted NF pellets.

Determination of the association of the catalytic subunit of PKA with NFs

NFs in medium A [1 mg of BSA (fatty acid; nuclease- and protease-free, from Calbiochem)/ml, 50 mM-NaCl, 20 mM-Hepes/Tris, pH 7.0] were combined with the free catalytic subunit, or combinations of regulatory and catalytic subunits, as indicated for individual experiments, in a final volume of 50 μ l.

Samples were mixed for 1 min at room temperature, placed on ice for 10–12 min with occasional stirring, and centrifuged at 4 °C in a Microfuge (16000 *g* for 20 min) to pellet the NFs. After centrifugation, the supernatants were transferred into separate tubes, and the sides of the tubes were dried with filter paper to remove any remaining supernatant. Pellets were then resuspended in medium A at a final volume of 50 μ l. Levels of associated and unassociated catalytic subunit were estimated by measuring kinase activities partitioned into pellets and supernatants respectively, as described below.

Kinase assay

The method of Roskoski [15] was employed, with certain modifications. The assay medium contained 10 mM-MgCl₂, 1 mM-EGTA, 1 mg of BSA/ml, 10 μ g of leupeptin/ml, 10 μ M-[γ -³²P]ATP (sp. radioactivity 500–2000 c.p.m./pmol), 200 μ M-kemptide, in 20 mM-Hepes/Tris, pH 7.0. The reaction was started by addition of 5 μ l of ATP and kemptide solution to fractions in 45 μ l of medium. Samples were incubated at 30 °C for 5 min. At the end of the incubation, 25 μ l samples were spotted on to squares of Whatman P81 phosphocellulose paper, which were transferred into 75 mM-H₃PO₄. The squares were washed four times with the same solution and radioactivity was measured by Cerenkov counting. A control experiment shows that, under the conditions employed, the amount of phosphate transferred is linear with respect to the amount of catalytic subunit of PKA present in the reaction mixture, up to 1.2 enzyme units of kinase per incubation, which is more than the maximal level that can be present in any assay sample tested in this study.

RESULTS

When the NF preparation from bovine spinal cord is incubated with ATP and an excess concentration of kemptide, a cyclic-AMP-regulated phosphorylation is observed. Wiptide blocks this cyclic-AMP-regulated phosphorylation (Table 1, control). These results indicate that the preparation contains PKA. The cyclic-AMP- and wiptide-independent basal phosphorylation observed must be due to the contribution of other protein kinases described to be present in the NF preparation, including the regulator independent endogenous kinase activity that phosphorylates NF proteins [10].

In order to determine the subunit responsible for the association of PKA with the NF fraction, the bond between the catalytic and regulatory subunits was disrupted by treatment with cyclic AMP, and any dissociated component was separated from the NF fraction by centrifugation. As shown in Table 1, the cyclic-AMP-washed preparation retains a similar level of wiptide-inhibitable total kinase activity to the control preparation which was washed with buffer only, although the cyclic AMP regulation is abolished (note that 'basal' and '+cAMP' groups are not significantly different in 'cAMP-washed' samples). The loss of cyclic AMP regulation is not due to residual cyclic AMP in the pellets, since it can be restored by the addition of exogenous regulatory subunit from bovine heart (Table 1, 'cAMP washed + RS'). Thus the data in Table 1 indicate that the catalytic subunit of PKA is attached to a component in the NF fraction, whereas the association of the regulatory subunit is via the catalytic subunit.

Fig. 1(a), lane 2, shows that the NF preparation also contains proteins other than NF subunits: To test whether the catalytic subunit of PKA is associated directly with the NF proteins, we studied the interaction of NFs reconstituted from purified subunits, NF-H, NF-M and NF-L, with the catalytic subunit of

PKA isolated from bovine heart. The reconstituted NFs were incubated with the catalytic subunit in medium containing 1 mg of BSA/ml (to block non-specific binding sites), and samples were subsequently centrifuged (see the Experimental section). The kinase activities that fractionated with NF pellets and in supernatants were determined with kemptide as substrate. Fig. 2 (●) shows the percentage of the kinase activity recovered in pellets as a function of the amount of NF protein included in the incubation mixture. The level of kinase activity that partitions into the pellets is linear with respect to the amount of NF protein, at least up to 25 μ g of protein/50 μ l final volume (linear regression analysis shows $r^2 = 0.952$ when data points in the 0–25 μ g range are taken). The contribution of residual supernatant (in pellets or on sides of tubes) to the values obtained is relatively small, as can be observed from the partitioning of another soluble protein, ovalbumin, shown in Fig. 2 as (○).

