Distribution of cAMP and cAMP-Dependent Protein Kinases in *Aplysia* Sensory Neurons

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Sensitization of the gill- and siphon-withdrawal reflex in *Aplysia* is considered a simple form of learning. Previous work has provided physiological and pharmacological evidence that cAMP-dependent protein phosphorylation within identified sensory neurons of the abdominal ganglion underlies the short-term form of this behavioral modification. Our main goal in this paper is to determine the subcellular distribution of cAMP and to measure the amounts and properties of the 2 types of subunits (regulatory and catalytic) that constitute the cAMP-dependent protein kinase. Do these biochemical parameters differ in sensory cells from those in other parts of nervous tissue? We found that the increased cAMP synthesized under conditions of sensitization is distributed in 3 compartments in the neuron: most of it is free in the cytoplasm; the remainder is bound either to cytoplasmic or to particulate proteins, which are believed to be regulatory subunits of the cAMP-dependent protein kinase. Binding of cAMP within the neurons is a measure of activation of the kinase. At rest, 17% of the binding sites in sensory cells were occupied. After brief electrical stimulation of the connective, which released endogenous transmitter, occupancy increased to 34%. This treatment increased the amount of cAMP bound to the various binding proteins differentially. The biochemical characteristics of cAMP binding were found to be the same in sensory neurons as in the rest of the nervous system but different from those in muscle. Thus, memory and learning are likely to be mediated by enzymes that are shared by other nerve cells. We found that sensory neurons have greater cAMP-dependent protein kinase activity than other neurons, however, and as a result may be more sensitive to small increases of cAMP.

There is now much evidence indicating that alterations in the synthesis of cAMP and consequent changes in protein phosphorylation within specific neurons underlie several simple forms of learning in invertebrates, for example, short-term sensitization of the gill- and siphon-withdrawal reflex in *Aplysia* (Kandel

and Schwartz, 1982). Although the ability to mediate learning is likely to be a specialized function of particular nerve cells, the enzymes that catalyze the reactions of the cAMP cascade are present in most if not all neurons. How then can ubiquitous molecules, which are universally distributed and are known to regulate many different metabolic pathways, be used by some neurons to accomplish so specialized a function as the modification of an animal's behavior?

Sensitization has been shown to be the consequence of presynaptic facilitation that results from the augmented release of transmitter from sensory neurons of the abdominal ganglion onto interneurons and motor neurons innervating the muscles that cause the gill and siphon to contract (Castellucci and Kandel, 1976; Klein and Kandel, 1978; Kandel and Schwartz, 1982). Reflex withdrawal of the tail is mediated by a population of sensory neurons in the pleural ganglion (Walters et al., 1983) with morphological and biophysical properties similar to those of the sensory cells in the abdominal ganglion (Pollock et al., 1985). Direct measurements in sensory cells have revealed that the enhanced synthesis of cAMP, which is produced by applying 5-HT or the small cardiac peptides (SCP) neuropeptides or by activating facilitating neurons with the release of the natural (endogenous) facilitating transmitter, persists for 15 to 30 min (Bernier et al., 1982; Abrams et al., 1984). Castellucci et al. (1980, 1982) have presented evidence that the sustained synthesis of cAMP in sensory cells, which maintains the protein kinase in an active form, accounts for the time course of the memory for short-term sensitization.

It has been shown that cAMP activates the kinase by binding to regulatory subunits (R) in the following reaction:

$$4cAMP + R_2C_2 \rightleftharpoons 2C + 2(R \cdot cAMP)$$
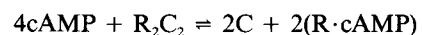
thereby releasing the catalytic subunits (C) from inhibition (Flockhart and Corbin, 1982; Nestler and Greengard, 1984). In vertebrate nervous tissue, the only proteins that bind cAMP are regulatory subunits of the cAMP-dependent protein kinase (Walter et al., 1977, 1978). In those animals, the extent to which cAMP is bound therefore reflects the activation of these kinases, and the intracellular distribution of bound cAMP can provide insight into the compartments of the cell affected by the transmitter. In *Aplysia* nervous tissue, Eppler et al. (1982) identified 5 major cAMP-binding proteins by photoaffinity labeling with ^{32}P-8-N_3cAMP. Although the subcellular distribution and the regional assortment of these binding proteins differ, the 5 proteins appear to be closely related in structure and to serve as regulatory subunits of the cAMP-dependent protein kinase (Eppler et al., 1986). In this paper, we determine how much cAMP
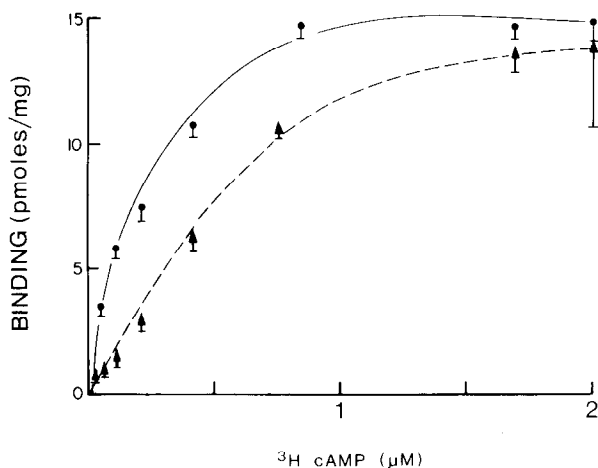
Figure 1. Binding of ^{3}H-cAMP in a homogenate of *Aplysia* neural components was measured after 10 min at room temperature in potassium phosphate (pH 6.8). Bound ^{3}H-cAMP was separated by filtration through nitrocellulose. The *solid line,* fit to the data points by eye, represents binding in homogenates from ganglia that had not been exposed to 5-HT. Each point is the average of 3 separate, triplicate determinations with 40 μg of protein. The *dashed line* represents binding in neural components from ganglia treated for 5 min with 2×10^{-4} M 5-HT before homogenization. Each point is the average of 2 determinations in triplicate. Maximum binding occurs at 3 min at room temperature. Incubation in the presence of a 20-fold excess of cold cAMP reduces the binding to values obtained on filters in the absence of added protein.

is bound to protein and how much is free in sensory neurons at rest and under conditions of sensitization. We also examine the distributions of cAMP binding and cAMP-dependent protein kinase activity in sensory neurons and in other *Aplysia* cells and tissues, and we study the characteristics of the binding reaction both *in vitro* and in intact sensory neurons within the isolated ganglion.

Materials and Methods

Animals and tissues. Aplysia californica weighing 90–125 gm (supplied from California by the Pacific Biomarine Company, Venice; Sea Life Supply, Sand City; Marine Specimens Unlimited, Pacific Palisades; and the Howard Hughes Medical Institute's Mariculture Resource Facility, Woods Hole Oceanographic Institute at Woods Hole, MA) were kept at 15°C in artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH). Abdominal and pleuropedal ganglia with nerves attached were removed through an incision in the foot from animals anesthetized by injection of a volume of isotonic $MgCl_2$ corresponding to one-half the weight of the animal. Ganglia were pinned to silicone plastic (Sylgard, Dow Corning Co., Midland, MI) in high Mg^{2+} seawater (Byrne et al., 1974) to minimize any effects of mechanical stimulation. After they were pinned, the ganglia were washed several times and then kept at 15°C for at least 30 min in artificial seawater supplemented with amino acid and vitamins (Eisenstadt et al., 1973) containing P_i at a concentration of about 5 μM (Bernier et al., 1982).

