0270-6474/85/0501-0151$02.00/0
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The Journal of Neuroscience
Vol. 5, No. 1, pp. 151–159
January 1985

CALCIUM- AND CYCLIC NUCLEOTIDE-DEPENDENT PROTEIN KINASES AND THEIR SUBSTRATES IN THE *APLYSIA* NERVOUS SYSTEM[1]

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Received March 9, 1984; Revised June 11, 1984; Accepted June 15, 1984

Abstract

Homogenates of the *Aplysia* nervous system contain protein kinase activities sensitive to cAMP, cGMP, and Ca^{2+}/calmodulin. The cAMP- and cGMP-dependent activities are either soluble enzymes or are only loosely bound to membranes, since they can be detected only in crude but not in washed membrane fractions, and are present in 20,000 or 100,000 × *g* supernatants prepared from homogenates. In contrast there are both soluble and tightly membrane-bound Ca^{2+}/calmodulin-dependent protein kinase activities. The three activities present in supernatant fractions can be separated by chromatography on DE-cellulose, indicating that they are due to distinct enzyme species. Substrates for these enzymes were analyzed by two-dimensional gel electrophoresis. Protein phosphorylation within the identified *Aplysia* neuron R15 *in vivo* was measured by the intracellular injection of [γ-^{32}P]ATP. cAMP stimulates the phosphorylation of nine proteins and decreases phosphorylation of two proteins in this cell. This *in vivo* pattern was compared with *in vitro* phosphorylation measured in homogenates of whole ganglion. Most of the phosphoproteins affected by cAMP in neuron R15 *in vivo* are also substrates for cAMP-dependent protein kinase *in vitro*. Thus, the *in vitro* system will be a useful tool for detailed biochemical analysis of phosphoproteins which have been identified as being physiologically relevant *in vivo*.

Recent physiological experiments with molluscan neurons have suggested a role for protein phosphorylation in the control of the electrical activity of these cells. It has been shown that intracellular injection of the catalytic subunit of cAMP-dependent protein kinase into several different *Aplysia* neurons increases the duration of the action potential (Kaczmarek et al., 1980; Castellucci et al., 1980). In *Helix* neurons, internal perfusion with the same enzyme increases a specific K^+ conductance (DePeyer et al., 1982). Furthermore the intracellular injection of a specific inhibitor of cAMP-dependent protein kinase blocks the effect of serotonin on another K^+ conductance in *Aplysia* neuron R15 (Adams and Levitan, 1982).

Most *in vivo* biochemical studies of protein phosphorylation in *Aplysia* have involved labeling of ganglia or bag cells with $^{32}P_i$ (Levitan and Barondes, 1974; Levitan et al., 1974; Paris et al., 1981; Jennings et al., 1982). More recently it has become possible to examine phosphorylation in single cells *in vivo* following the intracellular injection of [γ-^{32}P]ATP (Lemos et al., 1982, 1984). Among the advantages of this technique is the possibility of monitoring the cell's physiological properties during the labeling period, and directly relating changes in specific phosphoproteins to changes in membrane conductance. Using this method we have described several proteins in the identified neuron R15 whose phosphorylation state is affected by serotonin, concomitant with the serotonin-induced increase in K^+ conductance in this cell (Lemos et al., 1982).

Although this *in vivo* methodology is important for the identification of phosphoproteins which may be involved in the regulation of neuronal membrane properties, the small amounts of material available and the difficulty of the experiments preclude a detailed biochemical analysis of these phosphoproteins and their protein kinases *in vivo*, and for this purpose *in vitro* techniques are more appropriate. cAMP-dependent protein phosphorylation has been described in *Aplysia* nervous system extracts (Jennings et al., 1982; Levitan and Norman, 1980), and effects of cGMP on protein phosphorylation in homogenates (Levitan and Norman, 1980) and nerve root membranes (Ram and Ehrlich, 1978) have been published. In addition substrates for an endogenous membrane-associated Ca^{2+}/calmodulin-dependent protein kinase have been described in *Aplysia* ganglia (Novak-Hofer and Levitan, 1983; DeRiemer et al., 1984). In this report, *Aplysia* nervous system protein kinases and their substrates are studied in more detail. We show that a cGMP-dependent protein kinase can be separated from cAMP-dependent protein kinase by ion exchange chromatography. Furthermore, in addition to the membrane-bound Ca^{2+}/

[1] This work was supported in part by Grant NS17910 to I. B. L. from the National Institute of Neurological and Communicative Disorders and Stroke. We are grateful to M. Ackermann for photography, and to Drs. J. Martin-Perez, G. Thomas, and A. Wilks for critically reading the manuscript.

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calmodulin-dependent protein kinase described previously (Novak-Hofer and Levitan, 1983), there also exists a soluble Ca^{2+}/calmodulin-dependent enzyme. Finally, the membrane-bound and soluble phosphoproteins that are regulated by cAMP and Ca^{2+} *in vitro* are analyzed by two-dimensional gel electrophoresis, and are compared with phosphoproteins affected by cAMP within neuron R15 *in vivo*.

Materials and Methods

Materials

Adenosine 5′-[γ-^{32}P]triphosphate (>5000 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, U. K. Catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart according to Demaille et al. (1977). Calmodulin purified from bovine brain according to Dedman et al. (1977) was a gift from Dr. J. Norman (Ciba-Geigy Inc., Ardsley, NY). Regulatory subunit (type II) of cAMP-dependent protein kinase was generously provided by Dr. E. Fischer (University of Washington).

Marine snails, *Aplysia californica*, were maintained in artificial sea water as described previously (Levitan and Norman, 1980). Cerebral, pleural, and pedal ganglia were removed and kept in *Aplysia* medium at 4°C for 1 to 7 days prior to homogenization. Electrophysiological recordings indicate that the *Aplysia* nervous system survives well *in vitro* over this time period, and in addition the phosphoprotein pattern is not changed as compared with freshly dissected ganglia (not shown). Ganglia were incubated in fresh medium for 60 min at 20°C prior to homogenization.

Separation of protein kinases from Aplysia nervous system extracts

Separation of cAMP- and cGMP-dependent protein kinase. Aplysia ganglia were quickly rinsed in 10 mM EGTA, pH 7.0, and then in the homogenization buffer (20 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.0). They were then homogenized in this buffer with five strokes of a Teflon-glass grinder (1.5-ml volume), the connective tissue was removed, and the extract was further homogenized with five additional strokes. All operations were performed at 4°C. The homogenate was centrifuged for 20 min at 20,000 × *g*. The supernatant was then dialyzed overnight against 25 mM K_2HPO_4, pH 7.5, containing 1 mM EDTA, 50 mM β-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (KEM buffer). Protein concentrations were determined with the Bradford method (Bradford, 1976) using bovine serum albumin as the standard, and the protein concentration was adjusted to 1 mg/ml. The separation of the protein kinases by DE-cellulose chromatography was essentially as described by Bandle and Guidotti (1980). A DE52-cellulose column (1-ml volume) was equilibrated with KEM buffer and 3 mg of protein was loaded on the column. It was then washed with KEM buffer and eluted with a gradient of 0 to 0.3 M NaCl in KEM. cGMP-dependent protein kinase elutes in the wash without salt, whereas the cAMP-dependent protein kinase elutes with 0.125 M NaCl. The fractions were kept on ice and assayed for protein kinase activity within the next 2 days. They could be kept at 4°C for up to 1 week without loss of activity.

