0270-6474/82/0212-1692$02.00/0

The Journal of Neuroscience
Vol. 2, No. 12, pp. 1692-1704
December 1982

CHARACTERIZATION AND LOCALIZATION OF ADENOSINE 3':5'-MONOPHOSPHATE-BINDING PROTEINS IN THE NERVOUS SYSTEM OF *APLYSIA*[1]

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Received June 21, 1982; Revised August 30, 1982; Accepted September 3, 1982

Abstract

Earlier work in *Aplysia californica* has indicated that sensitization of the gill reflex, a simple form of learning, is produced by cAMP-dependent protein phosphorylation which regulates the flux of ions in sensory neurons of the abdominal ganglion. These changes in ion flux result in the enhanced release of neurotransmitter from synapses of the sensory neurons which, in turn, mediate the behavior. Because it can be presumed that protein phosphorylation regulates the functioning of ion channel proteins, we have characterized cAMP-binding proteins photoaffinity labeled with 8-N_3-cAMP and have found that, unlike other tissues, the nervous system contains a great variety of binding species. Also unlike other tissues, several of the binding proteins in neurons are associated with membrane, and these components are concentrated in fractions enriched in nerve endings. Selectivity of phosphorylation, not only between substrates in cytosol and membrane but also between different regions of the cell, is thus possible because of the variety of cAMP-binding proteins in neurons. We think that these membrane-associated binding proteins are the most likely candidates for the regulatory subunits of the cAMP-dependent kinases that control the functioning of ion channel proteins at the synapse.

Work in *Aplysia* demonstrates that certain simple forms of learning occur at synapses between specific identified nerve cell (Castellucci et al., 1970; Castellucci and Kandel, 1974, 1976). It now appears that behavioral training induces modification of specific ion channels (for example, those for Ca^{2+} and for certain K^+ channels) which, in turn, modulate the release of neurotransmitter (Klein and Kandel, 1980) and thereby constitute the molecular components underlying learning. Ca^{2+} and K^+ channels are thought to consist of specific proteins that are concentrated in the membrane of nerve endings. Thus, once it is recognized that certain forms of learning involve modification of specific membrane proteins, it becomes possible to investigate the biochemical basis of learning fruitfully by defining the mechanisms that regulate the functioning of specific ion channel proteins.

We undertook the identification and characterization of regulatory subunits of the cAMP-dependent protein kinase in *Aplysia* nervous tissue because the interaction of cAMP with the kinase appears to be a key biochemical step in the sequence of the reactions that underlie sensitization of the gill and siphon withdrawal reflex (Klein and Kandel, 1978, 1980; Kandel and Schwartz, 1982; Castellucci et al., 1980; 1982). The key question arises if we consider how cAMP can control synaptic activity selectively, when it is known that many general aspects of cellular metabolism also are regulated by the cyclic nucleotide. More precisely, how can phosphorylation be directed to the proteins that regulate ion flux?

Studies of mammalian cAMP-dependent protein kinases have revealed several features that might allow the enzyme to modulate different aspects of cellular metabolism selectively. The catalytic subunits of the kinase are inactive when bound to regulatory subunits: cAMP, by binding to the regulatory subunits, causes them to dissociate, thereby activating the catalytic subunits (Beavo et al., 1975; Rosen et al., 1977; Corbin et al., 1978). Only one type of catalytic subunit appears to exist in all tissues examined, but two general classes of regulatory subunits,

[1] This work was supported by National Institutes of Health Research Grant NS 15834. C. M. E. is supported by a National Institutes of Mental Health Training Program. We thank Ora Rosen and Charles Rubin for advice and helpful criticism. We also thank Thomas Abrams, Richard Axel, Hagen Bayley, Lise Bernier, Vincent Castellucci, Yadin Dudai, Eric Kandel, Flora Katz, James Roberts, and Tsunao Saitoh for reading the manuscript critically and Alice Elste for electron microscopy and help with the figures.

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types I and II, that have different molecular weights and different affinities for the catalytic subunit have been found (Hofmann et al., 1975; Reimann et al., 1971; Rubin et al., 1972). Because kinases with different regulatory subunits have similar substrate specificities and similar properties of activation *in vitro*, the functions of the two different classes of regulatory subunits in the cell are not yet known. It has been postulated, however, that function is determined, at least in part, by intracellular localization and that the subcellular distribution of a kinase depends on the nature of its regulatory subunits (Corbin et al., 1977; Cumming et al., 1981; Walter et al., 1978).

Recent reports of tissue-specific regulatory subunits (Rubin et al., 1979, 1981; Weber et al., 1981) and of isoelectric variants in regulatory subunits of the kinase in mammalian brain (Panter et al., 1981) suggested to us that specialization of function within a neuron could depend on the nature of the regulatory subunit. We therefore wanted to know how many kinds of regulatory proteins there are in neurons of *Aplysia*. If many species exist, how are they distributed among the various subcellular compartments; particularly, are there any regulatory subunits associated with membrane? How are the activities distributed regionally? Most important to ask, are there special kinases in nerve endings that might affect ion channel proteins selectively?

A preliminary report of this work has been published (Eppler et al., 1982).

Materials and Methods

Preparation of tissue homogenates

Neural components were dissected from central nervous tissue dissected from *Aplysia californica* weighing 140 to 180 gm, which were supplied by Pacific Bio-Marine Laboratories, Venice, CA, as described by Giller and Schwartz (1971). After the ganglia were washed in 0.3 M NaCl, 0.2 M sucrose, 1 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl (pH 7.4), nerve cell bodies and neuropil were scooped out into the buffer at 0°C through an incision in the connective tissue sheath that envelops the neural components. Each nervous system, consisting of five major central ganglia, yields about 0.5 mg dry weight of protein. Heart, body wall muscle, buccal mass muscle, and salivary glands also were washed in the buffer. In some experiments, the tissue was homogenized in glass/Teflon tissue grinders without further fractionation.

A region of neural components containing sensory neurons and comprising not more than 5% of the abdominal ganglion was dissected from the ganglion at −20°C as described by Bernier et al. (1982).

Fractionation procedures

All procedures were carried out at 0°C. For each experiment, we used the central nervous systems of four to nine animals. In some experiments, we included protease inhibitors (all from Sigma, St. Louis, Mo): soybean trypsin inhibitor, 1 mg/ml; leupeptin, 1 mg/ml; pepstatin, 1 mg/ml; phenylmethylsulfonyl fluoride, 0.5 mM; EGTA, 1 mM.

Hand dissection of neural regions. In most experiments, neural components were separated before homogenization into two fractions enriched either in cell bodies or in nerve endings with ultrafine forceps under a dissecting microscope. Hand dissection could be used to obtain a fraction from neuropil because of the organization of *Aplysia* ganglia. Cell bodies are located in ganglia around a central core of fibers called neuropil which contains axons and their terminals. Nearly all synapses occur in the central core. Thus, with the aid of a dissecting microscope, it is possible to dissect the ganglia into two fractions, one containing predominantly cell bodies and the other that is enriched in nerve endings. Typically, the neural components from four to six nervous systems can be sorted in 1.5 to 2 hr. It should be clear that the fractions obtained were by no means pure: glial cells were not removed from the cell body fraction, which contained a small proportion of axon terminals; the nerve ending fraction, in addition to axon terminals, also contained axons and glial cells. Fractions were homogenized by 20 strokes, in glass/Teflon tissue grinders at a protein concentration of 1 to 2 mg/ml. The resulting homogenates were centrifuged at 130,000 × *g* for 1 hr, and the pellets were resuspended and washed once in the buffer at pH 7.4.