Exposure to $^{32}P_i$. Ganglia were exposed at 15°C to 0.5 mCi of $^{32}P_i$ (Amersham, Arlington Heights, IL, orthophosphate, carrier free), each in a 0.5 ml droplet of the supplemented artificial seawater for 3 hr (Bernier et al., 1982). The ganglia were washed and maintained in fresh seawater for an additional 18–22 hr in order to label phosphorylated intermediates stably.

Stimulation. In one series of experiments, abdominal ganglia were exposed for 5 min to 0.2 mM 5-HT creatinine sulfate (Sigma Chemicals Co., St. Louis, MO). This saturating concentration has previously been used to ensure maximal stimulation of adenylate cyclase in *Aplysia* ganglia (Cedar and Schwartz, 1972) and sensory neurons (Bernier et al., 1982). In another series, facilitating interneurons (Hawkins et al., 1981) were stimulated extracellularly by shocking the left connective through

a suction electrode (10 sec train, 6 Hz, 3 msec, 6 V pulses) (Hawkins et al., 1981) while recording intracellularly from one sensory cell of the abdominal cluster. The efficacy of nerve stimulation was assessed by measuring how much the spike broadened in sensory cells; increased spike broadening accompanies presynaptic facilitation. A 2 sec train of suprathreshold intracellular pulses (20 Hz) was used to inactivate the nonmodulated K^+ currents of spikes in sensory neurons and thus enhance spike-broadening during facilitation (M. Klein, personal communication). The duration of the last spike in a train was measured both before nerve stimulation and 20 sec thereafter; in all successfully stimulated ganglia the spike broadened by more than 35%. The ganglia were frozen for dissection 10 sec later.

Methods of dissection. Ganglia were frozen quickly at $-20°C$ in propylene glycol:2 M NaCl (1:1, vol/vol); cell bodies of abdominal (Byrne et al., 1974) and pleural (Walters et al., 1983) sensory neurons were identified by their position and characteristic appearance, and were dissected out on powdered solid CO_2 as previously described by Bernier et al. (1982). In some experiments, the frozen neural components were sorted by dissection into 2 fractions enriched either in cell bodies or in neuropil (Eppler et al., 1982).

Preparations and assays

Membrane-cytoskeleton fraction. Neural components from a single ganglion were extracted in a 25 μl volume of 2 M glycerol, 1 mM $MgSO_4$, 2 mM EGTA, 0.2% saponin, 0.02% Na azide, and 0.1 M K-PIPES (pH 6.9), and centrifuged at 7000 × g for 15 min at 4°C (Saitoh and Schwartz, 1983). The resulting pellet was vortexed in another 25 μl volume and centrifuged again. The combined supernatants constitute the cytosol fraction. The pellet, suspended in 50 μl, constitutes the membrane-cytoskeleton fraction.

cAMP. Dissected neural components from ganglia that had been exposed to $^{32}P_i$ were transferred while still frozen to a glass–glass homogenizer (Micrometric Instruments, Cleveland, OH) containing 0.25 ml of 50% aqueous ethanol at 0°C with 0.2 μg of cAMP (sodium salt, Sigma) added to measure recovery. cAMP was isolated by thin-layer chromatography on polyethylimine plates, and ^{32}P-ATP and AMP were separated from each other and from P_i and PP_i by high-voltage electrophoresis (Bernier et al., 1982). The content of labeled cAMP is calculated as a percentage of ^{32}P-ATP: knowing the specific activity of $^{32}P_i$ in the seawater, it is possible to estimate the amount of labeled ATP, presuming that the labeled ATP is completely equilibrated (Ambron et al., 1975; Bernier et al., 1982).

Homogenates for the binding assay. Neural components from the central ganglia of one animal were suspended in 0.5 ml of 0.3 M NaCl, 0.2 M sucrose, 3 mM EDTA, 1 mM EGTA, 0.1% NP-40, and 20 mM Tris-HCl (pH 7.4) in a glass tissue grinder. After homogenization, the sample was centrifuged at 105,000 × g for 30 min. The supernatant is the soluble fraction. The pellet was suspended and homogenized again in an equal volume of the same buffer, to become the particulate fraction. The volume used for homogenization can be reduced for smaller samples: For example, 2 clusters of sensory neurons dissected from the pleural ganglion were homogenized in 0.1 ml. This procedure was also used to prepare a soluble fraction of buccal muscle.

Embryo homogenates. Aplysia egg masses were frozen in liquid nitrogen and pounded to powder in a mortar chilled to $-70°C$. The powder was suspended in 0.35 M sucrose, 0.17 M NaCl, 3 mM EDTA, 1 mM EGTA, 0.1% NP-40, 5 mM 2-mercaptoethanol, 20 mM benzamidine (Sigma), and 20 mM Tris-HCl (pH 7.4) and filtered through 3 layers of cheesecloth. The protein concentration in the filtrate was between 2 and 5 mg/ml.

cAMP-dependent protein kinase. The dissected neural components were homogenized in 0.1 M NaCl, 3 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl (pH 7.4). Kinase activity was determined by phosphorylation of a synthetic heptapeptide (Kemptide, Sigma), with slight modification from our previously described method (Eppler et al., 1986). To mitigate the effect of any endogenous inhibitors (Hofmann et al., 1977), kinase activity was determined at several dilutions of the homogenate in 0.5 mg/ml BSA (crystallized; Sigma) and 5 mM 2-mercaptoethanol. The specific activities expressed are derived from the plateau values obtained with dilution. Activity is calculated as the difference between ^{32}P-phosphorus incorporated into the heptapeptide in the presence of 10 μM cAMP and that in the presence of protein kinase inhibitor (0.1 mg/ml, Sigma; Type II).

cAMP-binding proteins. cAMP bound to protein was measured by filter assay (Sugden and Corbin, 1976): 50 μl homogenates of *Aplysia*

Table 1. Concentrations of cAMP in *Aplysia* neurons

Neurons	cAMP (pmol/mg protein)	
	Free	Bound
Sensory cells		
No treatment	16.3 ± 3.4	3.0 ± 0.03
5-HT	47.3 ± 9.6	6.0 ± 0.8
Extent of stimulation	2.9	2.0
Central ganglia		
No treatment	20.3 ± 2.9	2.1 ± 0.4
5-HT	47.8 ± 3.9	7.8 ± 0.6
Extent of stimulation	2.4	3.7

Values of cAMP both free and bound (soluble plus particulate) were calculated from the ^{32}P-ATP present in the samples, assuming complete equilibration of the label in ATP (Bernier et al., 1982) and values of ATP measured previously (Ambron et al., 1975). cAMP was measured in sensory cell clusters from 5 pleural ganglia and 5 abdominal ganglia for each condition.

neural components containing 5–100 μg of protein were incubated for 10 min at 22°C with 5,8-^{3}H-adenosine 3',5'-cyclic monophosphate (56 Ci/mmol, Amersham) in 0.1 ml containing 0.5 mg/ml histone IIa, 1 mM IBMX, 1 mM theophylline (all from Sigma), 2 M NaCl, 1 mM EDTA, and 50 mM potassium phosphate (pH 6.8). The reaction was stopped by adding 2.6 ml of cold 10 mM potassium phosphate, 1 mM EDTA (pH 6.8). A period of 10 min was chosen, although binding reached a maximum at 3 min and remained constant for 30 min, decreasing slightly thereafter (Bernier, 1984). After incubation, the mixture was filtered through a moistened 0.45 μm nitrocellulose filter (HAWP, Millipore Corp., Bedford, MA). The tubes were rinsed with 2.6 ml of the buffer and the filter washed 3 more times (total wash, 13 ml). The filters were dried overnight at room temperature, and radioactivity was measured by scintillation counting. To assay for total cAMP-binding activity in extracts we used 2.2 μM ^{3}H-cAMP because this concentration is saturating even after stimulation of endogenous cAMP synthesis by 5-HT (Fig. 1). The binding of ^{3}H-cAMP to protein in *Aplysia* homogenates and its retention by the filter was proportional to the amount of protein added from 5 μg to >140 μg. Addition of 0.1 mM AMP did not influence the binding of ^{3}H-cAMP. Nonspecific binding was assessed by determining the amounts of ^{3}H-cAMP retained in the presence of a 20-fold excess of unlabeled cyclic nucleotide, and was 1.0 pmol. This amount was retained by the filter both in the presence and in the absence of a protein sample.