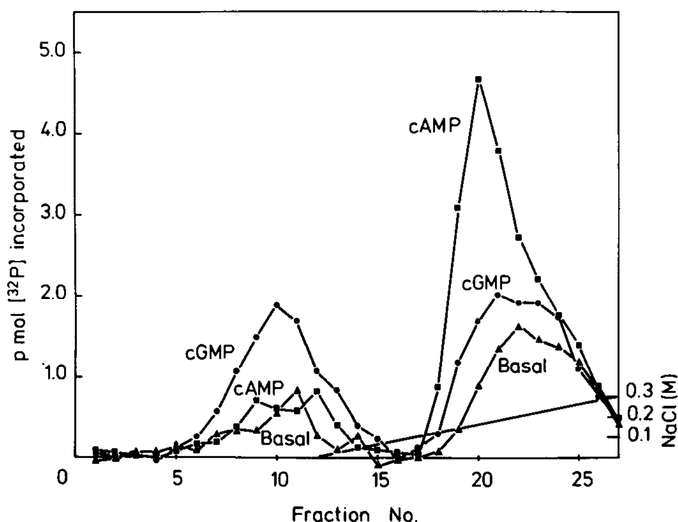


Figure 1. DE-cellulose chromatography of 20,000 × *g* supernatant from the *Aplysia* nervous system: separation of cAMP-dependent and cGMP-dependent protein kinase. Elution was with a NaCl gradient ranging from 0 to 0.3 M NaCl in KEM buffer. Protein kinase activity (expressed as picomoles of phosphate incorporated per assay) was measured as described under "Materials and Methods" with either 0.1 mM IBMX, 2.5 mM EGTA (▲), IBMX + EGTA + 0.7 μM cAMP (■), or IBMX + EGTA + 0.7 μM cGMP (●).

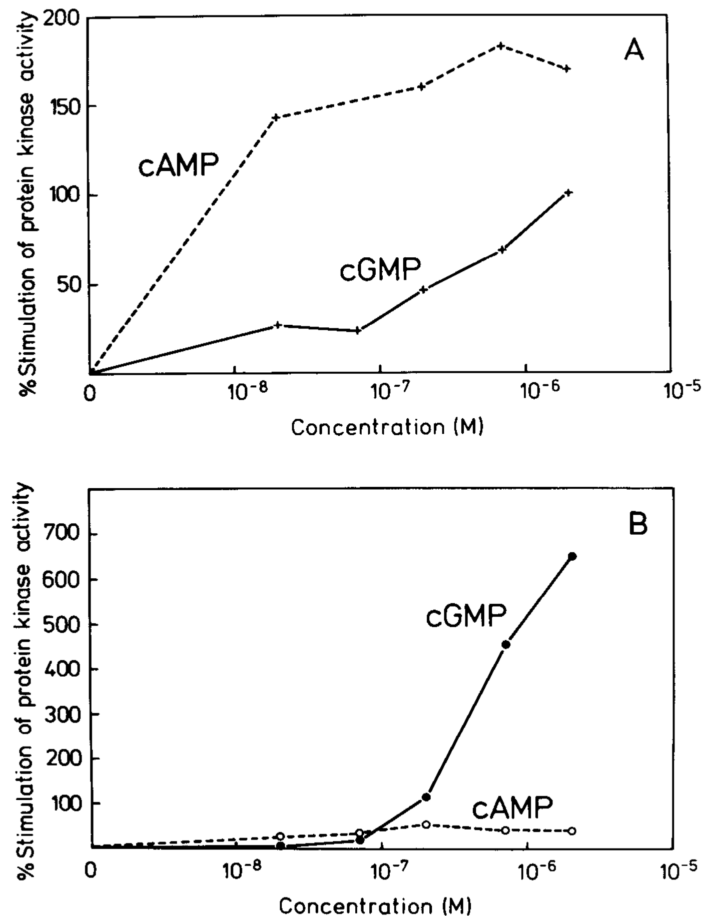


Figure 2. Concentration dependence of cAMP-dependent protein kinase (*A*) and cGMP-dependent protein kinase (*B*) activities on cAMP and cGMP. The two enzyme activities were separated on a DE-cellulose column and assayed with different concentrations of cyclic nucleotides in the presence of 0.1 mM IBMX as described under "Materials and Methods." Enzyme activity is expressed as per cent stimulation above the basal levels (0.47 pmol of phosphate incorporated/assay in *A* and 0.16 pmol of phosphate incorporated in *B*).

Protein kinase activity was measured with a filter assay as described by Levitan and Norman (1980). The reaction mixture contained in a final volume of 100 μl: 70 mM Mg acetate, 140 mM Na acetate, pH 7.3, 0.1 mM isobutylmethylxanthine (IBMX), 1 mM dithiothreitol 7 μM ATP, 20 μg of histone 2A (Sigma), 0.7 μM cAMP or cGMP, 0.25 μCi of [γ-^{32}P]ATP (7 × 10^5 cpm/nmol). These conditions are optimal for the assay of cGMP-dependent protein kinase (Bandle and Guidotti, 1980). The reaction was started by adding 30 μl of the column fractions and was continued for 10 min at 30°C. It was stopped by the addition of 1 ml of 20% trichloroacetic acid.

Separation of cAMP and Ca^{2+}-dependent protein kinases. Aplysia nervous system homogenates were prepared as described in the previous section. They were centrifuged for 60 min at 100,000 × *g* and the supernatants were dialyzed overnight against 20 mM MOPS, pH 7.3, containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF (MOPS/Ca^{2+} buffer). Because it was necessary to dialyze against this

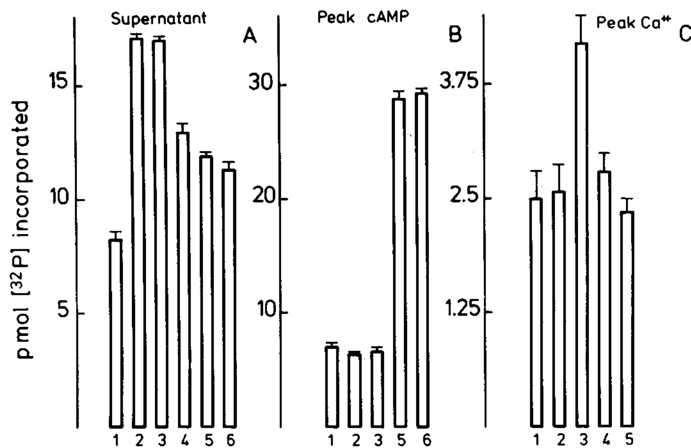


Figure 3. Influence of cAMP, Ca^{2+}, calmodulin, and *t*-flupenthixol (FP) on the protein kinase activity of 100,000 × *g* supernatant fractions (*A*), the cAMP-dependent protein kinase (*B*), and the Ca^{2+}-dependent protein kinase (*C*). The protein kinases were separated by DE-cellulose chromatography and their activity was measured with the following additions to the standard incubation mixture: *1*, 2.5 mM EGTA; *2*, 3 μM free Ca^{2+} (100 μM EGTA, 89 μM $CaCl_2$); *3*, Ca^{2+} + 156 nM calmodulin; *4*, Ca^{2+} + calmodulin + 50 μM FP; *5*, EGTA + 0.7 μM cAMP; *6*, EGTA + cAMP + FP.