Subcellular fractionation. Nerve ending fractions were obtained by a modification of the procedure of Dowdall and Whittaker (1973) for isolating synaptosomes from squid. The neural components were dissected in 0.17 M NaCl, 0.35 M sucrose, 5 mM 2-mercaptoethanol, and 10 mM Tris-HCl (pH 7.4) and homogenized by 11 gentle passes of a glass/Teflon tissue grinder driven by a motor at about 500 rpm. The homogenate was centrifuged at 1,000 × *g* for 12 min to obtain a pellet, which was washed by being resuspended by pipetting in the buffer and centrifuged again. In contrast to the results with squid, we found that most of the synaptosomes sedimented at 1,000 × *g*. The combined supernatants from the two centrifugations were centrifuged for 12 min at 17,000 × *g* to obtain another pellet. The 17,000 × *g* supernatant was centrifuged at 130,000 × *g* for 1 hr to obtain a microsomal pellet and a supernatant. The 1,000 × *g* and 17,000 × *g* pellets were resuspended in the buffer, placed on discontinuous sucrose density gradients with steps at 0.6, 0.8, 1.0, 1.2, and 1.6 M made up in the buffer, and centrifuged at 130,000 × *g* for 1.5 hr. Membranes were collected from each interface, concentrated by centrifugation at 100,000 × *g* for 15 min, and resuspended in the buffer. Fractions, concentrated by centrifugation at 2,000 × *g*, were examined by electron microscopy after fixation and embedment as described by Ambron et al. (1981).

Identification of cAMP-binding proteins. Photoaffinity labeling was carried out according to the procedures of Walter et al. (1977) and Walter and Greengard (1978) except that 0.1% Triton X-100 was added to all fractions and the ^{32}P-labeled affinity reagent sometimes was diluted with unlabeled 8-N_3-cAMP (Sigma). Maximal labeling occurred with 0.6 μM 8-N_3-[^{32}P]cAMP (50 to 80 Ci/mmol, ICN, Irvine, CA) and 3 to 30 μg of protein. After 45 min incubation at 4°C, the 50-μl reaction mixture was exposed to ultraviolet light for 10 min. Experiments with shorter periods of irradiation gave similar results (see "Results"). For electrophoresis on 8% polyacryl-

amide gels in SDS (Krueger et al., 1977), 13 μl of 15% SDS, 40% glycerol, 6% 2-mercaptoethanol, and 0.15 M Tris-HCl (pH 7.0) were added to the irradiated samples, which then were heated for 10 min at 100°C. For two-dimensional analyses (O'Farrell, 1975), 35 μl of the non-denaturing stop solution of O'Farrell (1975) was added and the samples were stored at −20°C if not analyzed immediately. Electropherograms were stained with Coomassie blue and dried, and the location of labeled proteins was determined by autoradiography enhanced by image intensifier screens.

Assay of cAMP-dependent protein kinase. Reaction mixtures contained, in a final volume of 0.1 ml, 0.01 μmol of cAMP, 1.0 μmol of HEPES (pH 6.5), 1.0 μmol of magnesium acetate, 0.8 μmol of NaF, 0.12 μmol of isobutylmethylxanthine, 0.5 μmol of 2-mercaptoethanol, 0.1 mg of Triton X-100, and 30 μg of *Aplysia* protein from the fraction to be assayed. Reaction mixtures were incubated at 15°C for 4 min before the addition of 0.04 μmol of γ-^{32}P-labeled ATP (5 μCi, ICN) and then for an additional 8 min. The reaction was stopped and the phosphoproteins were analyzed by polyacrylamide gel electrophoresis in SDS as described above. Radioactive phosphoproteins were located by autoradiography.

Other methods. Protein was measured colorimetrically (Lowry et al., 1951). Performic acid oxidation was carried out by the method of Hirs et al. (1956).

Results

Three major molecular weight classes of cAMP-binding proteins. The neural components (Giller and Schwartz, 1971) of *Aplysia* ganglia contain three size classes of cAMP-binding proteins with molecular weights of 45,000 to 47,000, 50,000 to 52,000, and about 105,000 (Fig. 1*A*). These proteins were photoaffinity-labeled with 8-N_3-[^{32}P]cAMP, denatured under reducing conditions, separated by SDS-polyacrylamide gel electrophoresis, and identified by autoradiography. The M_r = 45,000 to 47,000 and 50,000 to 52,000 classes correspond in size to the two types of regulatory subunits of mammalian cAMP-dependent protein kinases (Krebs, 1972; Rubin and Rosen, 1975). The M_r = 105,000 class, which constitutes 10% of the binding activity in *Aplysia* neural components (Table I), does not correspond to any of the major classes of mammalian binding proteins. Although small amounts of binding protein larger than the two well characterized classes of regulatory subunits have been observed in mammalian brain previously, they have not been present in such high proportion (Lohmann et al., 1978; Walter et al., 1978). The components with lower molecular weights (less than 40,000) probably are generated by proteolytic cleavage of the larger proteins as in mammalian tissues (Potter and Taylor, 1980; Takio et al., 1982). During purification, all of the binding activity