Photoaffinity-labeling of cAMP-binding proteins. Labeling with ^{32}P-8-N$_{3}$cAMP (50–80 Ci/mmol, ICN, Irvine, CA) was carried out by a modification of the procedure of Eppler et al. (1982). Equal portions of homogenate were made 0.2% saponin and first incubated with 1.0 μM ^{32}P-8-N$_{3}$cAMP at 0°C or 22°C for 10 min followed by a further 10 min equilibrium period at 0°C. The samples were then exposed at 0°C for 10 min to ultraviolet light. Proteins were separated by SDS-PAGE, stained with silver (Bio-Rad), and autoradiographed at −70°C (Eppler et al., 1986).

Separation of bound and free cAMP. Ganglia or isolated cells were homogenized for 5 sec at 0°C in a volume of 0.1–0.5 ml (depending on the amount of tissue used) containing 0.3 M NaCl, 0.2 M sucrose, 3 mM EDTA, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.4). Under these conditions, loss of bound cAMP is negligible: 3.5% in 30 min, with a half-life of 7 hr (Bernier, 1984). A sample was removed to measure total cAMP in order to evaluate any loss that might occur during the separation of bound and free cAMP by procedures modified from Terasaki and Brooker (1977). (The sum of free and bound cAMP equaled the total measured cAMP: in 10 experiments, the recovery was 103 ± 10% for abdominal ganglia and 88 ± 6% for sensory neurons.) The remainder was diluted to 3 ml and centrifuged at 105,000 × g for 30 min. cAMP bound to protein in the supernatant was separated from free cAMP by filtration through nitrocellulose. cAMP retained on the filter was eluted for 4–5 hr with 0.01 M HCl (2 ml). Particulate cAMP was extracted from the 105,000 × g pellet with 0.01 M HCl (0.5 ml). All samples were evaporated to dryness and resuspended in 50% aqueous ethanol (0.1 ml). The cAMP in all fractions was measured as described above.

Protein. Protein was determined using BSA as a standard (Lowry et al., 1951). The neuronal components from the 5 major central ganglia
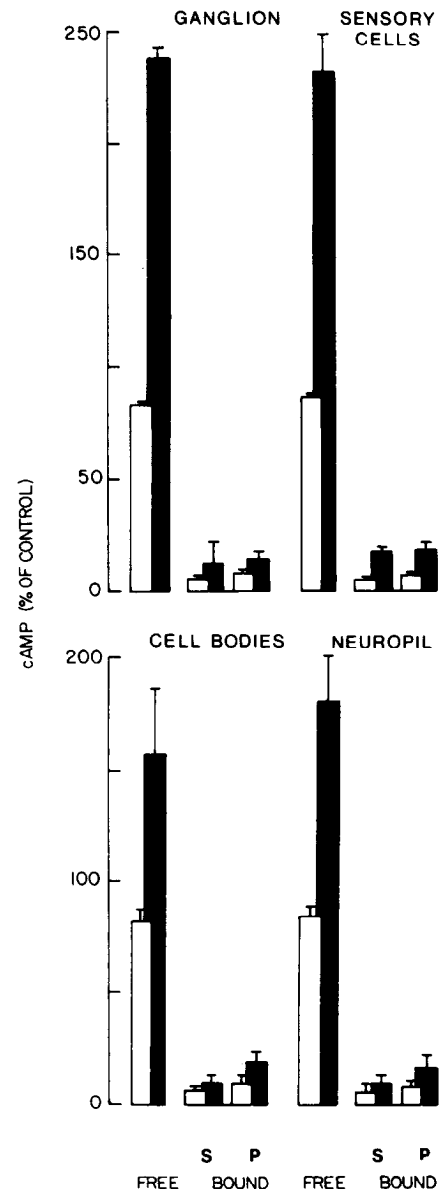

Figure 2. Distribution of cAMP in nervous tissue. cAMP was measured in neural components from abdominal ganglia labeled by exposure to ^{32}P$_{i}$ (Bernier et al., 1982). Free and bound cAMP were separated as described in Materials and Methods from ganglia treated for 5 min with 2 × 10^{-4} M 5-HT (*open bars*) and untreated controls (*solid bars*). The bound cAMP was distributed in 2 fractions: soluble (*S*) and particulate (*P*). The values, from 6 determinations, are expressed as a percentage of the total cAMP in the untreated ganglia.

of 1 animal's nervous system dissected from their connective tissue sheaths yielded 670 ± 52 μg (*n* = 8) dry weight of protein. The neuronal components of a single abdominal ganglion yielded 148 ± 32 μg (*n* = 9), and a cluster of sensory neurons from the pleural ganglion yielded 5.7 ± 0.6 μg (*n* = 10). For many of the determinations, we used sensory neurons from the pleural rather than from the abdominal ganglion because there are 10 times more cells in a pleural cluster.

Results

Distribution of cAMP in sensory neurons

Underlying sensitization of the gill- and siphon-withdrawal reflex is the elevation of cAMP within sensory neurons that results in the activation of cAMP-dependent protein kinase (see Bernier et al., 1982; Kandel and Schwartz, 1982). The concentration of

Table 2. cAMP-binding and kinase activity in various parts of the *Aplysia* nervous system

Component	Binding (nmol/mg)	Kinase (nmol/min/mg)	Ratio	n
Central ganglia				
Total neural components	12.8 ± 1.3	6.7 ± 0.7	1.9	7
Abdominal ganglion				
Cell bodies	11.0 ± 0.8	3.2 ± 0.2	3.4	6
Neuropil	9.9 ± 1.2	7.4 ± 0.7	1.3	6
Bag cells	15.6 ± 0.9	–	–	3
Pleural ganglion				
Sensory neurons	12.7 ± 0.6	13.1 ± 1.2	1.0	10
Remaining neural components	13.6 ± 2.0	6.9 ± 0.7	2.0	5

The maximum binding capacity present in the various parts was assayed at 22°C using a saturating concentration (2.2 μM) of ^{3}H-cAMP as probe. Kinase activity was determined by phosphorylation of the synthetic heptapeptide (Materials and Methods). Values are the means ± SE in *n* separate, duplicate determinations. An arbitrary *ratio* between cAMP-binding proteins and kinase activity was obtained by dividing the *binding* measurements by the values of kinase *activity*.

cAMP, normally 20 pmol/mg protein in unstimulated sensory neurons, increases maximally 2- to 3-fold 5 min after ganglia are exposed to 0.2 mM 5-HT (Table 1; and Bernier et al., 1982). What proportion of the elevated cAMP is bound to protein and how much is free? We found that most of the cAMP is free both in sensory neurons (Fig. 2A, Table 1) and in the total neuronal components from the abdominal ganglion (Fig. 2B). Less than 20% was associated with protein, and this proportion did not change appreciably after stimulation with 5-HT.