buffer for optimal assay and recovery of the Ca^{2+}/calmodulin-dependent protein kinase, it was not possible to separate all three enzyme activities on a single ion exchange column. The dialyzed supernatant (2 to 4 mg of protein) was then applied to a DE52-cellulose column (0.5-ml volume) equilibrated with MOPS/Ca^{2+} buffer. The column was eluted stepwise with 0.1, 0.15, 0.2, 0.25, 0.3, 0.5, and 1.0 M NaCl in MOPS/Ca^{2+} buffer. Under these conditions the cAMP-dependent activity elutes in the 0.1 M NaCl fraction and the Ca^{2+}-dependent activity elutes with 0.5 M NaCl. The fractions were kept on ice and assayed for protein kinase activity within 24 hr. Activity was assayed as described in the previous section using a reaction mixture containing in a final volume of 100 μl: 50 mM MOPS, pH 7.3, 10 mM $MgCl_2$, 1 mM DTT, 25 μM ATP, 20 μg of histone 2A, and 0.25 μCi of [γ-^{32}P]ATP. EGTA, Ca^{2+}, calmodulin, and flupenthixol were added to this mixture as indicated in the figure legends.

Preparation of membrane-bound Ca^{2+}/calmodulin-dependent protein kinase. EGTA-washed membrane fractions were prepared as described previously (Novak-Hofer and Levitan, 1983).

Measurement of Aplysia calmodulin. High speed (100,000 × *g*) supernatants or DE52-cellulose column fractions, prepared as previously described, were placed in a boiling water bath for 3 min and then centrifuged at 12,000 × *g* for 15 min to remove precipitated material. The 12,000 × *g* supernatants were dialyzed against H_2O, and were tested for their ability to stimulate the activity of a calmodulin-free cAMP phosphodiesterase partially purified from beef heart according to Ho et al. (1976). Phosphodiesterase activity was measured by a modification (Levitan and Norman, 1980) of the Thompson and Appleman (1977) procedure.

Phosphorylation of endogenous proteins from Aplysia nervous system extracts

Ganglia were homogenized and homogenates or 20,000 × *g* supernatants were prepared as described above; EGTA-washed membranes were prepared as described before (Novak-Hofer and Levitan, 1983). For cAMP- and cGMP-dependent phosphorylation, fractions were dialyzed against KEM buffer, whereas for Ca^{2+}-dependent phosphorylation, dialysis was against MOPS/Ca^{2+} buffer. The net incorporation of phosphate from [γ-^{32}P]ATP into proteins was measured at 30°C in 200 μl of a mixture containing 50 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 1 mM DTT, 30 μM ATP, and 5 μCi of [γ-^{32}P]ATP (1.5 × 10^6 cpm/nmol). Additions to the assay mixture are indicated in the legends to the figures. After 15-sec preincubation, the reaction was started by the addition of 100 to 200 μg of protein from supernatant or membrane fractions. After 5 min at 30°C, the reaction was stopped by solubilizing the proteins with 20 μl of 20% SDS and 50% β-mercaptoethanol. Then 50 μl of a solution containing 10% Ampholines, pH 2 to 11, 40% Nonidet P-40 (NP40), 9.5 M urea was added, together with 110 mg of solid urea (Ames-Ferroluzi and Nikaido, 1976). The dissolved protein mixture was kept for 2 min at 40°C and was then either loaded directly on an isoelectric focusing gel, or was kept at −70°C for later analysis.

Injection of [γ-^{32}P]ATP in Aplysia neuron R15

Intracellular injection of [γ-^{32}P]ATP was performed as described by Lemos et al. (1982). Beginning 30 min after the injection, 7 × 10^{-4} M 8-benzylthio-cAMP (8-BT-cAMP) was perfused over the ganglion for 20 min. The ganglia were quick-frozen with liquid N_2 and were homogenized in 50 μl of a SDS sample buffer consisting of 0.08 M Tris, pH 6.8, 1% glycerol, 2% SDS and 15% β-mercaptoethanol. Afterwards, 100 μl of a solution containing 10% NP40, 4% Ampholines, pH 2 to 11, and 9.5 M urea, together with 55 mg of solid urea, was added. The samples were kept 2 min at 40°C and were then stored at −20°C before they were applied to the isoelectric focusing gel.

Two-dimensional gel electrophoresis and autoradiography

The first dimension (isoelectric focusing) was performed in 11.0-cm long and 0.25-cm wide glass tubes essentially as described by O'Farrell (1975). Sample (150 μl; about 100 μg of protein) was loaded on the cathode side and isoelectric focusing was performed for about 15 hr at 300 V. The pH gradient was linear from pH 7 to 4. Afterward, the gels were equilibrated with a buffer containing 2% SDS, 5% β-mercaptoethanol in 0.06 M Tris, pH 6.8, for 20 min at 40°C. Then they were either loaded directly on the second dimension gel or were stored at −70°C. In order to determine apparent P_i values for the various proteins listed in Table 1, the isoelectric focusing gel was cut into 5-mm pieces, the pieces were eluted for 60 min with distilled water, and the pH of the eluate was measured.

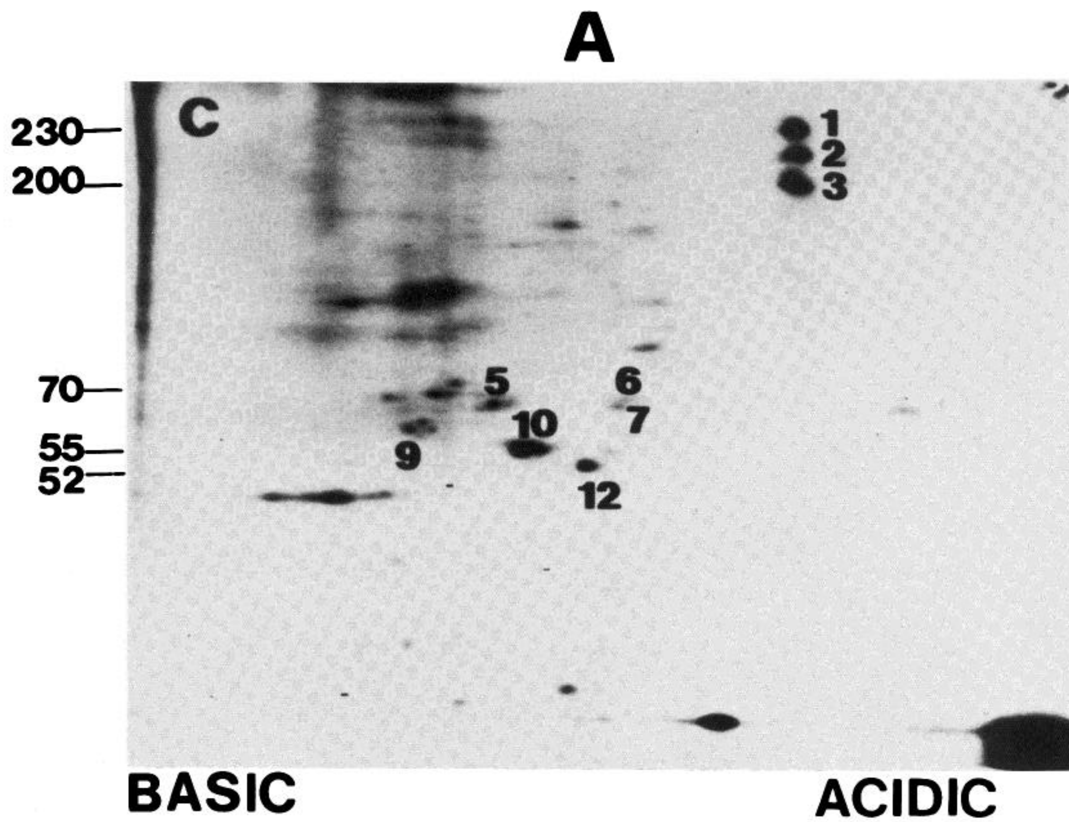
The second dimension (SDS electrophoresis) was performed in slab gels (11.0 cm long, 18.0 cm wide) with a separating gel consisting of 10% acrylamide, 0.13% bisacrylamide, and 0.1% SDS in 0.38 M Tris-HCl, pH 8.7, and a stacking gel consisting of 5% acrylamide, 0.13% bisacrylamide, and 0.1% SDS in 0.13 M Tris-HCl, pH 6.8. The running buffer was 0.025 M Tris-HCl, pH 8.3, containing 0.1% SDS and 0.19 M glycine. Electrophoresis was performed for about 7 hr at 100 V. After electrophoresis, the gels were stained and destained as described (Novak-Hofer and Levitan, 1983) and then were dried and exposed to Kodak X-Omat x-ray film using Kodak regular intensifying screens.

