BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **235,** 223–226 (1997)
ARTICLE NO. RC976764

Purification of a Regulatory Subunit of Type II cAMP-Dependent Protein Kinase from *Drosophila* Heads

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Received May 6, 1997

The cytosolic extract from *Drosophila* heads was separated using anion-exchange column chromatography. Two types of cAMP-dependent protein kinase (PKA), type I and type II, were detected, and type II PKA was found to be a major isozyme. The regulatory subunit of type II PKA (RII) was purified, and only one isoform was observed. The purified protein had an apparent molecular mass of 51 kDa on SDS gel electrophoresis. Partial amino acid sequences of the protein were almost identical with the RIIα subunit of human. Since PKA has been implicated to be especially important for learning and memory in *Drosophila*, the RII subunit may play an essential role in the regulation of neuronal activity in the brain of *Drosophila*, and possibly in human.

cAMP-dependent protein kinase (PKA) is an important participant in neuronal modulation. In *Aplysia* mechanosensory neurons, PKA plays roles in both short and long term presynaptic facilitation, although it is only a simple model for learning and memory (1, 2). In *Drosophila*, direct evidence of the role of cAMP and PKA in learning and memory has been obtained by the isolation and characterization of the mutants *dunce, rutabaga* and *DC0* (3, 4). These genes encode a cAMP-specific phosphodiesterase, a Ca2+/calmodulin-responsive adenylyl cyclase and a catalytic subunit of PKA, respectively. The PKA is, in its holoenzyme form, an inactive tetrameric complex, composed of two regulatory (R) and two catalytic (C) subunits (R_2C_2). cAMP activates the enzyme by causing dissociation of the inactive holoenzyme to yield free C subunits, which phosphorylate serine and threonine residues of specific protein substrates. Two types of PKA (type I and type II) can be distinguished based on their order of elution from anion-exchange resins (5). The different charge properties of the type I and type II holoenzymes are primarily due to differences in the R subunits (RI and RII). Four different R subunits (RIα RIβ, RIIα and RIIβ) and three separate gene products of C subunits (Cα, Cβ and Cγ) have been cloned from various mammalian species (6). In *Drosophila*, one RI subunit (DRI) and three C subunits (DC0, DC1 and DC2) have been cloned using mammalian probes (7). Although type II PKA has been purified from *Drosophila* (8), it has not yet been cloned. In this study, we showed that type I and type II PKA were present in *Drosophila* heads, and that type II was a major isozyme. The RII protein was purified from heads, from which was obtained partial amino acid sequences. The sequences were found to be almost identical to the RIIα subunit of human.

MATERIALS AND METHODS

Materials. Canton-S, a wild-type of *Drosophila melanogaster* was used. [^{32}P]ATP and [^{3}H]cAMP were purchased from DuPont NEN, and 8-N_3-[^{32}P]cAMP was from ICN. TSKgel DEAE-5PW (0.8 × 7.5 cm) was obtained from Tosho (Japan), and Superose 12 (HR 10/30) was from Pharmacia. cAMP-agarose (A7396) was purchased from Sigma.

Chromatography. All procedures were carried out at 0-4° C unless otherwise stated. Flies were decapitated and the heads were homogenized in 20 mM potassium phosphate, pH 7.4, containing 1 mM EDTA, 0.5 mM EGTA, 25 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 × g for 1 h and the cytosolic extract was obtained. For anion-exchange column chromatography, the sample was applied to a DEAE-5PW column, equilibrated with 20 mM potassium phosphate, pH 6.8, containing 1 mM EDTA (buffer A). After washing the column with buffer A, the column was eluted with a linear gradient of NaCl from 0 to 0.4 M in 20 ml of buffer A at a flow rate of 0.4 ml/min. For gel filtration, the sample was applied to a Superose 12 column, equilibrated with 50 mM potassium phosphate, pH 6.8, containing 1 mM EDTA and 0.1 M NaCl. The column was eluted with the same buffer at a flow rate of 0.4 ml/min. For affinity chromatography, the sample was added to cAMP-agarose, equilibrated with 20 mM potassium phosphate, pH 6.8, containing 1 mM EDTA and 1 mM DTT (buffer B). The slurry was rotated gently for 2 h, and the resin was washed with 0.5 M NaCl in buffer B, and then only with buffer B. After

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0006-291X/97 $25.00

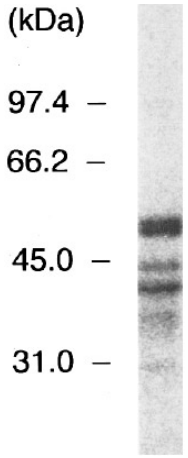

FIG. 1. Photoaffinity labeling of cAMP-binding proteins in *Drosophila* heads. The homogenate was labeled with 8-N_3-[^{32}P]cAMP. Proteins were separated on SDS-PAGE (10 % gel) and analyzed with a Molecular Imager. The protein markers used are phosphorylase b (97,400), serum albumin (66,200), ovalbumin (45,000), and carbonic anhydrase (31,000).

washing, cAMP-binding proteins were eluted with 10 mM cAMP in buffer B.