Some cAMP-binding proteins in *Aplysia* neural components have been shown to be associated with membrane or with cytoskeleton (particulate) fractions, and others to be unattached in the cytosol (soluble) (Eppler et al., 1982, 1986). We find that the cAMP bound to protein within the cell is distributed almost equally between the cytosolic and particulate fractions (Fig. 2A). We separated these 2 forms of bound cAMP in order to determine the distribution of cAMP between these 2 classes of binding proteins (see Materials and Methods). The bound cAMP in the cytosol was isolated by filtering the supernatant fraction through nitrocellulose: soluble cAMP-binding proteins adsorb to the filters. The bound cAMP associated with membranes was sedimented with the particulate fraction. The amounts of cAMP

Table 3. Regional distribution of cAMP-binding activity in the abdominal ganglion

Fraction	% Total activity in ganglion	
	Cell bodies	Neuropil
Soluble	28.5 ± 2.7	21.7 ± 0.8
Particulate	19.2 ± 1.6	29.0 ± 1.8

Cell bodies and neuropil from 6 abdominal ganglia were sorted by hand and fractionated as described (Eppler et al., 1982; see Materials and Methods). Binding of cAMP was assayed on nitrocellulose filters and is presented as the percentage of the total binding activity in each fraction. The total activity in the neural components of the ganglion, obtained by summing the activities measured in the fractions (cell body and neuropil) in each of the 6 determinations, was 10.5 ± 1 pmol cAMP bound/mg protein. This did not differ from 11.1 ± 0.9, the value measured in 14 determinations on the unfractionated neural components of the ganglia.

Table 4. Subcellular distribution of cAMP-binding proteins

Treatment	% Total binding activity			
	Abdominal ganglion		Sensory neurons	
	Soluble	Particulate	Soluble	Particulate
None	54.8 ± 3.7	44.8 ± 3.7	51.4 ± 1.1	48.6 ± 1.1
5-HT	52.4 ± 7.4	47.2 ± 7.4	51.0 ± 0.5	49.0 ± 0.5

Ganglia and cells were extracted with 0.2% saponin in 2 M glycerol and the soluble and particulate (membrane-cytoskeleton complex) fractions prepared as described in Materials and Methods. cAMP-binding activity in each fraction is given as the percentage of total binding activity (soluble and particulate) in the extract. Values are the means ± SE of 4 separate determinations, each with 1 abdominal ganglion; 3 clusters from the pleural ganglia were combined for each sensory neuron determination.

in Figure 2 are reported as a percentage of the total cAMP in the control; in the neural components of the abdominal ganglion, this value corresponds to 2.6 pmol, and in individual sensory neurons, to 0.6 fmol. These values have been estimated from the proportion of ^{32}P-ATP converted to cAMP assuming that complete equilibration of the alpha position of ATP had taken place (see legend to Table 1; and Bernier et al., 1982).

Distribution of cAMP in regions of the abdominal ganglion

The neural components from ganglia can be dissected by hand into 2 fractions, one enriched in neuronal cell bodies, the other in axons and nerve endings (neuropil) (Eppler et al., 1982). cAMP was partitioned similarly in fractions containing cell bodies (Fig. 2C) and neuropil regions of the abdominal ganglion (Fig. 2D). Only a small proportion of the cAMP in both of the fractions was bound to protein, and stimulation by 5-HT resulted in an increase in cAMP of the same extent in all of the fractions. Bound cAMP is similarly distributed between the soluble and particulate compartments in both regions.

Distribution of binding activity and cAMP-dependent protein kinase activity in nervous tissue

Our examination of cAMP's subcellular distribution within the neural components described above shows that there are proteins that bind cAMP within the cells, but it does not provide a measurement of how much total binding activity is actually present. We used nitrocellulose filters to determine the specific activity of cAMP binding in homogenates of sensory neurons and of the neural components from abdominal ganglia. These data are presented together with cAMP-dependent protein kinase activity in Table 2. Sensory neurons have the same specific binding activity as other parts of nervous tissue.

We fractionated neural components in 2 other ways: first regionally, into cell-body and neuropil fractions, and, second, into membrane-cytoskeleton and supernatant fractions (Materials and Methods). Although the total cAMP-binding activity is equally distributed between cell bodies and nerve endings, the nerve-ending fraction appeared to be enriched in particulate binding activity (Table 3). The distribution of cAMP-binding proteins is similar between the cytoplasm and the membrane-cytoskeleton fraction (Table 4). Similar distributions of photoaffinity-labeled binding proteins in *Aplysia* nervous tissue have previously been described (Eppler et al., 1982, 1986). Treatment of abdominal ganglia with 5-HT did not change the proportion of binding proteins present in either compartment, nor did it change the total amounts of the binding proteins in the tissue.
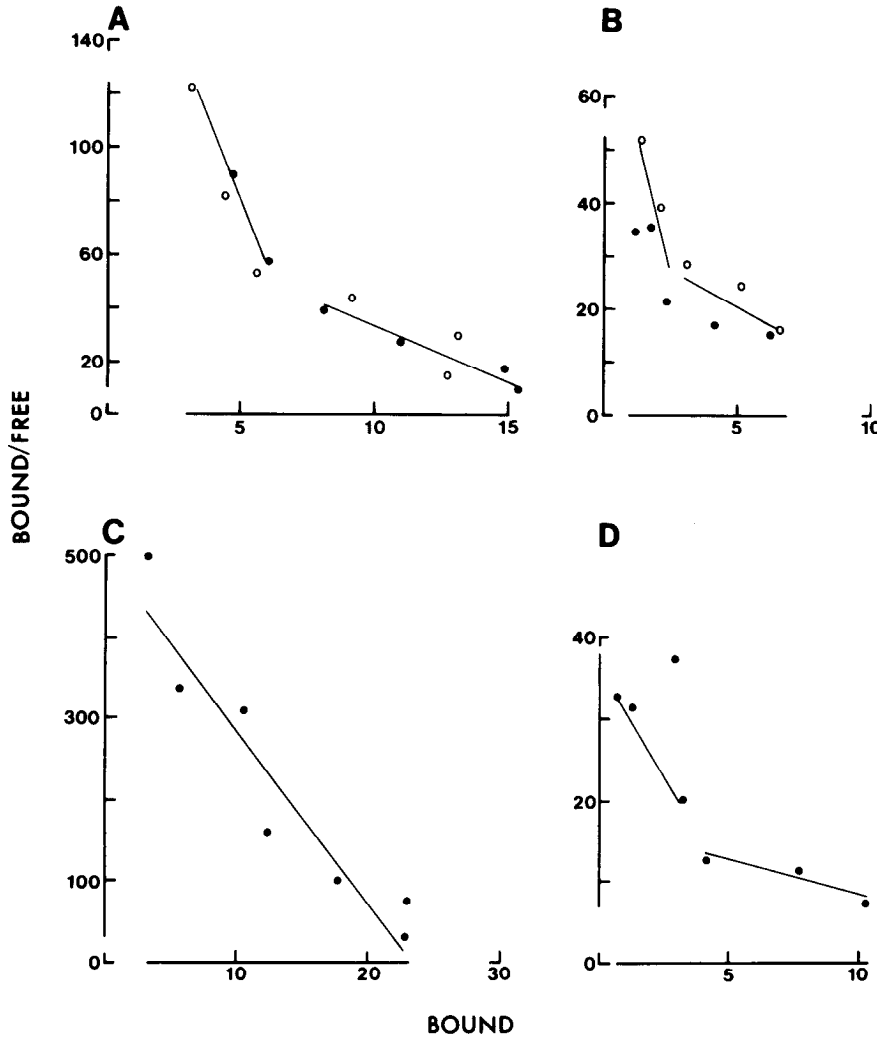
Figure 3. Scatchard plots of the dependence of binding on concentration of ^{3}H-cAMP. Samples were incubated for 10 min at room temperature. Bound cAMP is calculated as pmol/mg protein; free cAMP is the micromolar concentration. *A* and *B*, Soluble (*A*) and particulate (*B*) fractions generated from neural components of all the central ganglia (O) and from sensory neurons dissected from 34 pleural ganglia (●). Concentrations of cAMP varied from 0.26 to 1.7 × 10^{-6} M. *C*, Fibers from buccal muscle were homogenized under the same conditions used to prepare binding proteins of the nervous tissue. Since muscle has no particulate binding activity, only the soluble fraction was used. *D*, Extracts of *Aplysia* embryos were prepared as described in Materials and Methods. Particulate and soluble components were not separated in these determinations. For each tissue, binding was determined in triplicate at each concentration. For nervous tissue (*A*, *B*) and muscle (*C*), each determination was repeated 3 times, and the data points represent the average of the 3 determinations. Only 1 determination, in triplicate, was carried out with the embryo extracts (*D*). Lines generated by linear regression analysis were fitted to points chosen by eye to represent the highest and lowest plausible binding constants.