Results

Separation of cAMP-, cGMP-, and Ca^{2+}-dependent protein kinase activities from extracts of the Aplysia nervous system

It has been shown that cAMP and cGMP derivatives stimulate protein kinase activity in homogenates from *Aplysia* ganglia (Levitan and Norman, 1980). In order to determine whether the two nucleotides are activating the same or different protein kinases, soluble extracts (20,000 or 100,000 × *g* supernatants) were subjected to DE-cellulose chromatography and the fractions were assayed for protein kinase activity. Figure 1 shows the elution pattern of such a column, with a cGMP-dependent protein kinase activity eluting in the wash with KEM buffer, whereas a cAMP-dependent protein kinase activity elutes with 0.125 M NaCl. To determine the cyclic nucleotide specificity of the protein kinase activities, the two peaks were tested with different concentrations of cAMP and cGMP (Fig. 2). The cAMP-dependent protein kinase shows some stimulation by cGMP concentrations above 10^{-7} M (Fig. 2*A*), whereas the cGMP-dependent protein kinase is not stimulated by cAMP concentrations as high as 2 × 10^{-6} M (Fig. 2*B*). The cAMP-dependent enzyme shows maximal stimulation with about 20 nM cAMP, whereas the cGMP-dependent protein kinase activity has a lower affinity for cGMP (K_a about 1 μM) and maximal stimulation at about 60 μM cGMP. Both the cAMP- and cGMP-dependent activities were stable for at least 1 week at 4°C.

To confirm that the two activities represent two distinct enzymes, we tested the effects of the regulatory subunit of cAMP-dependent protein kinase added to the assay. The reg-



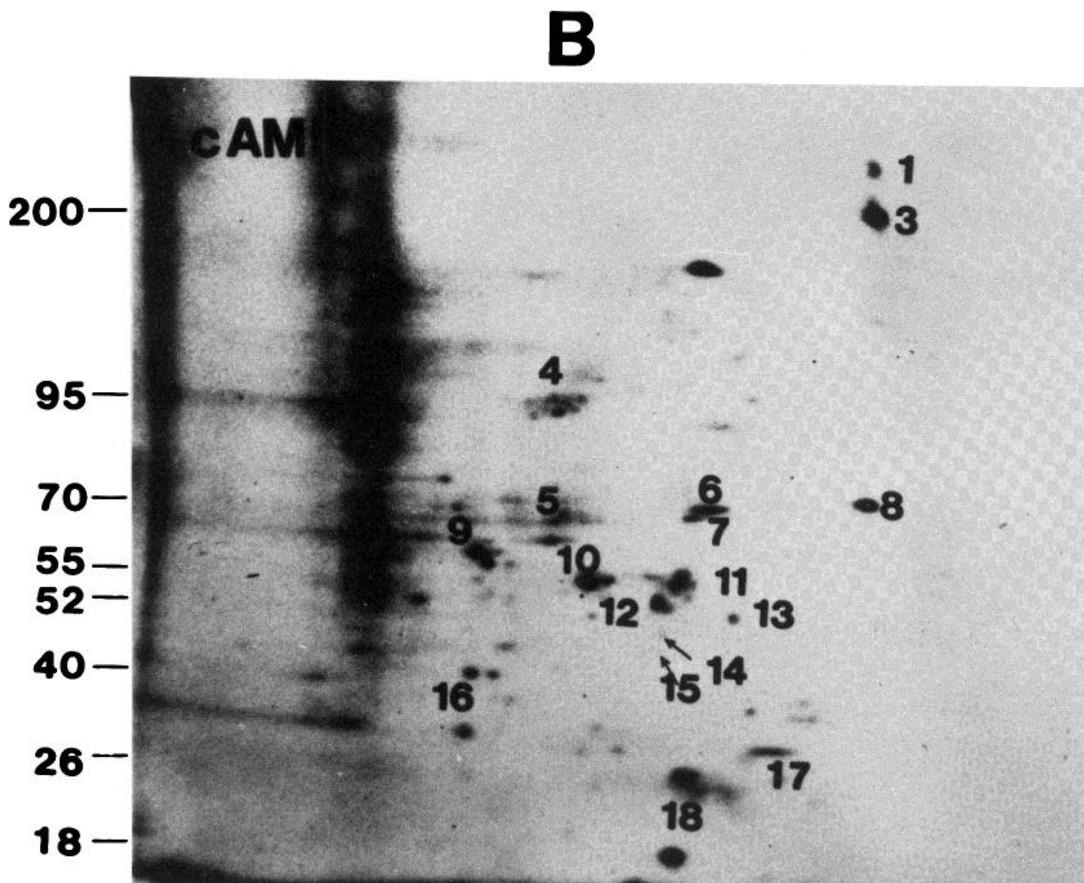


Figure 4. Effect of 8-BT-cAMP on *in vivo* phosphorylation in *Aplysia* neuron R15. Autoradiographs of two-dimensional separations of phosphoproteins. Neuron R15 was injected with [γ-^{32}P]ATP; after 30 min, the cell was perfused for an additional 20 min with either normal *Aplysia* medium (*A*), or medium containing 7.10^{-4} *M* 8-BT-cAMP (*B*). The molecular weights $\times 10^{-3}$ of standard proteins are listed at the *left side* of the gels. Phosphoproteins are designated by *numbers* (see Table I).

TABLE I

Substrates for cAMP-dependent protein kinase in vivo in Aplysia neuron R15

Comparison with the stimulation of *in vitro* phosphorylation by cAMP in homogenates from the *Aplysia* nervous system. An upward arrow represents an increase in phosphorylation. See Figures 4 to 6 for the numbering of the phosphoproteins. Data are from three *in vivo* and seven *in vitro* experiments.