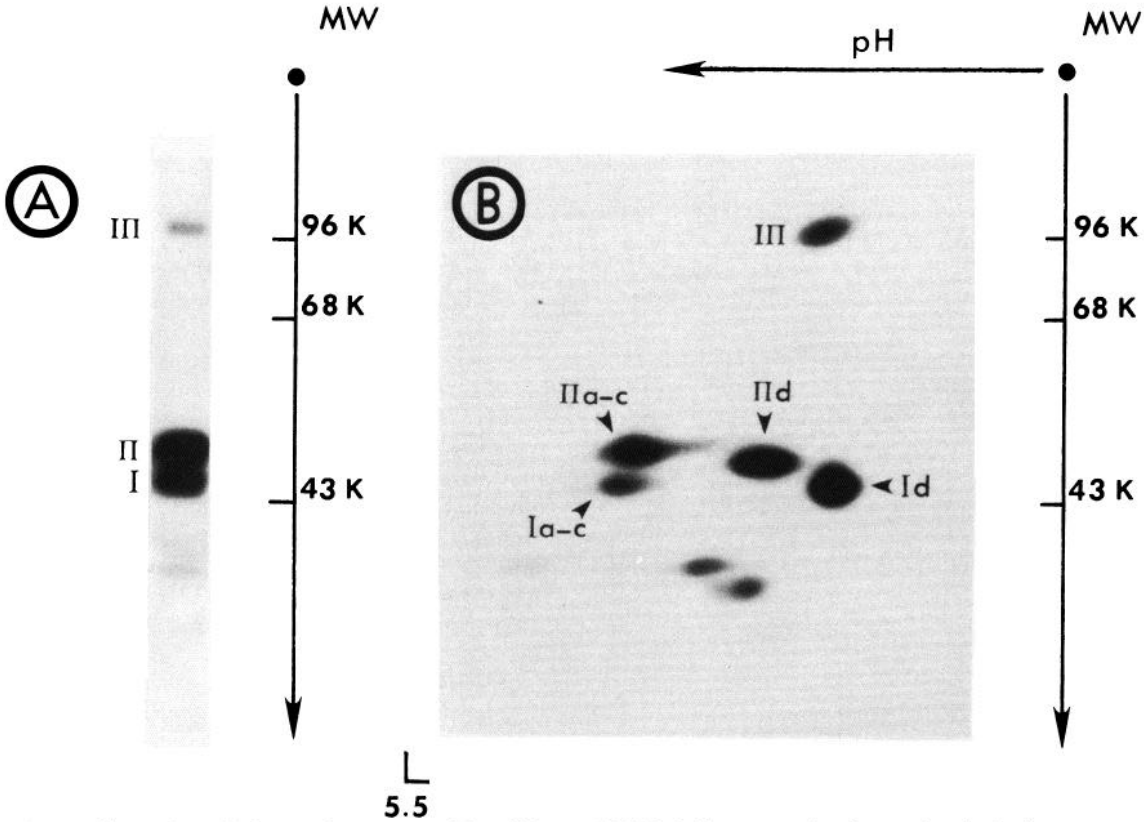

Figure 1. Autoradiographs of electropherograms identifying cAMP-binding proteins from the *Aplysia* nervous system. A fraction of the neural components enriched in nerve endings was obtained by hand dissection, homogenized, labeled with 8-N_3-[^{32}P]cAMP, and separated on polyacrylamide gels as described under "Materials and Methods." The distribution of proteins obtained from the neuropil fraction is shown because it contains all of the neural species of cAMP-binding proteins in large amounts (see Table I). *A*, Separation by electrophoresis on 8% polyacrylamide gels in SDS; *B*, two-dimensional separation by isoelectric focusing followed by electrophoresis on 8% polyacrylamide gels in SDS. The three size classes are indicated by *Roman numerals*; see the text for the designation of the components resolved within classes.

TABLE I

Distribution and specific radioactivity of proteins labeled with 8-N_3-[^{32}P]cAMP in neuropil (nerve ending) and cell body fractions sorted by hand dissection of the neural components of Aplysia ganglia

The preparation of fractions by centrifugation, photoaffinity labeling of samples containing 20 μg of protein, and polyacrylamide gel electrophoresis in SDS are described under "Materials and Methods" and "Results." After the labeled proteins were located by autoradiography, they were cut out of the gels and counted by liquid scintillation. The values for radioactivity correspond to 0.5 to 4.0 pmol of 8-N_3-[^{32}P] cAMP bound per mg of protein. The nerve ending fraction contained 33% of the total protein of the neural components; in both nerve ending and cell body fractions, protein in membrane was approximately equal to soluble protein.

Region	Fraction	Proportion of Total Binding Activity in Neural Components	Specific Binding by Size Class ($M_r \times 10^{-3}$)			
			III 105	II 50–52	I 45–47	<40
		%	*cpm/20 μg protein*			
Nerve endings	Particulate	31.5	846	2578	2275	303
	Soluble	22.6	239	2560	931	584
Cell bodies	Particulate	13.8	192	554	348	220
	Soluble	32.2	180	1571	736	581

was recovered as the low molecular weight forms unless protease inhibitors were included in the homogenate.

Further work attempting to discount an artifactual explanation for the M_r = 105,000 protein showed it to be distinct and not an aggregate of any of the lower molecular weight proteins. When the photoaffinity reaction was followed by oxidation with performic acid, which prevents disulfide linkage, the proportion of M_r = 105,000 protein was unchanged. Shortening the period of irradiation of the photoaffinity reaction did not reduce the proportion of the M_r = 105,000 class. It is therefore unlikely that its presence was produced by dimerization during exposure to ultraviolet light.

Heterogeneity within cAMP-binding protein classes. Separation of the photoaffinity-labeled proteins in a homogenate of neural components by two-dimensional gel electrophoresis revealed diversity within the size classes (Fig. 1*B* and also see Fig. 7*B*). The proteins with molecular weights of 45,000 to 47,000 (class I) can be separated into four major isoelectric species, Ia to Id. Upon exposing the electropherograms for long periods of time, two additional minor cAMP-binding proteins also could be detected (data not shown). Moreover, four species, IIa to IId, can be separated from the M_r = 50,000 to 52,000 proteins (class II). Resolution of these components (Ia to Ic and IIa to IIc) seemed to depend on the particular commercial batch of ampholytes for electrofocusing (compare Figs. 1*B* and 7*B*). The M_r = 105,000 protein (class III) appears to be a single polypeptide. The low molecular weight components, which probably are proteolytic cleavage products, are quite heterogeneous. The specificity of all of these proteins for cAMP was shown by inhibiting the labeling of each protein by the photoaffinity reagent in the presence of a 50-fold molar excess of cAMP (Walter et al., 1977). 5′-AMP failed to inhibit labeling.

cAMP-binding proteins localized to neural membranes. Particulate and soluble fractions yielded distinctive patterns of binding proteins; the two fractions shared only four of the nine major cAMP-binding activities that were separated by two-dimensional electrophoresis (Fig. 2). Thus, proteins Ia to Ic appeared only in the soluble fraction (Fig. 2*A*), while III and Id are essentially all particulate (Fig. 2*B*). The activities that appeared in both the soluble and particulate fractions are all in class II. The membrane fraction, however, contained substantially more IId activity than did the soluble fraction, which, in turn, was enriched in proteins IIa to IIc. The membrane proteins remain particulate after sonication (three 5-sec pulses at 0°C) but were solubilized completely by 0.5% Triton X-100.

cAMP-binding proteins in membrane fractions enriched in nerve endings. Using 8-N_3-cAMP, Walter et al. (1978) have shown that cAMP-binding protein is concentrated in mammalian synaptosomes. It is important to determine whether any of the membrane-specific cAMP-binding proteins are localized to nerve terminals in *Aplysia*. We obtained fractions enriched for nerve endings by two complementary techniques: hand dissection of the central nervous system (see "Materials and Methods") and subcellular fractionation by differential centrifugation, a method previously used to obtain synaptosomes from the central nervous system of squid (Dowdall and Whittaker, 1973).

Liquid scintillation counting of labeled proteins from particulate and soluble fractions of neuropil and nerve cell bodies separated on SDS gels showed that membrane-associated cAMP-binding proteins are concentrated in membranes enriched in nerve endings and axons (Table I). The extent of labeling with 8-N_3-[^{32}P]cAMP using equal amounts of soluble proteins from both cell body and neuropil fractions were similar. However, the specific binding activity of membrane proteins from the neuropil was about 5 times greater than that of membrane proteins from the cell body fractions.