Biochemical assays. The standard reaction mixture for the assay of protein kinase activity contained 50 m potassium phosphate, pH 6.8, 10 mM $MgCl_2$, 0.1 mM [^{32}P]ATP, 0.1 mM kemptide and 2 μM cAMP (when used) in a total volume of 40 μl. After 10 min incubation at 30°C, aliquots were spotted on 2 × 2 cm phosphocellulose squares (Whatman P-81). Filters were washed batchwise three times with 75 mM phosphoric acid, dried and the radioactivity was counted. cAMP-binding activity was assayed essentially by the same method as described by Kumon *et al.* (9). The standard mixture contained 50 mM sodium acetate, pH 4.5, 10 mM $MgCl_2$, 3 mM theophyline (buffer C) and 0.1 mM [^{3}H]cAMP in a total volume of 0.1 ml. After 1 h incubation at 0°C, the reaction mixtures were diluted with 1 ml of ice-cold 10 mM Tris/HCl, pH 8.0 containing 40 mM $MgCl_2$, followed by rapid filtration on cellulose filters. Filters were washed four times with 1 ml of the same buffer and the radioactivity was counted. For photoaffinity labeling by 8-N_3-cAMP, the samples were incubated in buffer C and 8-N_3-[^{32}P]cAMP in the dark on ice for 1 h, and then exposed to UV light for 5 min. Photolysis was stopped by adding SDS sample buffer, and boiling for 5 min. The proteins were then separated by SDS-polyacrylamide gel electrophoresis (PAGE). The labeled proteins were analyzed with a Model GS-250 Molecular Imager (Bio-Rad Laboratories).

Electrophoresis. SDS-PAGE was performed as described by Laemmli (10). For isoelectric focusing for 2-dimensional electrophoresis, Immobiline DryStrip (pH 3-10, Pharmacia) was used. The sample was prepared by adding urea to a final concentration of 9M, and then Triton X-100, pharmalyte (pH 3-10) and 2-mercaptoethanol to a final concentration of 2%. Isoelectric focusing was conducted for 20100 Vh using a DryStrip kit (Pharmacia). The gels were then equilibrated for 10 min in equilibration solution (50 mM Tris-HCl, pH 6.8, 30 % glycerol and 1% SDS) containing 16 mM DTT, and for 10 min in equilibration solution containing 0.24 M iodoacetamide. The gels were placed on 10 % SDS-PAGE gels for the second dimensional electrophoresis.

Sequence analysis. The main band corresponding to 51 kDa was excised from the SDS-PAGE gel, and was digested with lysyl endopeptidase, as described by Rosenfeld *et al.* (11). The peptide fragments were separated by HPLC, and amino acid sequences were analyzed, as described by Majima *et al.* (12).

RESULTS AND DISCUSSION

Identification of Regulatory Subunits of PKA in Drosophila Heads

To determine the number and size of cAMP-binding proteins in *Drosophila* heads, homogenates were subjected to photoaffinity labeling with 8-N_3-[^{32}P]cAMP and analyzed by SDS-PAGE. Four major radiolabeled bands with molecular masses of 53, 51, 44 and 41 kDa, and a few minor bands of approximately 37-36 and 31 kDa were observed (Fig. 1). Labeling of these proteins appears to be specific as it was blocked by the inclusion of an excess of unlabeled cAMP (data not shown), although some of the proteins may be degradation products of regulatory subunits of PKA.

To separate type I and type II PKA in *Drosophila* heads, cytosolic extracts were analyzed using DEAE chromatography, since type I holoenzyme elutes from DEAE at a low concentration of NaCl, whereas type II at a high concentration of NaCl (5). As shown in Fig. 2, three peaks of PKA activity (I, II and III), which

