Ca⁺⁺/CALMODULIN-REGULATED PROTEIN PHOSPHORYLATION IN THE *APLYSIA* NERVOUS SYSTEM¹

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Received April 30, 1982; Revised July 19, 1982; Accepted September 15, 1982

Abstract

Protein substrates for an endogenous Ca⁺⁺/calmodulin-dependent protein kinase were characterized in the *Aplysia* nervous system. Ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid-washed membrane fractions from *Aplysia* ganglia contain an endogenous Ca⁺⁺/calmodulin-dependent protein kinase which phosphorylates a number of membrane proteins. Such washed membrane preparations exhibit little or no adenosine 3':5'-cyclic phosphate (cAMP)-dependent protein kinase activity but do contain substrates for exogenously added catalytic subunit of cAMP-dependent protein kinase. Low concentrations of catalytic subunit rapidly stimulate the phosphorylation of a protein of *M_r* 70,000 and of a 52,000-dalton doublet, indicating that these proteins are major substrates for this enzyme. Phosphopeptide patterns obtained after limited proteolysis suggest that the 70,000-dalton protein and the 52,000-dalton doublet are similar in structure and that their phosphorylation is stimulated both by Ca⁺⁺/calmodulin and by catalytic subunit. The 52,000-dalton doublet consists of two closely spaced bands: the phosphorylation of the upper band is stimulated by catalytic subunit, whereas the phosphorylation of the lower band is stimulated by the endogenous Ca⁺⁺/calmodulin-dependent protein kinase. The results suggest that in *Aplysia* membranes a number of proteins can be phosphorylated by both Ca⁺⁺/calmodulin-dependent protein kinase and catalytic subunit of cAMP-dependent protein kinase. This convergence of biochemical effects of Ca⁺⁺ and cAMP may play a role in some of their physiological actions in molluscan neurons.

There is much current interest in the possibility that intracellular second messengers may mediate certain actions of neurotransmitters on nerve cells (Greengard, 1978). One putative second messenger which has received a great deal of attention is calcium, since it plays a role in a large number of intracellular phenomena (Rasmussen and Waismann, 1981). The early suggestion by Meyer et al (1964) that many actions of Ca⁺⁺ might require the presence of a binding protein was followed by the purification of calmodulin several years later (Cheung, 1971; Wolff and Siegel, 1972). Among the many enzyme activities regulated by Ca⁺⁺/calmodulin (reviewed by Means and Dedman, 1980) are specific protein kinases (Schulman and Greengard, 1978; Yamauchi and Fujisawa, 1980; Burke and De Lorenzo, 1981; Grab et al., 1981; Kennedy and Greengard, 1981), suggesting that at least some effects of Ca⁺⁺ result from phosphorylation of specific

proteins. Cyclic nucleotides also seem to act by stimulating protein kinase activity (cf. Greengard, 1978; Kuo and Greengard, 1969), and it has been found that some protein substrates can be phosphorylated by a Ca⁺⁺- as well as an adenosine 3':5'-cyclic phosphate (cAMP)-dependent protein kinase (Huttner and Greengard, 1979) at different phosphorylation sites. Protein phosphorylation may thus be a point at which the Ca⁺⁺ and cyclic nucleotide second messenger systems can interact.

Results from recent physiological experiments using molluscan neurons have suggested that cAMP-dependent phosphorylation may affect Ca⁺⁺-dependent responses. cAMP has been reported to activate a voltage-dependent Ca⁺⁺ channel in some *Aplysia* neurons (Pellmar, 1981). In others the intracellular injection of the catalytic subunit of cAMP-dependent protein kinase influences the cell's electrical activity, apparently by enhancing the Ca⁺⁺ component of the action potential (Kaczmarek et al., 1980; Castellucci et al., 1980). Furthermore, there is evidence that intracellular perfusion of snail neurons with catalytic subunit results in selective enhancement of the Ca⁺⁺-activated K⁺ conductance in these cells (De Peyer et al., 1982). In addition to effects of cAMP-dependent phosphorylation on Ca⁺⁺-dependent

¹ We are grateful to Dr. E. Fischer (University of Washington, Seattle) for his generous gift of catalytic subunit of cAMP-dependent protein kinase, to Dr. J. Norman (Ciba-Geigy Corp., Ardsley, NY) for kindly providing calmodulin, and to Mr. M. Ackermann for the photography.