Using subcellular fractionation, we found most cAMP-binding activity at two densities on the sucrose gradient, the interfaces between 0.8 and 1.0 M and 1.0 and 1.2 M (Fig. 3*A*, *lanes 8* and *9*). These fractions contained only 14% of the total particulate protein. All of the classes of binding proteins were present in these fractions which contained essentially all of the M_r = 105,000 protein. Electron microscopic examination of material from this region of the gradient showed that, although it was heterogeneous, this fraction was the one most enriched in synaptosomes (Fig. 4).

Evidence that cAMP-binding proteins are regulatory subunits of protein kinase. There are several indications that most of the cAMP-binding proteins in *Aplysia* are regulatory subunits of protein kinase. We assayed the amount of cAMP-dependent kinase activity in the subcellular fractions that are enriched for synaptosomes. Phosphorylation of endogenous substrate showed that fractions that have the highest concentrations of cAMP-binding proteins also contained the greatest concentration of cAMP-dependent protein kinase; conversely, fractions that contained little or no binding protein contained little kinase activity (Fig. 3*B*). In another experiment, cAMP-binding proteins were shown to sediment at the

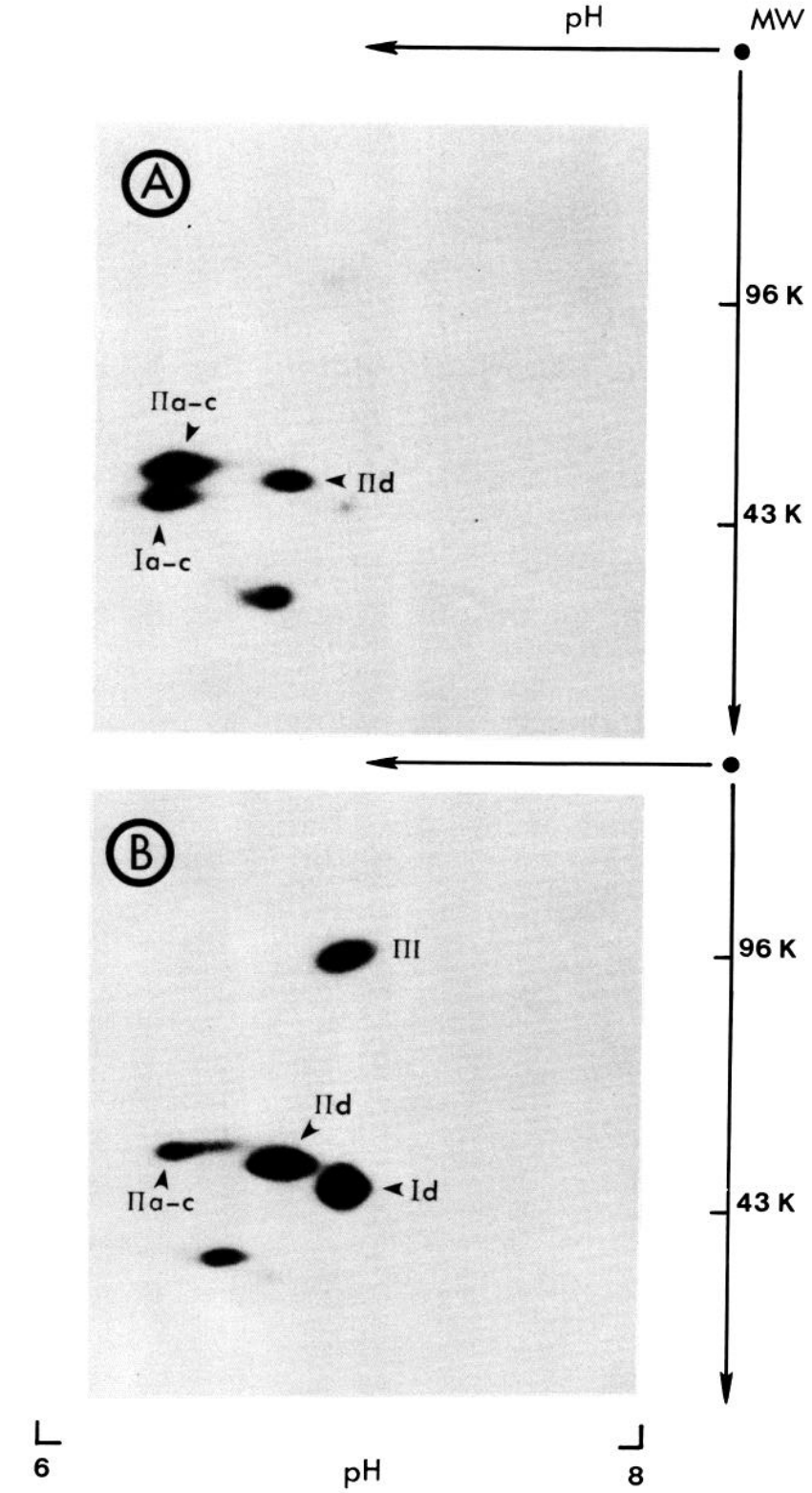

Figure 2. Distinctive particulate and soluble patterns of neural cAMP-binding proteins. A homogenate of neuropil was centrifuged for 1 hr at 130,000 × *g* to obtain a particulate and a soluble fraction. Photoaffinity labeling and two-dimensional gel electrophoresis of the fractions were carried out as described in the legend to Figure 1. *A*, Soluble binding proteins; *B*, particulate proteins.