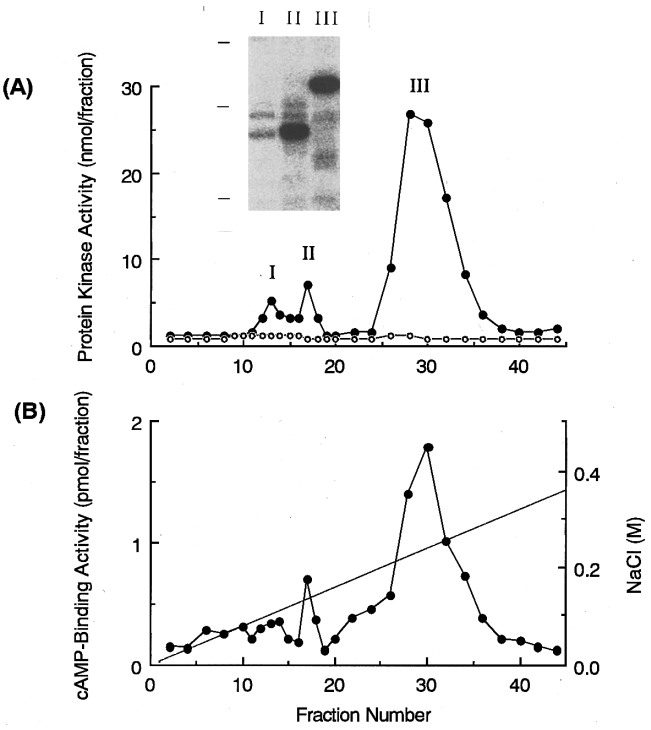

FIG. 2. DEAE-5PW column chromatography of cytosolic extract obtained from *Drosophila* heads. Fractions of 0.4 ml were collected and assayed for (A) PKA activity in the presence (●) and absence (○) of cAMP, and (B) cAMP-binding activity. The inset shows 8-N_3-[^{32}P]cAMP photolabeling of peak fractions (I, II, III). Bars indicate the position of protein markers: phosphorylase b (97,400), serum albumin (66,200), and ovalbumin (45,000).

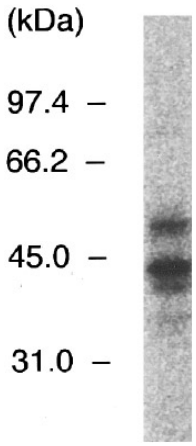

FIG. 3. Photoaffinity labeling of cAMP-binding proteins in third instar larvae of *Drosophila*. The homogenate was labeled with 8-N_3-[^{32}P]cAMP. Proteins were separated on SDS-PAGE (10 % gel), and analyzed with a Molecular Imager. The protein markers used are the same as those in Fig. 1.

were associated with a cAMP-binding activity, were obtained. To confirm isozyme positions, eluted fractions were photoaffinity labeled with 8-N_3-[^{32}P]cAMP (Fig. 2, inset). Peak I contained 44 and 41 kDa proteins, while peak II contained approximately 42 kDa and several minor proteins with molecular masses ranging from 45 to 41 kDa. Peak III contained approximately 51 kDa protein. Since Foster et al. purified type II PKA from *Drosophila* body, and found that the regulatory subunit existed in phospho and dephospho forms (8), it is conceivable that the 53 and the 51 kDa cAMP-binding proteins correspond to the phosphorylated and non-phosphorylated form of RII subunit, respectively. The apparent molecular mass of RI subunit was reported as 48 kDa (6) or 46 kDa (8), and full-length RI protein was shown to be present for all stages of development from embryo to adult (7). When homogenate of third instar larvae was photoaffinity labeled with 8-N_3-[^{32}P]-cAMP, 44 and 41 kDa proteins were detected (Fig. 3). Therefore, it is probable that the 44 and/or 41 kDa proteins may be RI, although the possibility that these proteins are degradation products of RII can not be excluded.

Purification of Regulatory Subunit of Type II PKA

To purify RII protein, peak III was concentrated and subjected to gel filtration chromatography. The cAMP-binding activity was eluted in a single peak corresponding to a molecular mass of approximately 260 kDa (data not shown), which agrees with R_2C_2 stoichiometry, since the molecular mass of the C subunit of *Drosophila* is 40 kDa (6). The active fractions were added to cAMP-agarose, and the bound proteins were eluted. SDS-PAGE analysis showed that the eluate contained a nearly homogeneous protein that shows an apparent molecular mass of 51 kDa (Fig. 4A). The result indicates that the RII subunit which exist in *Drosophila*