Protein	Apparent M_r $\times 10^{-3}$	Apparent P_i	Effect of 8-BT-cAMP in Neuron R15 *in vivo*	Stimulation of Phosphorylation by cAMP *in vitro*
6	70	5.8	↑	↑
7	68	5.9	↑	↑
8	70	4.7	↑	↑
9	60	6.7	↑	
11	55	5.9	↑	↑
13	50	5.6	↑	
16	40	6.7	↑	
17	29	5.5	↑	↑
18	26	5.9	↑	↑

ulatory subunit had little effect on the basal activity of either enzyme, nor did it prevent the stimulation of the cGMP-dependent activity by cGMP. In contrast, it did inhibit the stimulation of the cAMP-dependent protein kinase by cAMP (data not shown).

EGTA-washed membranes from *Aplysia* ganglia contain an endogenous Ca^{2+}/calmodulin-dependent protein kinase (Novak-Hofer and Levitan, 1983), and it was of interest to determine whether a soluble Ca^{2+}-dependent protein kinase is also present as has been reported to be the case in brain (Schulman and Greengard, 1978; Kennedy and Greengard, 1981). When soluble fractions (20,000 or 100,000 × *g* supernatants) are subjected to DE-cellulose chromatography, a Ca^{2+}-dependent protein kinase eluting at 0.5 M NaCl can be separated from the cAMP-dependent protein kinase. A similar ion exchange column was used as the initial step in the purification of a soluble Ca^{2+}/calmodulin-dependent protein kinase from rat brain (Bennett et al., 1983). To investigate the dependence of the enzyme activities on calmodulin, we examined the effects of Ca^{2+}, calmodulin, and the calmodulin-binding drug *t*-flupenthixol on the protein kinase activity of soluble fractions and of the cAMP-dependent and Ca^{2+}-dependent protein kinase peaks after DE-cellulose chromatography. Both Ca^{2+} and cAMP stimulate the protein kinase activity in soluble fractions (Fig. 3*A*, *2* 0.6r3,and *5*). Fifty μM flupenthixol inhibits the Ca^{2+}-dependent activity by about 50% (Fig. 3*A*, *4*), and does not affect cAMP-dependent activity (Fig. 3*A*, *6*). If calmodulin is added in addition to Ca^{2+}, no further stimulation is seen (Fig. 3*A*, *3*), suggesting the presence of endogenous calmodulin in this crude preparation. This was further tested by examining the effects of boïled *Aplysia* extracts on a partially purified cAMP phosphodiesterase from beef heart. The results (not shown) indicate that *Aplysia* does contain a soluble heat-stable factor which can stimulate phosphodiesterase activity in the presence of Ca^{2+}, and which is inhibited by flupenthixol. If this factor is indeed calmodulin, its concentration in *Aplysia* ganglia is in the range 0.5 to 2.5 μM, comparable to that in other species. Saitoh and Schwartz (1983) have recently described the purification of calmodulin from the *Aplysia* nervous system.

Figure 3*B* shows that Ca^{2+} (*2*) and Ca^{2+}/calmodulin (*3*) do not affect the activity of the cAMP-dependent protein kinase peak, whereas cAMP (*5*) stimulates its activity. Fig. 3*C* shows activities of the Ca^{2+}-dependent protein kinase peak. In contrast to the cAMP- and cGMP-dependent protein kinases, the Ca^{2+}-dependent enzyme is quite unstable; there are activity losses during chromatography and the activity cannot be stored longer than 24 hr after chromatography. Overall activity is about 10 times lower than in the cAMP-dependent peak (note the different scales in 3*B* and 3*C*), and cAMP has no effect on the activity of this protein kinase (*5*). Ca^{2+} alone (*2*) does not stimulate the enzyme, whereas Ca^{2+} plus calmodulin (*3*) stimulates about 2-fold. This stimulation is completely blocked by 50 μM flupenthixol (*4*). These findings indicate that the Ca^{2+}/calmodulin-dependent protein kinase has been separated from endogenous calmodulin by the ion exchange chromatography. In fact no calmodulin-like activity, measured in the phosphodiesterase assay, could be detected in the 0.5 M NaCl fractions containing the Ca^{2+}/calmodulin-dependent protein kinase (data not shown).

Endogenous substrates for in vivo and in vitro protein phosphorylation

Protein phosphorylation can be measured within individual identified *Aplysia* neurons *in vivo* following the intracellular injection of [γ-^{32}P]ATP (Lemos et al., 1982). Although this technique is a powerful method for identifying phosphoproteins which are involved in the regulation of neuronal activity, detailed characterization of such phosphoproteins will have to be carried out *in vitro*. Accordingly, we have compared phosphoproteins which are affected by cAMP within the *Aplysia* neuron R15 *in vivo* with substrates for cAMP- and Ca^{2+}/calmodulin-dependent protein kinases *in vitro*.

Phosphoproteins affected by cAMP within neuron R15 in vivo. Figure 4*A* shows basal protein phosphorylation after injection of [γ-^{32}P]ATP into neuron R15. A large number of phosphoproteins can be detected after 50 min of labeling. When 7.10^{-4} M 8-BT-cAMP is present during the last 20 min of the labeling period, the phosphoprotein pattern is changed, the phosphorylation of some proteins being stimulated while others (proteins 1 and 2) are inhibited (Fig. 4*B*). Table I lists nine proteins that are *in vivo* substrates for cAMP-dependent protein kinase in this cell, ranging in isoelectric points from 4.7 to 6.7 and with M_r values between 230,000 and 26,000. Many of these substrates are also affected *in vivo* by application of neurotransmitters or synaptic stimulation of neuron R15 (Lemos et al., 1982; 1984). Proteins 8 and 17 are particularly interesting, because they correspond to the serotonin-dependent *in vivo* phosphoproteins S70 and S29, which are associated with serotonin activation of a specific potassium channel in neuron R15 (Lemos et al., 1984).

Substrates for cAMP-dependent protein kinase in vitro in homogenates from the Aplysia nervous system. The effects of cAMP on protein phosphorylation in homogenates were studied in preparations that were dialyzed against KEM buffer as described in "Materials and Methods," and the phosphoproteins were separated by two-dimensional gel electrophoresis to allow direct comparison with the phosphoproteins detected in neuron R15 *in vivo*. Dialysis was necessary in order to obtain stimulation by cyclic nucleotides, and the degree of stimulation was nevertheless somewhat variable. Only effects that were seen consistently are included in Table I. Basal phosphorylation in such homogenates (Fig. 5*A*) shows many of the major *in vivo* phosphoproteins such as 1, 2, 3, 5, 10, and 12; however, other spots are present that do not correspond to *in vivo* substrates.