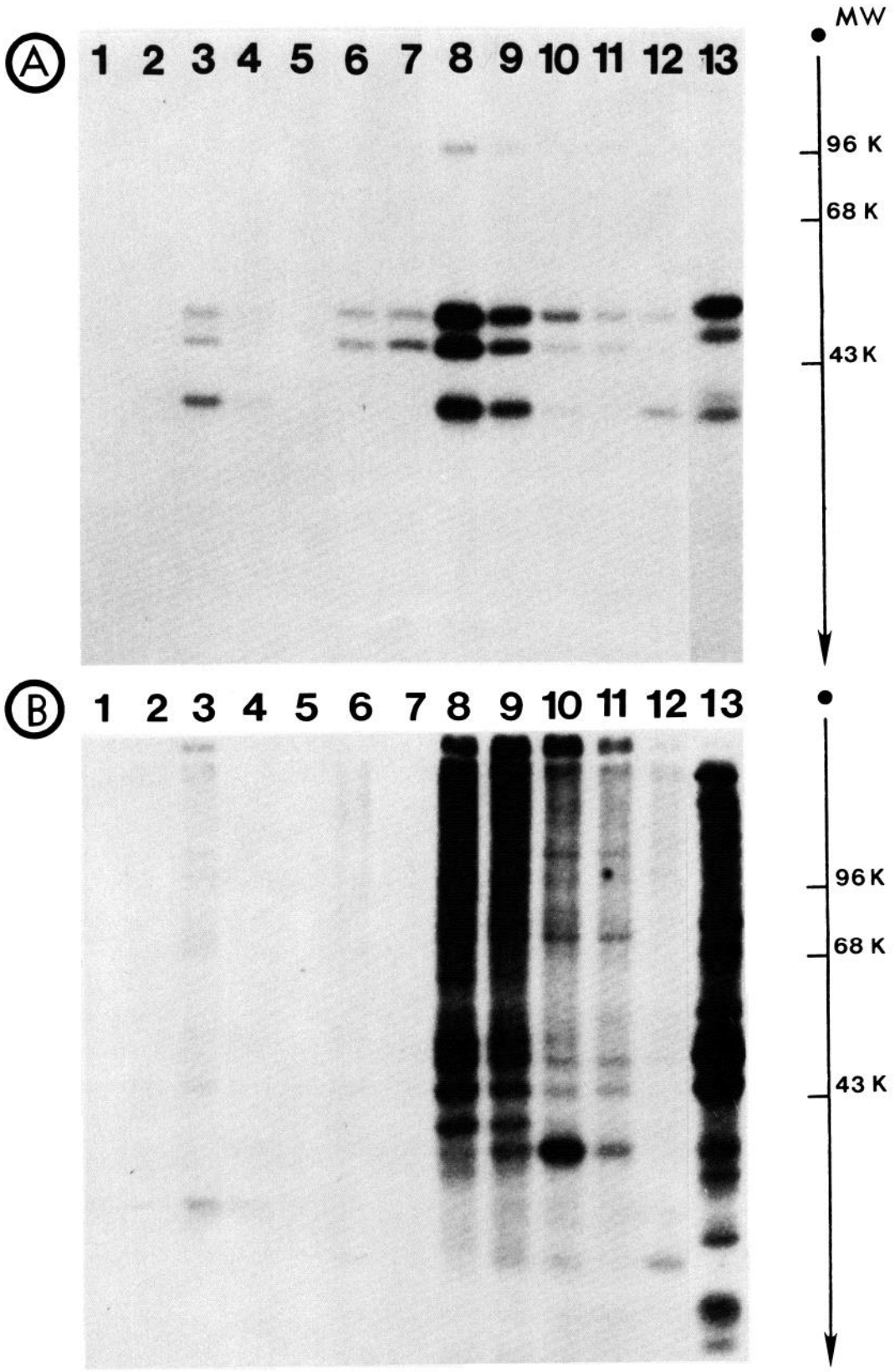

Figure 3. Subcellular fractionation of membranes from homogenates of *Aplysia* neural components and distributions of cAMP-binding proteins and cAMP-dependent protein kinase activity using endogenous substrates. The neural components were homogenized and fractionated by differential and sucrose density gradient centrifugation (Dowdall and Whittaker, 1973; see "Materials and Methods" for details). Two pellets, collected at 1,000 × *g* and 17,000 × *g*, were placed on identical step sucrose density gradients for fractionation at 130,000 × *g*. The fractions recovered from the gradients were assayed for (*A*) cAMP-binding proteins and (*B*) protein kinase activity with endogenous substrates in the presence of cAMP (see "Materials and Methods"). *Lanes 1* to *5* show fractions obtained from the 17,000 × *g* pellet at the following sucrose density interfaces: *1*, 0.35 and 0.6 M; *2*, 0.6 and 0.8 M; *3*, 0.8 and 1.0 M; *4*, 1.0 and 1.2 M *5*, 1.2 and 1.6 M *Lanes 6* to *11* show fractions from the 1,000 × *g* pellet: *6*, 0.35 and 1.6 M; *7*, 0.6 and 0.8 M; *8*, 0.8 and 1.0 M *9*, 1.0 and 1.2 M *10*, 1.2 and 1.6 M. *Lane 11* shows proteins that passed through the 1.6 M sucrose layer to the bottom of the tube, and *lane 12* shows the membranes that sedimented at 100,000 × *g* after obtaining the 17,000 × *g* pellet. The soluble proteins in the 100,000 × *g* supernatant are shown in *lane 13*.

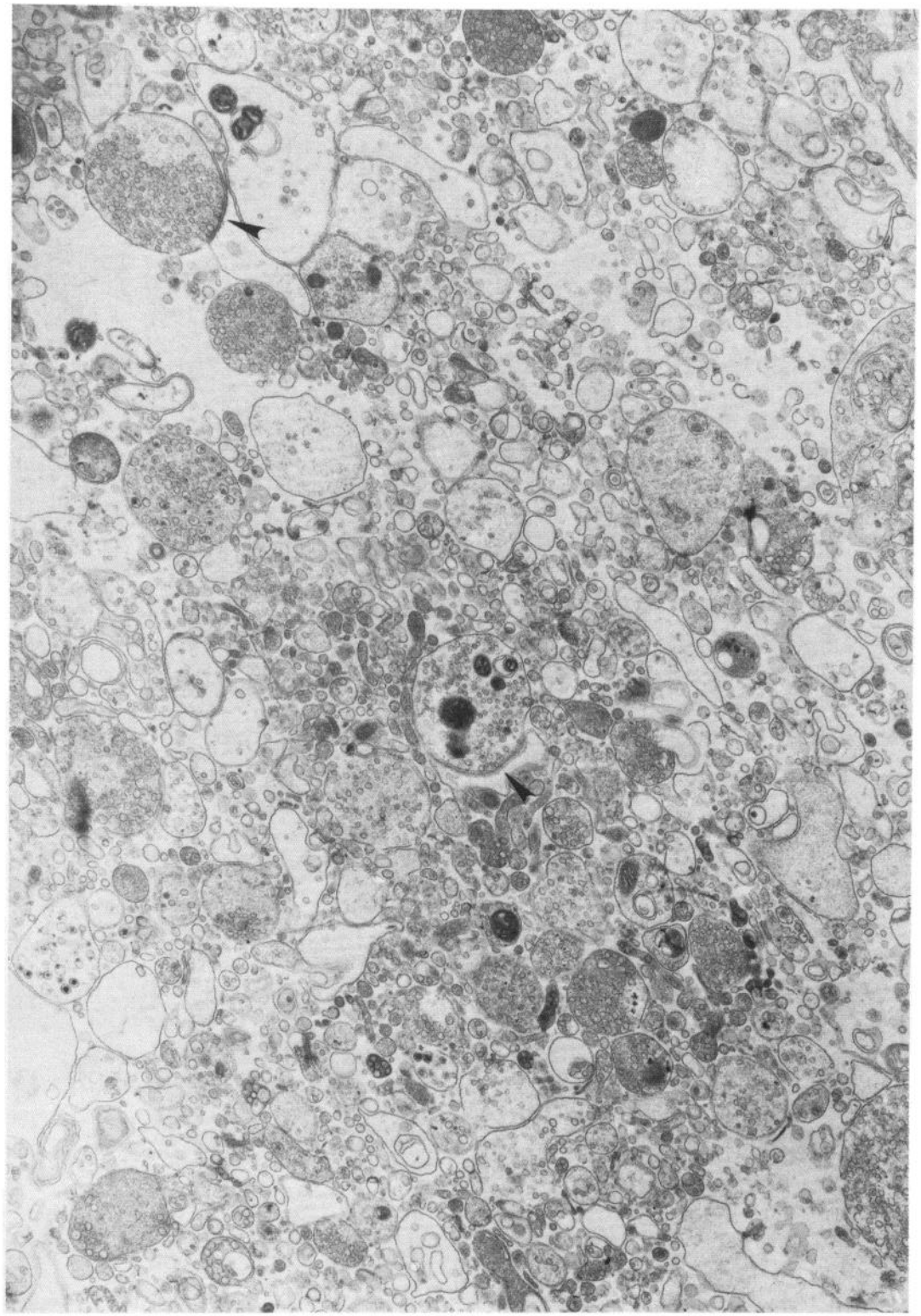

Figure 4. Electron microscopic examination of membranes enriched in nerve endings. A fraction from a sucrose step gradient layering between the 0.8 and 1.1 M sucrose interface (corresponding to fractions in *lanes 8* and *9* of Fig. 3) was collected and prepared for electron microscopic examination as described under "Materials and Methods." The field, magnified × 15,400 shows a variety of membranous profiles, some of which (*arrowheads*) have the features of synaptosomes (concentration of small vesicles, mitochondria, and an area of membrane specialization associated with the active zone). These structures were much rarer or completely lacking in fields obtained from the fractions collected from the gradient at other sucrose densities.