The activity of cAMP-dependent protein kinase, as measured by phosphorylation of the exogenous heptapeptide substrate, varies among the components of the nervous system tested (Table 2). It was greater in sensory cell bodies from the pleural ganglion than in the remainder of the ganglion's neural components ($p < 0.001$), and greater in the neuropil of the abdominal ganglion than in cell bodies ($p < 0.001$). Thus, the ratio of cAMP binding to cAMP-dependent kinase activity was not constant in all parts of the nervous system. We found it to be lowest in sensory neurons and in neuropil.

Heterogeneity in cAMP-binding proteins

The characteristics of the cAMP-binding reaction were determined for sensory neurons and for the neural components of all central ganglia, as well as for muscle and embryos. The binding activity in both sensory neurons and total neural components appeared to be heterogeneous (Fig. 3, *A, B*). In view of the work of Eppler et al. (1982) showing that *Aplysia* nervous tissue contains at least 5 distinct binding proteins, this result is expected. The Scatchard plots derived from the binding data indicate the presence of high- and low-affinity binding sites, with constants ranging from 0.04 to 0.25 μM for soluble fractions and from 0.04 to 0.33 μM for the particulate fractions. These values are comparable to binding constants measured in vertebrate ner-

vous tissue (Walter et al., 1977, 1978). No differences in these parameters were observed between sensory neurons and the rest of the nervous tissue. In contrast, the binding activity in muscle seems less complex (Fig. 3*C*). It is soluble and behaves as a single population of binding sites with an estimated constant of 0.05 μM, consistent with the observation that only 1 major binding protein can be identified in buccal muscle by photoaffinity labeling (Eppler et al., 1982). The binding is heterogeneous in a homogenate of embryos (Fig. 3*D*), which is similar to neurons in having multiple ^{32}P-8-N$_3$cAMP-binding proteins (Palazzolo, 1985).

Occupancy: Extent of binding by endogenous cAMP

The subcellular distribution studies with cAMP indicated that endogenous cAMP binds to proteins within the cell. The foregoing assays measure how much total cAMP-binding capacity is present in neurons. We next determined the proportion of cAMP-binding sites within the cell that are actually occupied by cAMP under various conditions of stimulation. To make this kind of measurement, we had to show, first, that the cAMP bound in the cell will remain associated with the protein during the extraction procedure, and, second, that after extraction, this endogenously bound cAMP can be made to exchange with a ^{3}H-cAMP probe.
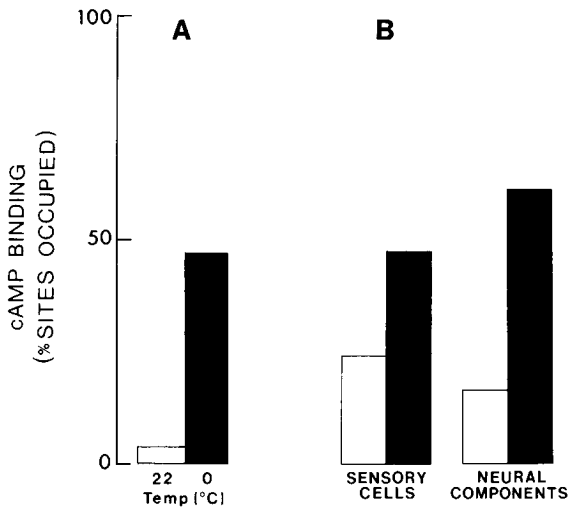
Figure 4. Occupancy of cAMP-binding sites in neural components. *A*, ³H-cAMP binding activity was measured in homogenates of untreated ganglia and of ganglia after exposure for 5 min to 10 μM dibutyryl cAMP. One aliquot of the extracts was kept at 0°C (*solid bar*) and the other warmed to room temperature (*open bar*) before carrying out the binding reaction. Values represent the fraction of the control ³H-cAMP binding that is blocked after treatment with dibutyryl cAMP and are the means of 3 independent determinations. The SD of the measurements used to derive these values was less than 15% of their means. *B*, The extent of cAMP binding *in vivo* at rest (*open bars*) or after treatment with 5-HT (*solid bars*) was obtained by dividing the values in Table 1 for bound cAMP by those in Table 2 for the ³H-cAMP-binding capacity of sensory cells and the neural components of nervous tissue.

We treated intact abdominal ganglia with N₆,O₂'-dibutyryl cAMP in order to increase the occupancy of the cAMP-binding proteins in the cells. Application of this analog of cAMP has previously been shown to enhance the release of transmitter from sensory neurons (Brunelli et al., 1976), presumably because the drug can penetrate and interact effectively with regulatory subunits of the cAMP-dependent protein kinase within the cells. We found that treatment with dibutyryl cAMP halved the amount of the ³H-cAMP probe bound, provided the homogenates were maintained at 0°C after extraction (Fig. 4*A*). This result indicates that dissociation of endogenously bound cAMP is negligible under these cold-trapping conditions, since dibutyryl cAMP is thought to bind preferentially only to 1 of the 2 cAMP-binding sites on the regulatory subunit (Rannels and Corbin, 1980; Doskeland et al., 1983). Consistent with this explanation, we find that 10 μM dibutyryl cAMP only weakly inhibits photolabeling with ³²P-8-N₃cAMP (unpublished observation), an analog thought to be selective for the other cAMP binding site.

In contrast, when the homogenate from ganglia treated with the drug is incubated at 22°C, the dibutyryl cAMP that had been bound within the cell exchanges with ³H-cAMP that is added in a saturating (excess) concentration: under these conditions no difference in ³H-cAMP binding was observed between the treated and control ganglia (Fig. 4*A*). Incubation at 22°C appears to result in equilibration of the sites with the ³H-cAMP probe, and therefore these conditions can be used to measure the cell's total cAMP-binding capacity.

Since the values of bound cAMP in Table 1 were obtained under the cold-trapping conditions, and those of ³H-cAMP-binding capacity presented in Table 2 were determined at 22°C, these data can be used to calculate the fraction of the cell's cAMP-binding sites that are occupied by cAMP *in vivo*. As

shown in Figure 4*B*, exposure to 5-HT results in increased binding to a similar extent both in sensory cells and total neuronal components: the fraction of sites occupied is raised from approximately 20 to 60% of the total cAMP-binding capacity.

Like the ³H-cAMP probe, the photoactive analog of cAMP, ³²P-8-N₃cAMP, can attach to unoccupied cAMP-binding sites (see Hoyer et al., 1980). Upon irradiation with ultraviolet light, the bound 8-N₃cAMP becomes covalently linked; it therefore can also be used to assay the occupancy of individual kinase regulatory subunits. Eppler et al. (1982) previously used this photoaffinity label in *Aplysia* neural components to identify and characterize 5 distinct cAMP-binding proteins. These proteins are separated into 3 molecular-weight classes by 1-dimensional SDS-PAGE: an M_r 47,000 and an M_r 52,000 class, each consisting of 2 distinct species, and a single M_r 105,000 protein (Eppler et al., 1982, 1986). As in the experiments with dibutyryl cAMP, we expect that, under cold-trapping conditions, endogenously bound cAMP will protect binding sites from subsequent *in vitro* labeling with the photoaffinity reagent and that 8-N₃cAMP will react only with the sites that had been unoccupied in the cell.