The effect of cAMP on protein phosphorylation in homogenates (Fig. 5*B*) was compared with its effect on phosphoproteins in neuron R15 (Fig. 4*B*). This comparison shows that a number of proteins are substrates for cAMP-dependent protein kinase both *in vivo* and *in vitro* (proteins 6, 7, 8, 11, 17, and 18). In addition, the inhibition of phosphorylation of protein 2 is also observed both *in vivo* and *in vitro* (compare Figs. 4*B* and 5*B*). These results indicate that it will be possible to further characterize a number of *in vivo* targets of cAMP-dependent

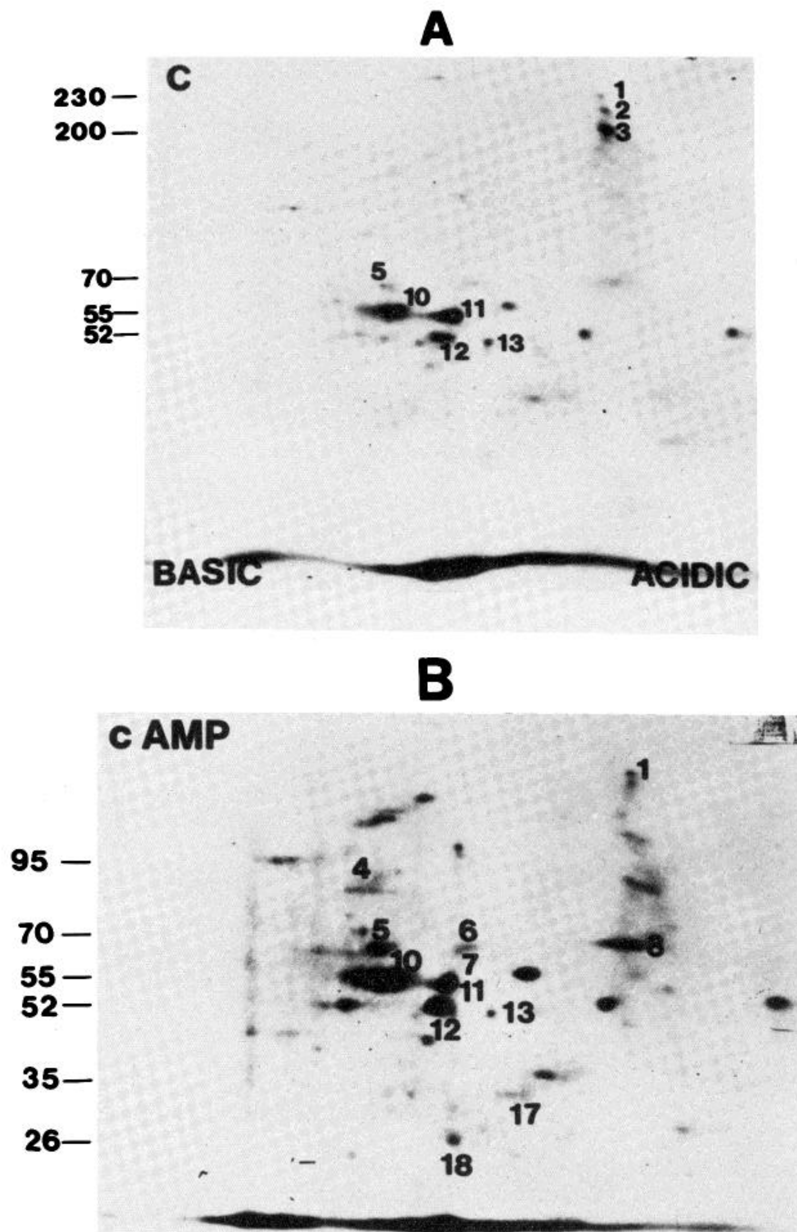


Figure 5. Effects of cAMP on endogenous phosphorylation in KEM-dialyzed homogenates: autoradiographs of two-dimensional gel electrophoretic separations of phosphoproteins. The phosphorylation assay was performed with 200 μg of protein as described under "Materials and Methods." Additions to the standard incubation mixture were 2.5 mM EGTA in *A* and EGTA + 10 μM cAMP in *B*. Samples were analyzed by two-dimensional gel electrophoresis as described under "Materials and Methods." The *numbers* on the *left side* of the gels are the apparent molecular weights × 10^{-3} of standard proteins.

protein kinase *in vitro* in homogenates or subcellular fractions. cGMP-dependent protein phosphorylation in homogenates is more restricted than cAMP-dependent protein phosphorylation. Fewer proteins are affected by cGMP, and the phosphorylation of all of them is also changed by cAMP (data not shown).

Substrates for Ca^{2+}/calmodulin-dependent protein kinases in vitro in Aplysia homogenates and subcellular fractions. We have previously described an endogenous Ca^{2+}/calmodulin-dependent protein kinase in membranes from the *Aplysia* nervous system (Novak-Hofer and Levitan, 1983). Some of its substrates are also substrates for cAMP-dependent protein kinase. Figure 6 shows effects of Ca^{2+} on protein phosphorylation in homogenates (*A* and *B*) and soluble fractions (*C* and *D*), as well as effects of Ca^{2+}/calmodulin on membrane protein phosphorylation (*E* and *F*). As can be seen by comparing Figs. 4 and 6, many phosphoproteins present in R15 can be substrates for Ca^{2+}/calmodulin-dependent protein phosphorylation *in vitro*. Ca^{2+}- and cAMP-dependent protein phosphorylation often overlap *in vitro* as well (Figs. 5 and 6). There are however some substrates specific for Ca^{2+}-dependent protein kinases such as proteins 9, 13, 14, 15, and 16.

Discussion

In the *Aplysia* nervous system, biochemical studies of protein kinases have focused on the cAMP-dependent enzyme (Levitan and Norman, 1980; Jennings et al., 1982; Eppler et al., 1982; Novak-Hofer and Levitan, 1983) since physiological experiments have suggested a role for cAMP-dependent protein phosphorylation in the regulation of certain ion channels (Kaczmarek et al., 1980; Castellucci et al., 1980; DePeyer et al., 1982; Adams and Levitan, 1982). In addition, *Aplysia* ganglia contain protein kinase activity sensitive to cGMP (Levitan and Norman, 1980) and to Ca^{2+}/calmodulin (Novak-Hofer and Levitan, 1983; DeRiemer et al., 1984). We show here that the kinase activities stimulated by cAMP, cGMP, and Ca^{2+}/calmodulin can be separated by ion exchange chromatography, and thus are due to distinct enzyme species. The stimulation of the cAMP-dependent enzyme by high concentrations of cGMP (Fig. 2*A*) has also been observed in other systems (Kuo and