same rate as kinase activity during centrifugation on continuous sucrose density gradients (Martin and Ames, 1961) (data not shown).

Some type II regulatory subunits in mammalian tissue are substrates for their own catalytic subunits (Rosen and Erlichman, 1975). To analyze which of the *Aplysia* cAMP-binding proteins can be phosphorylated, we incubated homogenates of neural components with [^{32}P] ATP and the catalytic subunit of bovine protein kinase. Comparison of two-dimensional gel patterns of the phosphoproteins with patterns of cAMP-binding proteins showed that Id and IId are phosphorylated (arrowheads in Fig. 5).

Possibility of glial contamination. Even though the

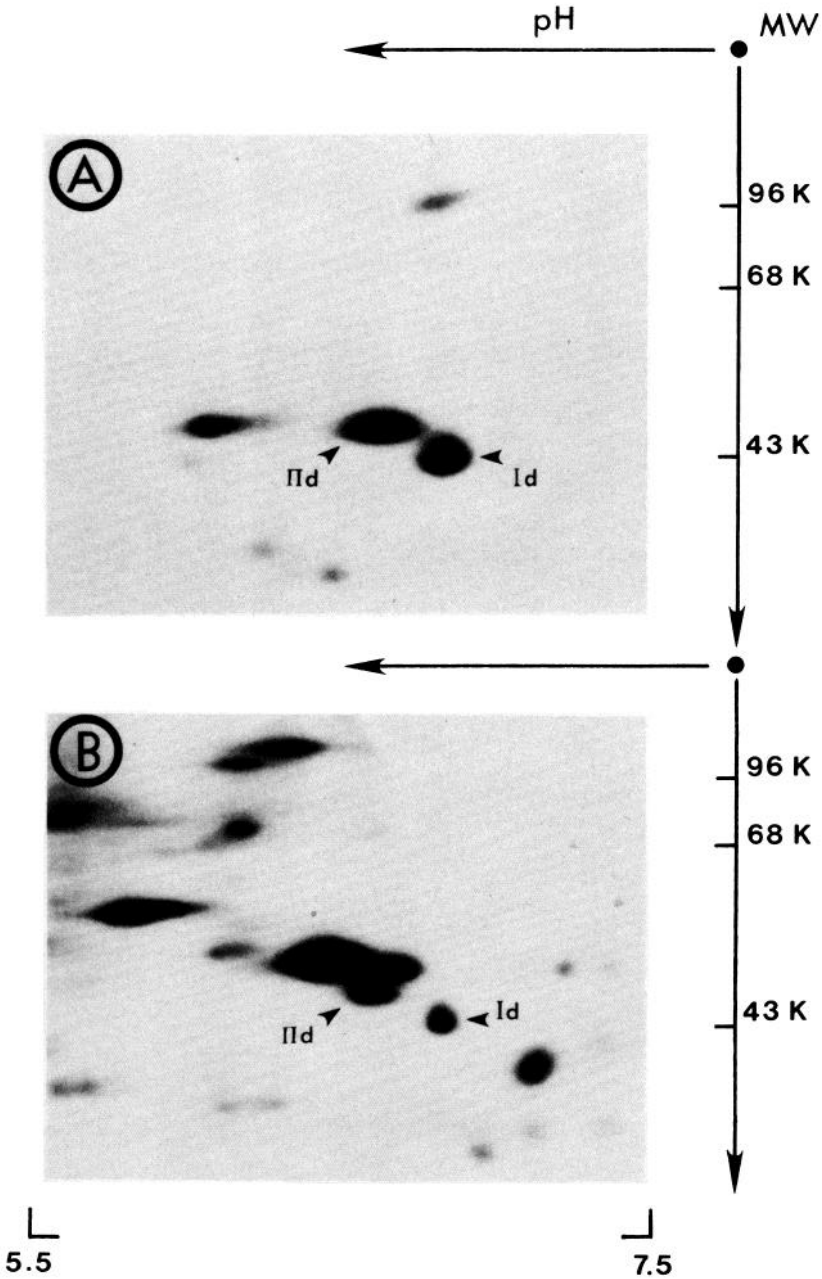

Figure 5. Phosphorylation of neural cAMP-binding proteins by cAMP-dependent protein kinase. The soluble and particulate fractions obtained by hand dissection of the neuropil were reconstituted for this experiment and photoaffinity-labeled by either ^{32}P-labeled or nonradioactive 8-N_3-cAMP. After photoaffinity labeling, the proteins were phosphorylated by adding the catalytic subunit of bovine cAMP-dependent protein kinase in the presence of 40 μM ATP (see "Materials and Methods"). The samples labeled with 8-N_3-[^{32}P] cAMP were phosphorylated with unlabeled ATP, while the samples photoaffinity-labeled with non radioactive reagent were phosphorylated with [γ-^{32}P] ATP. Two-dimensional electropherograms of (*A*) cAMP-binding proteins (radioactive 8-N_3-cAMP followed by ATP) and (*B*) phosphoproteins (8-N_3-cAMP followed by [^{32}P]ATP) are shown. The components that were labeled both by 8-N_3-[^{32}P]cAMP and by [γ-^{32}P]ATP are designated by *arrowheads*.

neural components used in these experiments contain both neurons and glial cells, all of the classes of cAMP-binding proteins can be shown to be present in neurons. Axoplasm, which is extruded from the pleuroabdominal connective by a method that yields material essentially free of glial contamination (Sherbany, 1981), gave the same two-dimensional electrophoretic patterns as did the neuropil fraction. The high content of membrane-associated binding proteins within axons could be explained if these components were being transported to nerve terminals. Moreover, cytoplasm extruded from single isolated cell bodies of R2 and the left pleural giant cell (Lasek and Dower, 1971) contained the same cAMP-binding proteins as did the nerve cell body fraction from the entire nervous system (data not shown).

cAMP-binding proteins in non-neuronal tissues of Aplysia. Membrane-associated cAMP-binding proteins in high concentrations were found only in nervous tissue. We examined particulate and soluble fractions from several non-neuronal tissues. Almost all of the binding activity in *Aplysia* muscle and salivary gland occurred in soluble fractions. As examples, the binding proteins from heart, body wall, and buccal mass muscle are shown in Figure 6. The membrane-specific M_r = 105,000 (class III) protein was completely absent in these tissues.