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reactions, Ca^{++} /calmodulin might in turn affect cAMP-mediated responses. It is also possible that, by phosphorylating the same protein substrate, Ca^{++} /calmodulin and cAMP could modulate each other's physiological actions.

In order to investigate the biochemical basis of the physiological effects of cAMP and Ca^{++} in the *Aplysia* nervous system, we have characterized cAMP- and Ca^{++} -dependent protein kinases and their substrates. In this report we identify substrates for Ca^{++} /calmodulin-dependent protein kinase in membrane fractions and describe the effect of catalytic subunit of cAMP-dependent protein kinase on these substrates.

Materials and Methods

Materials. Adenosine 5'-[γ - ^{32}P]triphosphate (>5000 Ci/mmol) was obtained from the Radiochemical Center, Amersham; adenosine 5'-0- (3-thio-) triphosphate ([γ - ^{35}S] ATP) (287.3 Ci/mmol) was from New England Nuclear Corp.; *t*-flupenthixol was obtained from H. Lundbeck & Co. (Copenhagen); *Staphylococcus aureus* V8 protease was from Miles Laboratories, Inc. Calmodulin purified from bovine brain according to the method of Dedman et al. (1977) was a gift from Dr. J. Norman (Ciba-Geigy, Inc., Ardsley, NY), and catalytic subunit of cAMP-dependent protein kinase purified from bovine heart (Peters et al., 1977) was kindly provided by Dr. E. H. Fischer (University of Washington, Seattle). Marine snails *Aplysia californica* were maintained in artificial sea water as described previously by 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 from identified neurons (not shown) confirmed the previous report (Strumwasser, 1971) that the *Aplysia* nervous system survives well *in vitro* over this time period. Prior to homogenization the ganglia were incubated in fresh medium for 60 min at 20°C.

Preparation of *Aplysia* membranes. Ganglia were quickly rinsed in 10 mM ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and then in homogenization buffer (20 mM *N*-morpholino propane sulfonic acid, (MOPS) pH 7.0, 10 mM NaF, 2.5 mM EGTA), and were homogenized in buffer (0.5 ml of buffer/animal) with five strokes of a Teflon-glass grinder. All operations were carried out at 4°C. The connective tissue sheath was removed, and the extract was further homogenized with five additional strokes. The homogenate was then centrifuged for 30 min at $12,000 \times g$. The pellet was washed once in the homogenizing buffer and twice in 20 mM MOPS, pH 7.0, 10 mM NaF in order to remove the EGTA. It was then resuspended in the same buffer to a concentration of 5 to 10 mg of protein/ml and kept in aliquots at -70°C. Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Phosphorylation. The net incorporation of phosphate into membrane proteins was assayed at 30°C in 200 μl of an assay mixture containing 20 mM MOPS, pH 7.0, 10 mM MgCl_2 , 1 mM dithiothreitol (DTT), 30 μM ATP, and 10 μCi of [γ - ^{32}P]ATP (4.10^6 cpm/nmol) or 5 μCi of [γ - ^{35}S] ATP (8.10^5 cpm/nmol). Where indicated EGTA, Ca^{++} (as a Ca^{++} /EGTA buffer), *t*-flupenthixol, calmodulin, and

catalytic subunit at the indicated final concentrations were included in the assay mixture. After 30 sec preincubation, the reaction was started by the addition of the membrane suspension (100 μg of protein). After 5 min the reaction was stopped by the addition of 100 μl of a stop buffer containing 80 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol. Samples were boiled for 2 min, and 100- μl aliquots were run on SDS-polyacrylamide gels.