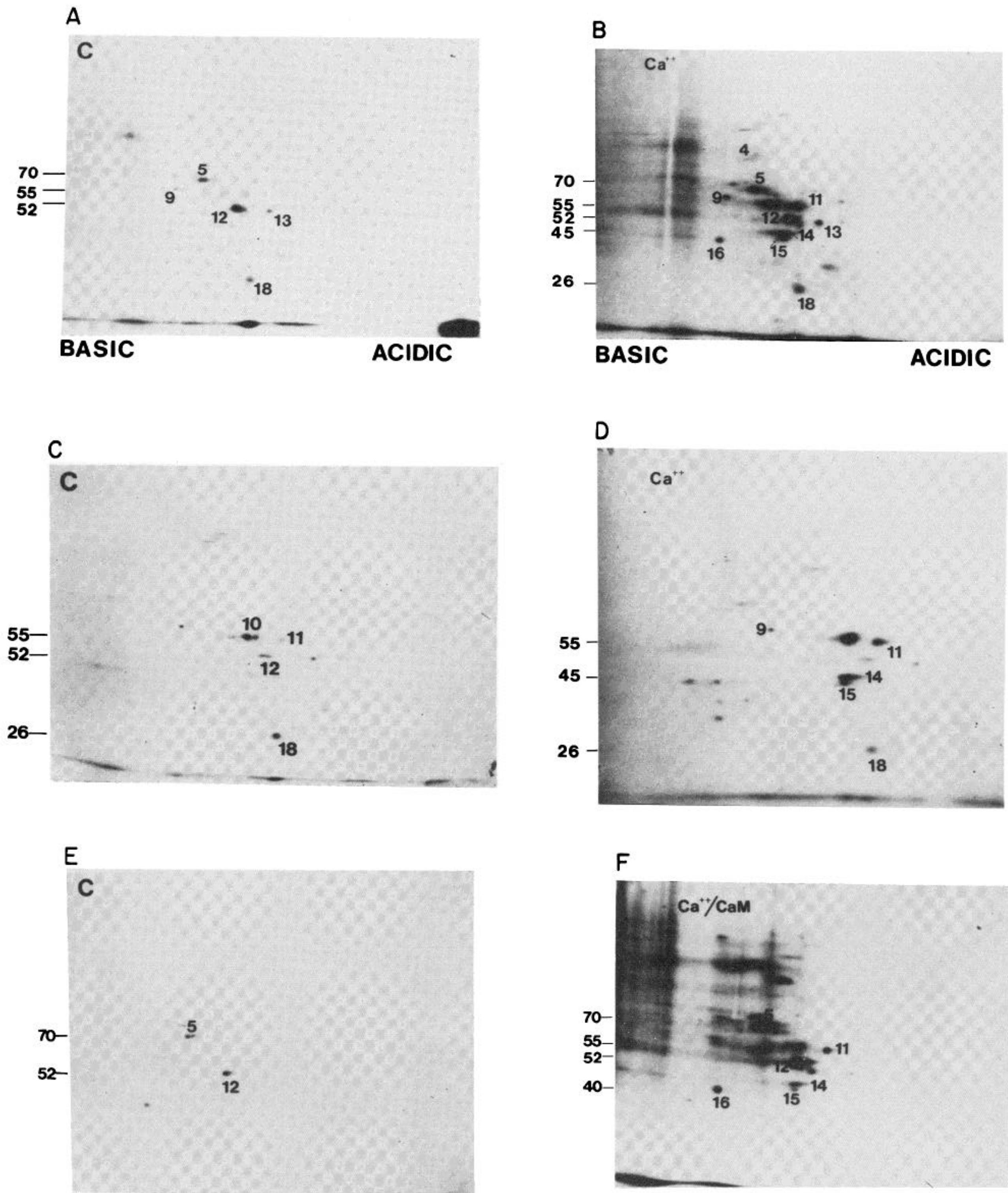


Figure 6. Effects of Ca^{2+}/calmodulin on protein phosphorylation in homogenates (*A* and *B*), soluble fractions (*C* and *D*), and EGTA-washed membranes (*E* and *F*): autoradiographs of two-dimensional separations of phosphoproteins. One hundred μg of protein was phosphorylated with the following additions to the standard incubation mixture: *A*, *C* and *E*, 2.5 mM EGTA (controls); *B* and *D*, 3 μM free Ca^{2+} (100 μM EGTA, 89 μM $CaCl_2$); *F*, 3 μM free Ca^{2+} + 156 nM calmodulin. Samples were analyzed by two-dimensional gel electrophoresis. The molecular weights × 10^{-3} of standard proteins are listed at the *left side* of the gels.

Greengard, 1974). On the other hand, the selective inhibition of the cAMP-dependent activity by the regulatory subunit of cAMP-dependent protein kinase argues strongly that it is distinct from the cGMP-dependent activity.

The subcellular distribution of the cAMP-dependent kinase activity is somewhat surprising. In contrast to the situation in vertebrate brain (Walter and Greengard, 1981), the activity is either soluble or is only loosely bound to membranes. A similar finding has been reported for the nervous system of the land snail *Helix* (Bandle and Levitan, 1977). Although it is possible

that our homogenization conditions lead to solubilization of an enzyme which is normally membrane-associated *in vivo*, a Ca^{2+}/calmodulin-dependent kinase remains tightly bound to the membranes under identical conditions (Novak-Hofer and Levitan, 1983). Furthermore these membranes contain a number of substrates for exogenously added catalytic subunit (Novak-Hofer and Levitan, 1983). Thus, it is possible that the phosphorylation state of ion channels, which are integral membrane proteins, may be regulated by a cytoplasmic protein kinase in this system. The subcellular distribution of the Ca^{2+}/calmodulin-dependent protein kinase activity is more similar to that in vertebrate brain (Kennedy and Greengard, 1981), since we were able to detect both soluble and tightly membrane-bound activities. It is not yet clear whether these two activities are due to two distinct enzyme species.

The two-dimensional gel analysis demonstrates that a large number of membrane and soluble proteins can act as substrates *in vitro* for the endogenous cAMP-dependent and Ca^{2+}/calmodulin-dependent protein kinases. In contrast, the substrates for the cGMP-dependent enzyme are more limited. We were unable to detect any specific substrates for cGMP-dependent protein kinase, that is, proteins whose phosphorylation is stimulated by cGMP but not cAMP, as has been found in brain (Schlichter et al., 1978). It is possible that cAMP is affecting these substrates by stimulating the cGMP-dependent kinase under the conditions of this assay, although this is not supported by our finding that the enzyme is selective for cGMP after ion exchange chromatography. In any event it seems likely that specific substrates for cGMP-dependent kinase do exist, since cGMP can have effects on neuronal electrical activity very different from those of cAMP (Levitan and Norman, 1980).

In comparing Ca^{2+}/calmodulin-dependent phosphorylation with cAMP-dependent phosphorylation, many common substrates for the two protein kinases are found in the *Aplysia* nervous system (Novak-Hofer and Levitan, 1983; DeRiemer et al., 1984). These observations are confirmed by analysis of Ca^{2+} and cAMP-dependent phosphoproteins with two-dimensional gel electrophoresis (compare Figs. 5 and 6). Such substrates may be a point where the cAMP and Ca^{2+} second messenger systems interact, and in view of these findings it will certainly be of interest to investigate Ca^{2+}/calmodulin-dependent protein phosphorylation *in vivo*. However, some substrates specific for each of the two kinases can also be seen, such as proteins 14 and 15, which are only phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase and proteins 2, 3, 6, 7, 8, and 17, which are selective substrates for cAMP-dependent protein kinase.

Proteins phosphorylated in cell-free extracts *in vitro* are not necessarily substrates for protein kinases *in vivo*. Homogenization may eliminate compartmentalization, allowing proteins to be phosphorylated by kinases to which they are not exposed *in vivo*. On the other hand some proteins may be substrates *in vivo* but not *in vitro*. Thus we compared the phosphoprotein pattern *in vitro* with that observed in a single neuron (cell R15) *in vivo* following the intracellular injection of [γ-^{32}P]ATP. Of the 11 phosphoproteins whose phosphorylation is altered *in vivo* by application of 8-BT-cAMP to the injected cell, at least seven are also affected by cAMP *in vitro* under the present conditions. In addition, if homogenates are dialyzed against MOPS/Ca^{2+} buffer (not shown) instead of KEM buffer (Fig. 5), at least two more of the *in vivo* phosphoproteins (13 and 16) also are sensitive to cAMP *in vitro*. However several high molecular weight proteins which are observed routinely *in vivo* in neuron R15 (Fig. 4; Lemos et al., 1982, 1984) do not appear to be phosphorylated *in vitro*, either in the presence or absence of cAMP. These discrepancies between the *in vivo* and *in vitro* patterns may be due in part to the fact that the *in vivo* experiments were done on a single cell, whereas the *in vitro* experiments involved the entire nervous system. In addition, compartmentalization (as discussed above) may play a role. On the other hand, the similarities between the two systems indicate that the *in vitro* experiments will indeed be useful in testing the hypothesis (Greengard, 1978) that neuronal activity can be regulated by protein phosphorylation. It should be possible to identify phosphoproteins important for physiological regulation *in vivo*, and subject these phosphoproteins to detailed biochemical analysis *in vitro*.