Comparisons of cAMP-dependent protein kinase from mammalian tissues have been drawn most frequently between the brain and muscle enzymes (Rubin et al., 1981). Examination of *Aplysia* muscle showed differences other than the relative paucity of membrane-associated components. In muscle, the predominant protein belongs to a single size class with a molecular weight of 54,000. Two-dimensional gel analyses of muscle-binding proteins reveal two components, neither of which overlaps precisely with the proteins of the nervous system (Fig. 7). The closest neural binding activities (class II) reproducibly had higher electrophoretic mobilities. Thus, cAMP-binding proteins appear to be tissue specific.

Discussion

Physiological implications of the regional and subcellular distribution of cAMP-binding proteins. We undertook our studies of cAMP-binding proteins in *Aplysia* nervous tissue because biophysical and pharmacological evidence strongly suggests that sensitization, a simple form of learning, involves phosphorylation of a protein which, in turn, regulates the functioning of specific synaptic ion channels (Klein and Kandel, 1978, 1980; Klein et al., 1982; Castellucci et al., 1980; Kandel and Schwartz, 1982). Maeno et al. (1971) and Ueda et al. (1973) suggested that membrane proteins in neurons can be modified functionally by cAMP-dependent protein phosphorylation. Jennings et al. (1982) recently have presented experiments that relate specific phosphorylation to neural activity in the neuroendocrine bag cells of *Aplysia*. Our main interest in examining kinases was to reveal the characteristics of these enzymes that might account for any selectivity for substrate, for example, for ion channel proteins.

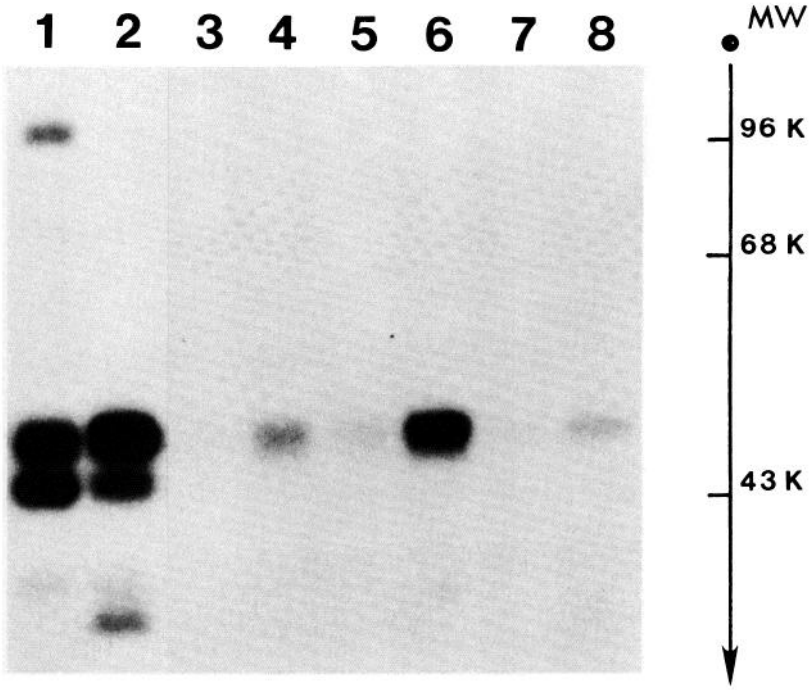

Figure 6. Membrane-associated cAMP-binding proteins are found in high concentrations only in nerve cells. Soluble and membrane fractions from neuropil and three kinds of muscle were photoaffinity-labeled and electrophoresed on 8% SDS gels. The *designations* are: *lane 1*, neuropil, particulate fraction; *2*, neuropil, soluble; *3*, heart muscle, particulate; *4*, heart muscle, soluble; *5*, body wall, particulate; *6*, body wall, soluble; *7*, buccal mass, particulate; *8*, buccal mass, soluble.