A similar procedure to that described in Fig. 2 was used to study the effect of the regulatory subunit on the association of the catalytic subunit with NFs. A fixed amount of catalytic subunit was pre-mixed with increasing levels of regulatory subunit from bovine heart to achieve regulatory-subunit/catalytic-subunit ratios of 0, 0.5, 1.0, 2.0 and 3.0. These were then incubated with a heat-inactivated NF preparation. After centrifugation, kinase activities partitioned into pellets and supernatants were assayed in medium containing cyclic AMP. Fig. 3 shows that the regulatory subunit causes a dose-dependent decrease in the percentage of kinase that partitions into pellets. At a regulatory-subunit/catalytic-subunit ratio of 2:1, the kinase

Fig. 2. Co-sedimentation of the catalytic subunit of PKA with NFs reconstituted from purified subunits

●, Catalytic subunit of PKA (2.5 enzyme units, 0.07 μ g of protein) was added to reconstituted NFs in medium A. The samples were mixed, incubated, and centrifuged as detailed in the Experimental section. In order to eliminate any effect of varying NF content during kinase assays, reconstituted NF preparation was added to the pellets as needed to make the NF content in all tubes 50 μ g. The pellets were then resuspended in a final volume of 50 μ l. Samples (20 μ l) from supernatants and pellets were assayed for kinase activity, with kemptide as substrate. Under the conditions employed, phosphorylation of NFs and other proteins in the medium was insignificant compared with that of kemptide (results not shown). Percent kinase activity partitioned into pellets was calculated as: $100 \times [\text{kinase activity in pellet} / (\text{kinase activity in supernatant} + \text{kinase activity in pellet})]$, where kinase activity is the number of pmol of P_i transferred in the assay. Each point represents one experiment with duplicate kinase assays. ○, Control experiment showing partitioning of ovalbumin. [¹⁴C]Methylated ovalbumin (2.5 μ g) was mixed with NFs, incubated and centrifuged as above. After centrifugation, pellets were dissolved in urea and radioactivity in supernatants and pellets was measured by using Aquasol. Percentage ovalbumin partitioned in pellets was calculated as: $100 \times [\text{radioactivity in pellet} / (\text{radioactivity in supernatant} + \text{radioactivity in pellet})]$.

Fig. 3. Effect of the regulatory subunit (RS) on the co-sedimentation of catalytic subunit (CS) with NFs

Heat-inactivated NF preparation (100 μ g of protein) was mixed with 2.5 units of catalytic subunit alone, or with 2.5 units of catalytic subunit previously combined with 1.25, 2.5, 5 or 7.5 units of regulatory subunit, in a final volume of 50 μ l in medium A + 1 mM-EGTA. After incubation and centrifugation, kinase activities in pellets and supernatants were measured in media containing 5 μ M-cyclic AMP. Percentage of kinase activity in pellets was calculated as in Fig. 2. Each point is the mean of four experiments with duplicate kinase assays; error bars represent S.D.

Table 2. Effect of cyclic AMP on co-sedimentation of kinase activity with NFs

Catalytic subunit (2.5 enzyme units) was combined with regulatory subunit (7.5 enzyme units) and incubated with either 100 μ g of heat-inactivated NF preparation or 40 μ g of reconstituted NFs in medium A + 1 mM-EGTA, in the absence (control) or presence of 5 μ M-cyclic AMP. The samples were centrifuged and pellets were resuspended in the same medium. Kinase activities in pellets and supernatants were measured in media containing 5 μ M-cyclic AMP. Percentage of activity in the pellet was calculated as shown in Fig. 2 legend. Values represent means $\pm$ S.E.M. from (n) experiments, each with duplicate kinase assays.

Preparation	Kinase activity in pellet (measured in the presence of cyclic AMP) (%)	
	Control	+ cyclic AMP
NF preparation	9.4 $\pm$ 0.83 (4)	54.5 $\pm$ 4.33 (3)
Reconstituted NFs	17.3 $\pm$ 0.88 (4)	38.7 $\pm$ 2.97 (4)

recovered in pellets is only 10 % of the total recovery, compared with 50 % when the regulatory/catalytic-subunit ratio is zero, i.e. when free catalytic subunit is used.