In order to examine the extent of occupancy within sensory cells, we stimulated the neurons by the 2 procedures used by Bernier et al. (1982) to study elevations of cAMP. We first examined homogenates of sensory neurons dissected from abdominal ganglia that had been exposed to a saturating concentration of 5-HT. Binding proteins from these cells were labeled less intensely by ³²P-8-N₃cAMP than were those from untreated ganglia. This inhibition of labeling (presumed to reflect enhanced intracellular occupancy of binding sites by the cAMP induced by 5-HT) was similar for each of the 3 molecular-weight classes of binding proteins, the average decrease being 66 ± 9% (*n* = 3). One of these experiments is shown in Figure 5. In another set of experiments, we produced facilitation of sensory neurons by stimulating the connective electrically. Bernier et al. (1982) showed that this treatment, which is presumably more physiologically relevant, elevated cAMP in sensory neurons. In these experiments, we labeled aliquots of the homogenate under cold-trapping and equilibration conditions, and calculated the percentage occupancy of each binding protein as the extent to which its labeling is reduced in extracts maintained at 0°C (cold-trapping) compared to that obtained at 22°C (equilibrium conditions). All sensory cell clusters included in the stimulated group were shown to be physiologically sensitized: the duration of their action potential was increased by more than 35% (see Materials and Methods). An autoradiogram of one of these experiments is shown in Figure 6. Again, the stimulated cells showed significant occupancy of all 3 classes of binding protein (Fig. 6*A*). Labeling in the control ganglia was similar whether extracts were kept at 0 or at 22°C (Fig. 6*B*), indicating that the sites in these unstimulated cells are not occupied with endogenous cAMP.

In 6 experiments, the cAMP-binding sites in stimulated sensory cells showed an average occupany of 34% compared with 17% for control neurons (Fig. 7*A*). Two of the extracts from control ganglia showed high occupancy values, possibly because these particular groups of animals had been partially sensitized by previous experiments; nevertheless, connective shock increased occupancy within each of the groups of animals tested (Fig. 7*B*). Variation in the previous experience of animals is likely to complicate biochemical studies of the closely regulated molecules underlying memory and learning.

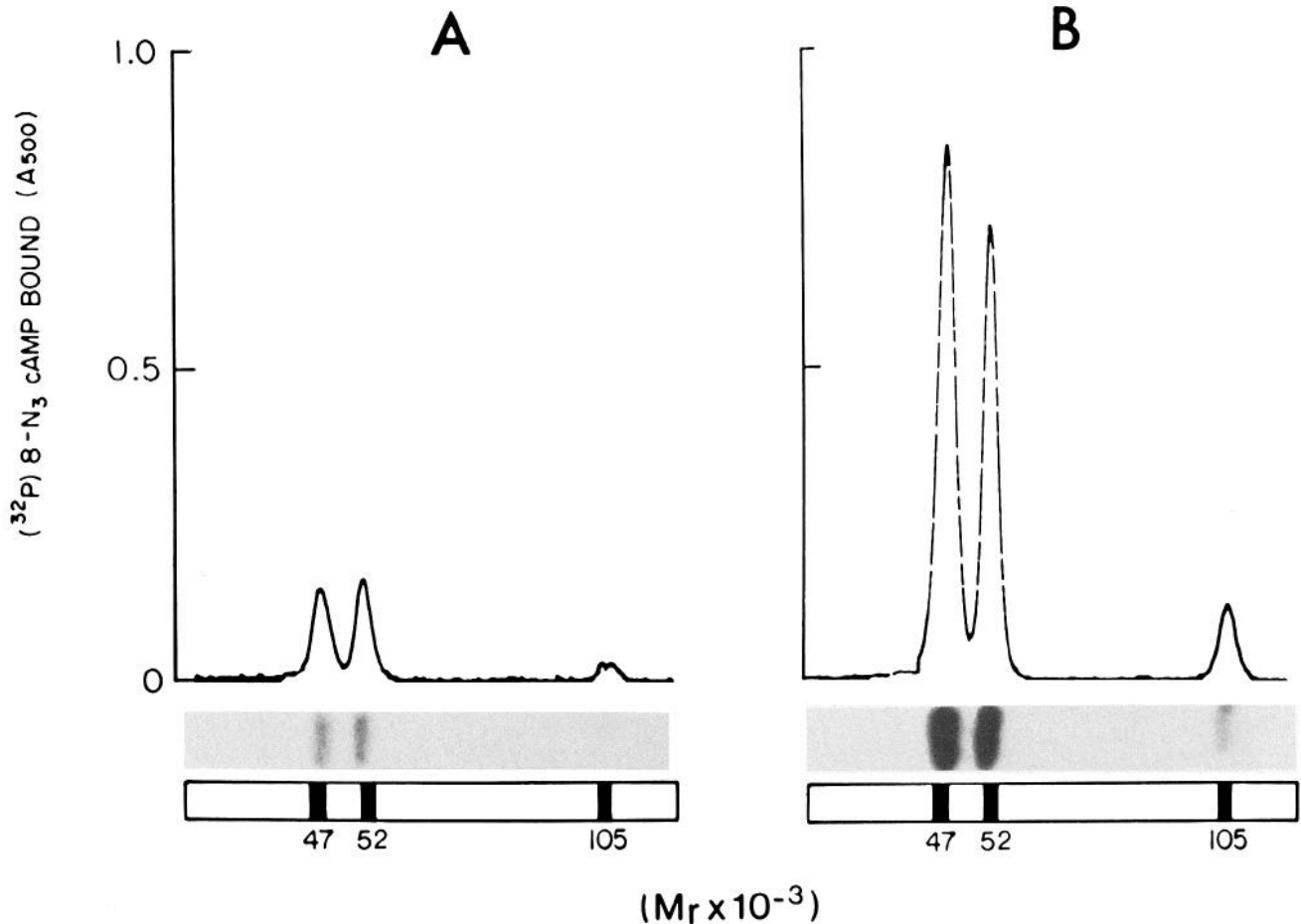The extent of occupancy after the connective was stimulated

Figure 5. Effect of treating abdominal ganglia with 5-HT on subsequent ^{32}P-8-N$_3$cAMP labeling in sensory neurons. The proteins were labeled with ^{32}P-8-N$_3$cAMP at 0°C and separated by electrophoresis on a 6–12% gradient gel. Autoradiographs and densitometric traces of cAMP-binding proteins from the sensory neurons are shown. Identical samples of protein from 3 abdominal sensory neuron clusters were placed in each lane. *A,* Cells treated with 0.2 mM 5-HT. *B,* Control cells.

was similar (31–39%) for each of the 3 molecular-weight classes of cAMP-binding proteins (Fig. 7C). In cells from the control ganglia, however, the occupancy of binding sites varied considerably, ranging from an average of 3% for the M_r 47,000 species to 33% for the M_r 105,000 protein. Stimulation of the connective significantly elevated binding of intracellular cAMP to the M_r 47,000 and 52,000 species but not to the M_r 105,000 protein. Thus, in ganglia from animals that have not been stimulated experimentally (controls), cAMP appears to be bound differentially, with preferential binding to the high-molecular-weight protein; stimulating the connective selectively elevates binding of cAMP to the other proteins.