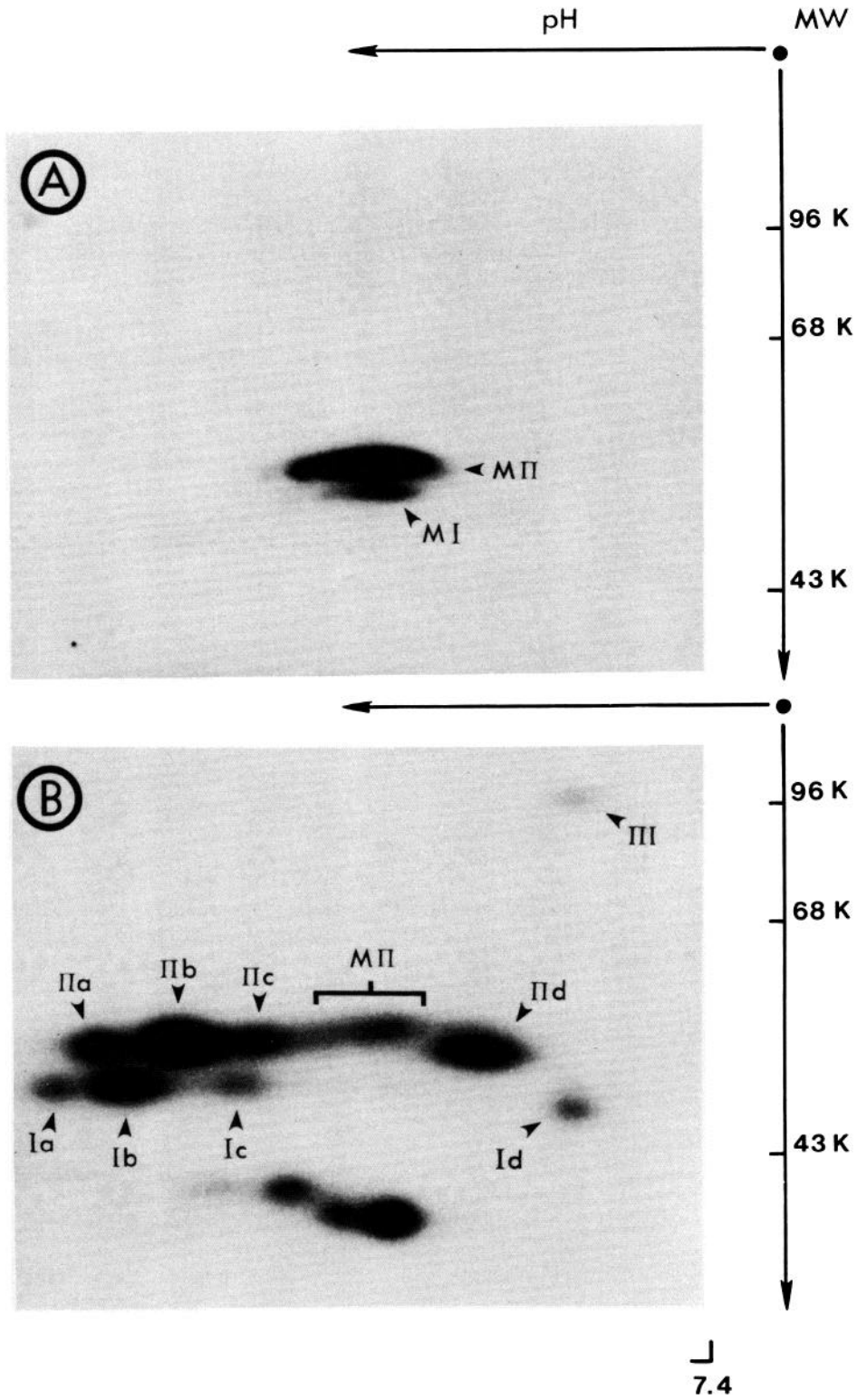

Figure 7. Comparison of soluble cAMP-binding proteins from buccal mass muscle (*A*) and from the cell body fraction of the neural components. In *B*, homogenates from both tissues were mixed and electrophoresed together in order to show that the binding proteins from the muscle (*M*) can be separated from the proteins from nervous tissue. The electropherogram in *A* has been exposed to x-ray film for 7 days in order to detect minority components; *B* was exposed for only 3 days.

One mechanism frequently proposed for achieving selectivity is compartmentalization of the kinase within the cell (Corbin et al., 1977; Brunton et al., 1981). We found a great variety of cAMP-binding proteins in *Aplysia* nervous tissue: at least nine major species can be separated, but only a few are enriched in the membranes from fractions containing nerve endings (Fig. 2). An attractive working hypothesis is that membrane-associ-

ated binding proteins belong to the kinases that are most likely to be involved in the modification of ion channels that underlies short term sensitization. We have not shown a direct link between a specific kinase and a specific substrate, but, in preliminary experiments, we found the same three classes of cAMP-binding proteins that are present in the neural components of whole ganglia in a small region (see "Materials and Methods" and Bernier et al., 1982) of the abdominal ganglion that contains the cluster of sensory cells and their processes. Earlier neurophysiological experiments have shown that a K^+ channel in these neurons is regulated by cAMP-dependent protein phosphorylation (Klein and Kandel, 1978; Castellucci et al., 1980; Kandel and Schwartz, 1982). Because this region of the ganglion contains neurons other than sensory cells, however, we are now attempting to extend the analysis to individual sensory cells.

Some of the heterogeneity in cAMP-binding proteins that we have observed might be artifactual because the techniques of photoaffinity labeling and two-dimensional gel analysis of proteins can result in the generation of extra components. For example, the affinity reagent could react with several types of charged residues at the cAMP-binding site of a single protein. If this were to occur, several isoelectric species might be generated from one polypeptide. Charge heterogeneity also could result from deamidation of asparagine or glutamine residues (Robinson, 1974). It is important to point out, however, that only the heterogeneity within groups Ia to Ic and IIa to IIc could be the result of these kinds of artifact. Thus, even if some of the heterogeneity is artifactual, it does not invalidate our major conclusion that there is a subset of cAMP-binding proteins that is concentrated in membrane fractions enriched in nerve endings.

Differences in the subcellular localization of classes of cAMP-dependent protein kinases have been observed previously in many mammalian cells and tissues. In those studies, molecular differences within the two recognized classes (R_I and R_{II}) usually were not examined. In a few studies, it was shown that differences can exist within a class, however. Thus, Rubin et al. (1981) found that R_{II}s from different tissues differ immunologically, and Panter et al. (1981) reported that nervous tissue contains isoelectric variants of both the R_I and R_{II} size classes. Our results extend these earlier studies by showing that different regions have characteristic distributions of cAMP-binding proteins. This suggests that regulatory subunits with different molecular structures might be directed to specific sites within the cell where they can phosphorylate local substrate proteins. Alternatively, a cell might direct a common precursor molecule to all of its regions; post-translational modifications then might alter this kinase differentally. Whatever the mechanism by which the heterogeneity in kinases observed is produced, selectivity of phosphorylation, not only between substrates in membrane and cytosol but also between different membrane proteins, is possible because of the great variety of cAMP-binding proteins in the neuron.

Although regional assortment of proteins within the cell is required for the distribution and supramolecular organization of some gene products (Sabatini et al., 1982), it is not obvious that targeting of regulatory subunits of cAMP-dependent protein kinase is necessary for modifying ion channels. Experiments in which the catalytic subunit of the bovine kinase was injected into the sensory neuron demonstrated that the activity of a soluble enzyme, if present in adequate concentration, can catalyze the phosphorylation which results in increased release of transmitter (Castellucci et al., 1980). Moreover, when a neuron is stimulated so that the release of transmitter is augmented for a period of 20 min to 1 hr because cAMP is elevated within the neuron (Bernier et al., 1982), it is conceivable that substrates other than those related to ion channels might be phosphorylated. Sugar metabolism is an obvious example. Thus, the stimulus that produces the changes in transmitter release which underlie short term sensitization might activate all cAMP-dependent kinases in the sensitized cell concomitantly, thereby elevating the extent of phosphorylation generally. General activation of the cell would not necessarily imply indiscriminate phosphorylation, however. We prefer to think that cytosolic pathways, such as sugar metabolism, would be controlled by cytosolic kinases and that membrane-specific pathways are controlled by membrane-associated kinases. Indeed, some observations in mammalian tissues strongly support the idea that the molecular components of certain cAMP cascades (receptor, cyclase, kinase, and kinase substrates) which act on particular cellular functions can be compartmentalized within the cell (Cumming et al., 1981; Earp and Steiner, 1978; Honeyman et al., 1979; LeCam et al., 1981). For example, Brunton et al. (1981) found that two agonists can stimulate the synthesis of cAMP in rat heart to the same extent, but each activates different physiological processes.

A hypothesis for long term sensitization. The variety of cAMP-binding proteins that we have observed in *Aplysia* nervous tissue encourages us to propose a mechanism for the conversion of short term sensitization to the long term form. A single noxious stimulus produces a change in behavior that lasts for 20 min to 1 hr, but 16 or more stimuli can prolong the memory for several weeks (Pinsker et al., 1973). Preliminary experiments indicate that elevated concentrations of cAMP do not persist in sensory neurons for these long periods of time (Bernier et al., 1982). While a site-specific regulatory subunit might not be implicated in the molecular mechanism underlying short term sensitization, it is attractive to think that long term memory results when prolonged stimulation of a sensory neuron induces the expression of a new regulatory subunit that endows the kinase with greater selectivity for substrate and greater sensitivity to cAMP (Kandel and Schwartz, 1982). As a result, slight elevations of cAMP of the sort that accompany normal activity of the sensory neuron (but inadequate to produce the short term process in the untrained terminal) would be sufficient to enhance transmitter release through phosphorylation and modification of the target, for example, the ion channel protein. Induction of a site-specific regulatory subunit would, for example, avoid changes in carbohydrate metabolism that need not persist for long periods of time. Similar mechanisms might underlie other forms of long term memory and provide a simple and testable explanation for the molecular events that underlie memory in higher animals.

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