The results presented in Fig. 3 indicate that the regulatory subunit has an inhibitory effect on the association of the catalytic subunit with neurofilaments. In a series of experiments to test whether this effect of the regulatory subunit is due to its capacity to bind to the catalytic subunit, the bond between the two subunits was disrupted by inclusion of cyclic AMP in the incubation medium before centrifugation. The experimental procedures followed in Table 2 were identical with those described for Fig. 3, a regulatory-subunit/catalytic-subunit ratio of 3:1 being used, except that in one group (+cAMP) 5 μ M-cyclic AMP was also included in the incubation mixtures containing the kinase subunits and the NF preparation, or kinase subunits and NFs reconstituted from purified proteins. Again, all kinase assays were done in cyclic-AMP-containing media. Table 2

shows that, when cyclic AMP is included in the initial incubation mixture, the percentage of activity that partitions into pellets increases drastically. In fact, percentages of kinase activities that co-sediment with the NF preparation and with reconstituted NFs in the presence of cyclic AMP in these experiments are very close to the values obtained when using catalytic subunit only, as can be verified from Figs. 2 and 3, implying that cyclic AMP abolishes the inhibitory effect of the regulatory subunit. These results suggest that the free catalytic subunit of PKA associates more readily with NFs than does the holoenzyme, i.e. the association of the catalytic subunit with the regulatory subunit inhibits its association with NFs. The inhibitory effect of the regulatory subunit is more pronounced when using the NF preparation than that observed using reconstituted NFs. This may be due to differences in the state of polymerization or in NF-protein stoichiometry which may exist between the native and reconstituted NFs.

DISCUSSION

To summarize, evidence obtained in this study indicates that the catalytic subunit of PKA associates with NFs, and further suggests that this association is regulated by cyclic AMP. It is shown that the NF preparation from spinal cord contains the PKA holoenzyme and that dissociation of the regulatory subunit from the catalytic subunit in the presence of cyclic AMP releases the regulatory subunit, but not the catalytic subunit, from the fraction (Table 1). Moreover, the isolated catalytic subunit from bovine heart co-sediments with NFs reconstituted from purified proteins (Fig. 2). In experiments where catalytic subunit pre-mixed with various amounts of regulatory subunit are incubated with NFs, it is observed that the regulatory subunit causes a dose-dependent inhibition of the association (Fig. 3). The inhibition of catalytic-subunit-NF association by the regulatory subunit must be directly related to its capacity to bind to the catalytic subunit, since inclusion of cyclic AMP completely abolishes the effect (Table 2). These observations on the inhibitory role of the regulatory subunit support the proposition that the association of the catalytic subunit with NFs is specific.

Since the regulatory subunit employed in this study was from heart tissue, which contains both type I and type II enzymes [16], we could not determine whether both type I and type II regulatory subunits have the same effect. Detection of cyclic AMP regulation in the NF preparation from bovine spinal cord would suggest that at least one isoform of the regulatory subunit does not interfere with the association of the catalytic subunit with NFs. Alternatively, it is possible that the regulatory subunit is able to attach to the catalytic subunit, perhaps with a different affinity, once it associates with NFs.

If the findings *in vitro* described in this paper can be verified in the intact cell, their implications in terms of the subcellular distribution of the PKA would be significant. Association of the catalytic subunit with NFs would be a factor in directing the kinase to specific sites within the neuron. Moreover, regulation of this association by cyclic AMP could provide a mechanism to modify the subcellular distribution of the kinase in response to extracellular signals. The regulatory and catalytic subunits of PKA, unlike other regulator-dependent kinases, are distinct proteins which physically dissociate in the presence of cyclic AMP. This means that the two subunits, once dissociated, may potentially remain separated from each other even after the cyclic AMP signal is over, provided that they attach to separate cellular structures. The widely documented association of the type II regulatory subunit with MAP2 [4-6] and the association of the catalytic subunit with NFs proposed in the present study may provide a mechanism for such a differential localization of

the two subunits under certain conditions, such as a sustained cyclic AMP signal.

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