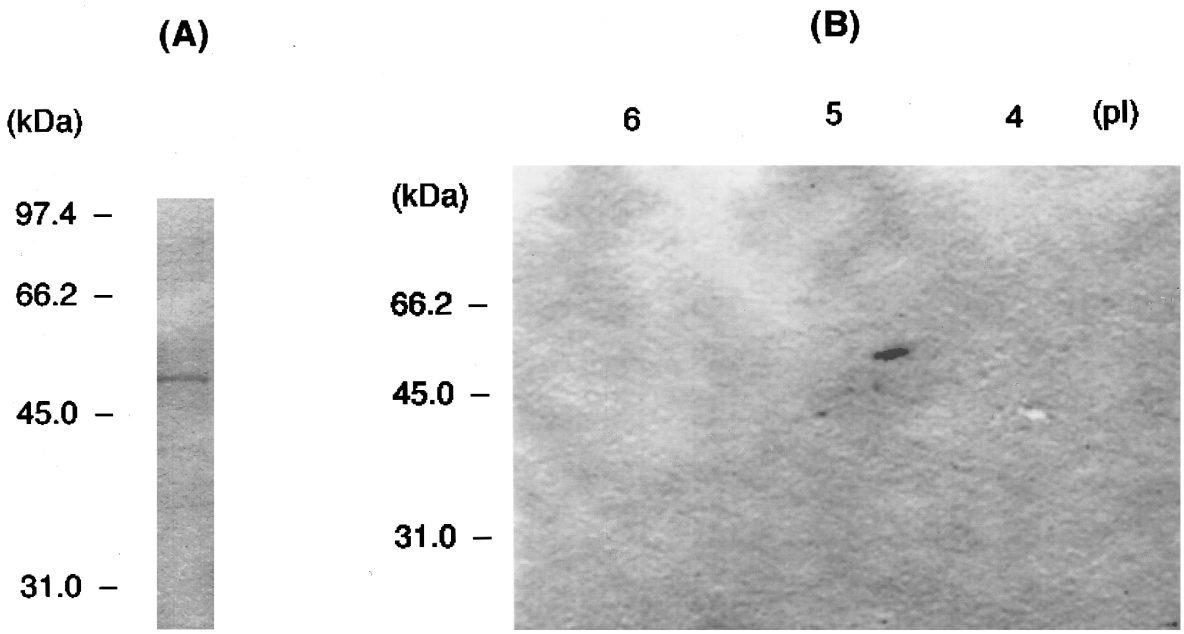

FIG. 4. Analysis of purified cAMP-binding protein of *Drosophila* heads by electrophoresis. (A) RII protein was purified by sequential chromatography on DEAE-5PW, Superose 12 and cAMP-agarose, and separated by SDS-PAGE (10 %). (B) cAMP-binding proteins were purified directly from the cytosolic fraction with cAMP-agarose, and separated by 2-dimensional electrophoresis as described in Material and Methods. The proteins were stained with Coomassie Blue. The protein markers used are the same as those in Fig. 1.

head is only one isoform, although it is probable that a small amount of another isoform is present.

To obtain sufficient RII protein for amino acid sequence determination, cytosolic extracts from *Drosophila* heads were incubated with cAMP-agarose. The affinity resin was washed, and proteins were eluted. The resulting proteins were analyzed by 2-dimensional electrophoresis. As shown in Fig. 4B, one major protein corresponded to approximately 50 kDa, and three minor proteins corresponding to 45 (two spots) and 40 kDa were observed. When purified RII protein was analyzed by 2-dimensional electrophoresis, the protein was seen as a spot with pI 4.8 (data not shown). Therefore, the 50 kDa protein may be the RII subunit.

Partial Amino Acid Sequences of Isolated Peptide Fragments

After SDS-PAGE, the band corresponding to 50 kDa was excised from the gel. Peptide fragments were generated by treating the protein with lysyl endopeptidase, and the amino acid sequences were determined. Two sequences, EQMNQVLDAMFERK and RNIDDYESQLVK, were obtained. These sequences were almost identical (75-85%) with those of residues 146-159, EQLSQVLDAMFERI, and 380-391, RNISHYEEQLVK, of human RIIα, respectively (13). Good agreement of amino acid sequences between *Drosophila* and human confirm similarity of the biological function of the enzyme. Recent studies have shown that the homologous *Drosophila* genes have similar roles in mammals (reviewed in 14).

In conclusion, we have shown that type II PKA is the major isozyme in *Drosophila* heads, and that only one isoform of type II R subunit was present. From gel filtration studies, R_2C_2 tetramer was deduced, which is the same as described in all species except *Dictyostelium* (15). Since the involvement of PKA in learning and memory in *Drosophila* has been established in many studies (3, 4,16), the RII subunit may play an essential role in the regulation of *Drosophila* neuronal activity. The structural similarity in RII subunit between *Drosophila* and human will contribute to the development of new insight into the study of the molecular mechanism of learning and memory of human brain, and elucidate the cause of brain disease in humans.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in Aid for Scientific Research on Priority Areas 07279105 from the Japanese Ministry of Education, Science and Culture and was also supported by the Research Future Program of the JSPS.

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