Discussion

Castellucci et al. (1982) showed that the duration of short-term facilitation in *Aplysia* sensory neurons is determined by a persistently active protein kinase: injection of the specific kinase inhibitor from rabbit muscle abruptly ended the facilitation once the sensitization process had been initiated. Bernier et al. (1982) showed that the content of cAMP in sensory cells, which was initially elevated after a sensitizing stimulus, remained elevated over a period of 15 min and declined with a time course that paralleled the duration of the facilitation. Here we have shown that 85–90% of the cells' cAMP exists in a free (unbound) form both at rest and after stimulation. The elevated amounts of

cAMP in sensory cells are not present in a form protected from degradation, and this implies that the time course of the elevation is determined solely by the balance of adenylate cyclase and phosphodiesterase activities. Evidence that the persistent synthesis of cAMP by an activated cyclase complex produces this time course has been presented previously (Schwartz et al., 1983).

We are interested in cataloging the intracellular properties of the cAMP cascade that determine its role in mediating neuronal plasticity. In unstimulated sensory neurons, we found comparable amounts of cAMP (19.3 pmol/mg, Table 1) and its intracellular binding proteins (12.7 pmol/mg, Table 2). Stimulating treatments increased the cAMP bound to protein in sensory cells 2- to 3-fold according to 2 independent methods of assay: nitrocellulose filter binding (Fig. 4B) and inhibition of photoaffinity labeling (Fig. 7A). Since the elevation of *bound* cAMP revealed by these methods is similar in magnitude to the elevation of *total* cellular cAMP previously found to be induced by these treatments (Bernier et al., 1982), the cAMP-binding proteins within the cell must be operating over a linear portion of their binding curves.

This observation indicates that the regulatory subunits bind cAMP less avidly in the cell than *in vitro*. The concentration of cAMP in unstimulated sensory neurons can only be roughly estimated because of uncertainty in evaluating cell volume, but
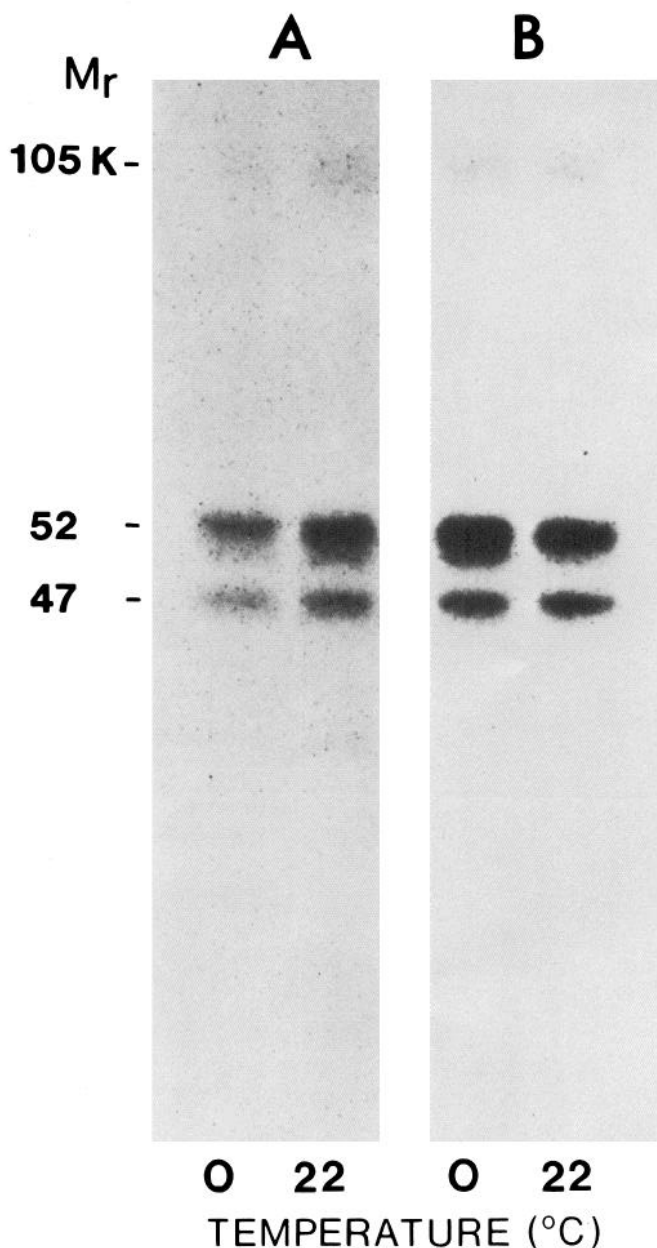
Figure 6. Binding of endogenous cAMP in sensory neurons. Less than 1 min after an electrical shock was delivered to the connective, ganglia were rapidly frozen in 50% propylene glycol. Sensory clusters from 3 stimulated (lanes 1 and 2) or control (lanes 3 and 4) ganglia were dissected and homogenized at 0°C in 5 mM 2-mercaptoethanol, 0.2% saponin, 0.35 M sucrose, 0.17 M NaCl, 3 mM EDTA, 1 mM EGTA, and 20 mM Tris-HCl (pH 7.4). Aliquots were first incubated for 10 min with 0.6 μM ^{32}P-8-N$_3$cAMP either at 0°C (lanes 1 and 3) or at 22°C (lanes 2 and 4). Both samples were then kept for another 10 min at 0°C, followed by a 5 min period of photolabeling with ultraviolet light. (Samples that had been kept at 0°C were subsequently warmed to 22°C for an additional 10 min to compensate for any proteolysis that might have occurred at the higher temperature.) Proteins were separated by gel electrophoresis, stained with the BioRad silver reagent, and autoradiographed for 96 hr at -70°C. Decreased photolabeling at 0°C relative to 22°C represents an increase in binding of endogenous cAMP.

it appears to be in the micromolar range (see Hockberger and Yamane, 1984), a value exceeding the highest *in vitro* binding constant that we measured in neuronal extracts (Fig. 3). Beavo et al. (1974), in discussing the similar values that they found

for the concentration and the binding of cAMP in rabbit muscle, suggested that the effective (*in vivo*) binding constant is higher than the apparent (*in vitro*) constant. On explanation offered was that a significant amount of the total cAMP in the cell is taken up by the binding proteins. The amount of cAMP that is bound at saturation in *Aplysia,* however, does not greatly diminish the content of the free cyclic nucleotide within the cell (compare the values for free and bound cAMP in Table 1). Rather, it would appear that the high concentration of kinase and relatively low concentration of substrate in the cell promote reassociation of the subunits to the holoenzyme with release of cAMP; thus both the effective binding and activation constants are raised under intracellular conditions (see, for example, Granot et al., 1980).

We find that different cells and different subcellular components from the *Aplysia* nervous system vary substantially in their relative amounts of cAMP-binding and cAMP-dependent protein kinase activity. Though it has been reported that untransformed cells contain equimolar amounts of regulatory and catalytic subunits (Corbin et al., 1977; Hofmann et al., 1977), the ratio of the subunits has not, to our knowledge, been determined for the various regions and subcellular components of vertebrate nervous tissues. Walter et al. (1978) found that regions of normal rat brain vary from one another with respect to amounts of R_I and R_{II} regulatory subunits; in addition, transformed cells (Walter et al., 1979; Erlichman et al., 1984), as well as differentiating granulosa cells (Richards et al., 1984), maintain a molar excess of regulatory to catalytic subunit under certain conditions. In *Aplysia* nervous tissue we found that sensory cells had the lowest ratio of binding to kinase activity, while the neuropil had a lower ratio than did the cell bodies. We suggest that a stoichiometric excess of regulatory subunits to catalytic subunits would result in a cell less sensitive to cAMP. At any subsaturating concentration of cAMP, free regulatory subunits would recombine with catalytic subunits to diminish the extent of kinase activation. Although we have not shown this enhanced sensitivity in *Aplysia* sensory neurons directly, Schwechheimer and Hofmann (1977) used the enzyme purified from rabbit skeletal muscle to demonstrate that the presence of greater than stoichiometric amounts of regulatory subunit raises the activation constant of the kinase.