References

Adams, W. B., and I. B. Levitan (1982) Intracellular injection of protein kinase inhibitor blocks the serotonin-induced increase in K^+-conductance in *Aplysia* neuron R15. Proc. Natl. Acad. Sci. U. S. A. *79:* 3877–3880.

Ames-Ferroluzi, G., and K. Nikaido (1976) Two dimensional gel electrophoresis of membrane proteins. Biochemistry *15:* 616–623.

Bandle, E., and A. Guidotti (1980) A simple and rapid method for the assay of cGMP-dependent protein kinase. J. Neurosci. Methods *2:* 419–427.

Bandle, E., and I. B. Levitan (1977) Cyclic AMP-stimulated phosphorylation of a high molecular weight endogenous protein substrate in sub-cellular fractions of molluscan nervous system. Brain Res. *125:* 325–331.

Bennett, M. K., N. E. Erondu, and M. B. Kennedy (1983) Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. J. Biol. Chem. *258:* 12735–12744.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72:* 248–254.

Castellucci, V. F., E. R. Kandel, J. H. Schwartz, F. Wilson, A. C. Nairn, and P. Greengard (1980) Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase stimulates facilitation of transmitter release underlying behavioural sensitization in *Aplysia*. Proc. Natl. Acad. Sci. U. S. A. *77:* 7492–7496.

Dedman, J. R., J. D. Potter, R. L. Jackson, J. D. Johnson, and A. R. Means (1977) Physiochemical properties of rat testis Ca^{2+}-dependent regulator protein of cyclic nucleotide phosphodiesterase:relationship of Ca^{2+}-binding, conformational changes and phosphodiesterase activity. J. Biol. Chem. *252:* 8415–8422.

Demaille, J. G., K. A. Peters, and E. H. Fischer (1977) Isolation and properties of the rabbit skeletal muscle protein inhibitor of adenosine 3′,5′-monophosphate dependent protein kinase. Biochemistry *16:* 3080–3086.

DePeyer, J., A. Cachelin, I. B. Levitan, and H. Reuter (1982) Ca^{2+}-activated K^+ conductance in internally perfused snail neurons is enhanced by protein phosphorylation. Proc. Natl. Acad. Sci. U. S. A. *79:* 4207–4211.

DeRiemer, S. A., L. K. Kaczmarek, Y. Lai, T. L. McGuinness, and P. Greengard (1984) Calcium/calmodulin-dependent protein phosphorylation in the nervous system of *Aplysia*. J. Neurosci. *4:* 1618–1625.

Eppler, C. M., M. J. Palazzolo, and J. H. Schwartz (1982) Characterization and localization of adenosine 3′5′-monophosphate-binding proteins in the nervous system of *Aplysia*. J. Neurosci. *2:* 1692–1704.

Greengard, P. (1978) Phosphorylated proteins as physiological effectors. Science *199:* 146–152.

Ho, H. C., T. S. Teo, R. Desai, and J. H. Wang (1976) Catalytic and regulatory properties of two forms of bovine heart cyclic nucleotide phosphodiesterase. Biochim. Biophys. Acta *429:* 461–473.

Jennings, K. R., L. K. Kaczmarek, R. M. Hewick, W. J. Dreyer, and F. Strumwasser (1982) Protein phosphorylation during afterdischarge in peptidergic neurons of *Aplysia*. J. Neurosci. *2:* 158–168.

Kaczmarek, L. K., K. R. Jennings, F. Strumwasser, A. C. Nairn, U. Walter, F. D. Wilson, and P. Greengard (1980) Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture. Proc. Natl. Acad. Sci. U. S. A. *77:* 7487–7491.

Kennedy, M., and P. Greengard (1981) Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites. Proc. Natl. Acad. Sci. U. S. A. *78:* 1293–1297.

Kuo, J. F., and P. Greengard (1974) Purification and characterization

of cyclic GMP-dependent protein kinases. Methods Enzymol. *38:* 329–350.

Lemos, J. R., I. Novak-Hofer, and I. B. Levitan (1982) Serotonin alters the phosphorylation of specific proteins inside a single living nerve cell. Nature *298:* 64–65.

Lemos, J. R., I. Novak-Hofer, and I. B. Levitan (1984) Synaptic stimulation alters protein phosphorylation *in vivo* in a single *Aplysia* neuron. Proc. Natl. Acad. Sci. U. S. A. *81:* 3233–3237.

Levitan, I.B., and S. Barondes (1974) Octopamine- and serotonin-stimulated phosphorylation of specific protein in the abdominal ganglion of *Aplysia californica.* Proc. Natl. Acad. Sci. U. S. A. *71:* 1145–1148.

Levitan, I. B., and J. Norman (1980) Different effects of cAMP and cGMP derivatives on the activity of an identified neuron: biochemical and electrophysiological analysis. Brain Res. *187:* 415–429.

Levitan, I. B., C. Madsen, and S. Barondes (1974) Cyclic AMP and amine effects on phosphorylation of specific protein in abdominal ganglion of *Aplysia californica:* localization and kinetic analysis. J. Neurobiol. *5:* 511–525.

Novak-Hofer, I., and I. B. Levitan (1983) Ca^{++}/calmodulin-regulated protein phosphorylation in the *Aplysia* nervous system. J. Neurosci. *3:* 473–481.

O'Farrell, P. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. *250:* 4007–4021.

Paris, C. G., V. Castellucci, E. Kandel, and J. Schwartz (1981) Protein phosphorylation, presynaptic facilitation and behavioral sensitization in *Aplysia.* Cold Spring Harbor Conf. Cell Prolif. *8:* 1361–1375.

Ram, J. L., and Y. H. Ehrlich (1978) Cyclic GMP-stimulated phosphorylation of membrane-bound proteins from nerve roots of *Aplysia californica.* J. Neurochem. *30:* 487–491.

Saitoh, T., and J. H. Schwartz (1983) Serotonin alters the subcellular distribution of a Ca^{++}/calmodulin binding protein in neurons of *Aplysia.* Proc. Natl. Acad. Sci. U. S. A. *80:* 6708–6712.

Schlichter, D. J., J. E. Casnelli, and P. Greengard (1978) An endogenous substrate for cGMP-dependent protein kinase in mammalian cerebellum. Nature *273:* 61–62.

Schulman, H., and P. Greengard (1978) Ca^{2+}-dependent protein phosphorylation system in membranes from various tissues, its activation by calcium-dependent regulator. Proc. Natl. Acad. Sci. U. S. A. *75:* 5432–5436.

Thompson, W. J., and M. M. Appleman (1977) Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochemistry *10:* 311–316.

Walter, U., and P. Greengard (1981) Cyclic AMP-dependent and cyclic GMP-dependent protein kinases in nervous tissue. Curr. Top. Cell. Regul. *19:* 219–256.