Sensory neurons are capable of mediating a variety of simple forms of learning (both short- and long-term sensitization and habituation, as well as associative, classical, and operant conditioning), and 2 of these forms, sensitization and classical conditioning, involve changes in the cAMP-dependent protein phosphorylation (Kandel and Schwartz, 1982; Hawkins et al., 1985). Are sensory neurons endowed with a special cAMP-dependent kinase to mediate these learning processes? If the lower ratio of regulatory to catalytic activities that we found in sensory cells results in a more sensitive kinase system, we would argue that sensory cells are poised to respond to smaller changes in cAMP. In other cells, where the concentration of regulatory subunits is in excess, relatively large amounts of the second messenger would need to be accumulated to bring about changes in protein phosphorylation.

The properties of the cAMP-binding proteins in sensory cells do not appear to differ from those in other neurons. cAMP is bound within sensory and other neurons to a similar extent (Figs. 2, 4B), and with similar affinities and capacities *in vitro* (Fig. 3, Table 2). Sensory neurons also contain the same set of ^{32}P-N$_3$-cAMP-labeled proteins that can be separated by 2-dimensional
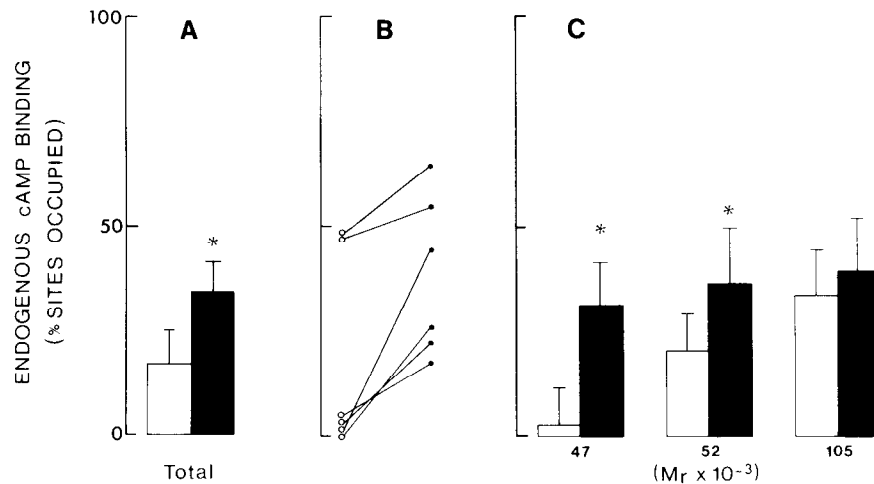
Figure 7. Effect of electrical stimulation on binding of cAMP in sensory neurons. Autoradiographs of ^{32}P-8-N$_3$cAMP-labeled samples were obtained as described in the legend to Figure 6, with exposures sufficiently long to allow for accurate quantitation by scanning. The amount of label in each band was measured as the integrated area of a densitometric scan of the autoradiogram. The ratio of label incorporated in samples maintained at 0°C to that in samples that had previously been incubated at 22°C was subtracted from 100% to produce the percentage occupancy value. *Solid bars* and *circles* represent binding in cells from stimulated ganglia, *open bars* and *circles* from control ganglia. *A,* Total occupancy of all ^{32}P-N$_3$cAMP-labeled proteins in the sample. *B,* Percentage of occupancy in each of the batches of animals tested. The values obtained from stimulated and unstimulated animals from a single batch are connected. *C,* Binding of cAMP by the separate molecular-weight classes of binding proteins. *, $p <$ 0.01; *t* test for paired determinations.

gel electrophoresis as does the rest of the nervous system (unpublished observations). cAMP regulates different K$^+$ channels in several *Aplysia* neurons: the K$_s^+$ channel in sensory neurons (Siegelbaum et al., 1982; Pollock et al., 1985), several K$^+$ channels in the neurosecretory bag cells (Strong and Kaczmarek, 1986), and an anomalously rectifying K$^+$ channel in R15 (Benson and Levitan, 1983). If one or more of the neuron-specific kinases are indeed specialized for modulating ion channels, it is not surprising that they are present in both sensory and other neurons. The learning that sensory cells mediate is only one possible behavioral outcome of the regulation of ion channels and would not necessarily require a unique type of kinase.

The 3 molecular-weight classes of cAMP-binding proteins that are resolved by 1-dimensional gel electrophoresis all bind cAMP after exposure to 5-HT or connective stimulation (Figs. 5, 6). Neither treatment results in total occupancy of all protein binding sites with cAMP. We suspect that the binding falls short of saturation because these treatments do not elevate the intracellular cAMP enough at the times tested. It is also possible that some endogenously bound cAMP exchanges with the ^{32}P-N$_3$cAMP probe, although exchange at 0°C has been reported to have a half-time on the order of days for vertebrate regulatory subunits (Hoyer et al., 1980). Moreover, bound dibutyryl cAMP did not appear to exchange with ^{3}H-cAMP under our conditions of isolation (Fig. 4*A*). In addition, loss of endogenously bound cAMP from *Aplysia* proteins is negligible at 0°C (see Materials and Methods).

A question of physiological importance is whether cAMP-dependent kinases formed from the different regulatory subunits can be selectively activated. It has been shown that certain drugs that elevate cAMP can increase the binding and the kinase activity of only 1 of the 2 vertebrate types of kinase (R$_I$ or R$_{II}$) (Litvin et al., 1984; Chew, 1985) or of the enzyme situated in a particulate rather than in a soluble compartment (Buxton and Brunton, 1983). This selectivity may result from compartmentalization of the newly formed cAMP. We have presented evi-

dence that the elevated cAMP in *Aplysia* sensory neurons that results from stimulating the connective can also be bound selectively: This input raises the occupancy of the proteins in the M$_r$ 47,000 and 52,000 classes but does not affect the occupancy of the M$_r$ 105,000 protein (Fig. 7*C*). Because the M$_r$ 47,000 and 52,000 bands on the gels each contain 2 distinct protein species (Eppler et al., 1982), we could not measure the occupancy of each of the 5 cAMP-binding proteins separately; some additional selectivity thus must be obscured. Our data indicate that the M$_r$ 105,000 species binds substantial amounts of cAMP in the sensory neurons from unstimulated animals but does not bind more cAMP in response to connective shock. The cAMP produced in response to the treatment with a saturating concentration of 5-HT, in contrast, was bound nonselectively by all 3 molecular-weight classes of binding protein (Fig. 5), implying that the 2 treatments differ with respect to their effects on adenylate cyclase.

It is attractive to think that only 1 type of cAMP-dependent kinase is the effector in the sensory neuron terminal for the specific protein modification underlying short-term sensitization. Another task for a distinctive form of regulatory subunit might be to serve as the intracellular messenger for instructing the nucleus of the sensory cell to alter its gene expression. A change in gene expression has frequently been proposed as the mechanism for converting short-term memory to the long-term form (see Kandel and Schwartz, 1982). The enhanced potential for protein phosphorylation postulated to account for long-term sensitization in sensory neurons (Kandel and Schwartz, 1982) might be produced by changes in the cell's population of regulatory subunits. Changes in populations of regulatory subunits have been reported in many cell types (Lohmann and Walter, 1984).

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