Gel electrophoresis and autoradiography. The solubilized proteins were separated by electrophoresis in an SDS-polyacrylamide gradient (7.5% to 17.5% acrylamide) slab gel. Stacking gel, running, and gel buffers were as described by Laemmli (1970). The gels were stained with 0.1% Coomassie Brilliant blue R 250 in 45% ethanol/10% acetic acid (vol/vol). Destaining was by diffusion in 25% ethanol/10% acetic acid (vol/vol). The gels were dried and exposed to Kodak XOMatik x-ray film for about 4 hr using a Kodak Regular Intensifying screen in the case of experiments with [γ - ^{32}P]ATP. In the experiments using [γ - ^{35}S]ATP, gels were exposed without the intensifying screen for about 3 weeks.

Phosphoprotein bands localized by autoradiography were cut out from the dry gel and their radioactivity was determined in a liquid scintillation counter using 5 ml of Aquasol as scintillant.

Partial peptide mapping by limited proteolysis. Limited proteolysis of isolated protein bands was performed according to Sieghart et al. (1979) using *Staphylococcus aureus* V8 protease and chymotrypsin. EGTA-washed *Aplysia* membranes were phosphorylated either with Ca^{++} /calmodulin or with a low concentration (10 nM) of catalytic subunit in the presence of EGTA as described above (see "Materials and Methods" and legend to Fig. 1). The phosphoproteins were separated by SDS-electrophoresis and detected by autoradiography as described. The 70,000-dalton protein as well as the 52,000-dalton doublet were cut out from the dried gel and counted in the liquid scintillation spectrophotometer (under the chosen conditions the proteins incorporate, on a molar basis, a similar amount of phosphate using either Ca^{++} /calmodulin-dependent protein kinase or catalytic subunit). The gel slices were then swollen for 1.5 hr in 0.5 ml of 0.125 M Tris, pH 6.8, 0.1% SDS, 1 mM EDTA ("SDS-buffer"). The pieces were then transferred to an SDS-gel consisting of a 4 cm long 5% stacking gel and an 8 cm long 15% separating gel. The gel slices were overlaid with 20 μl of a mixture of SDS-buffer and glycerol 4:1 (vol:vol). Then 20 μl of a 9:1 mixture of the SDS-buffer and glycerol containing either 0.1 μg of *Staphylococcus aureus* V8 protease or 5 μg of chymotrypsin were added. Electrophoresis was performed for about 16 hr at 40 V, the gel was then fixed and dried without previous staining and the separated phosphopeptides were detected by autoradiography. The autoradiographs were scanned using a Joyce-Loebl densitometer.

Results

Effect of Ca^{++} /calmodulin on the phosphorylation of membrane proteins. Figure 1 shows an autoradiograph of proteins phosphorylated in EGTA-washed *Aplysia* membranes. In the absence of Ca^{++} several phosphoprotein bands can be seen, and two with apparent M_r 70,000

Figure 1. Effect of Ca^{++} /calmodulin and catalytic subunit on phosphorylation of membrane proteins from *Aplysia* ganglia. Autoradiograph of a gel containing proteins phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as substrate, as described under "Materials and Methods." Additions to the assay tubes were: (1) 2.5 mM EGTA; (2) 3 μM free Ca^{++} (100 μM EGTA, 89 μM CaCl_2); (3) Ca^{++} + 500 μM *t*-flupenthixol; (4) Ca^{++} + 156 nM calmodulin; (5) Ca^{++} + calmodulin + EGTA; (6) Ca^{++} + calmodulin + *t*-flupenthixol; (7) EGTA + 100 nM catalytic subunit. The numbers to the right of the autoradiograph are molecular masses in daltons as determined from standard proteins run on the same gel. The protein designated as M_r 52,000 consists of two closely spaced bands.

and 52,000 predominate (Fig. 1, track 1). The 52,000-dalton region in fact consists of two closely spaced phosphoproteins (52A,B). Addition of 3 μM free Ca^{++} results in a slight increase of phosphorylation and bands with apparent M_r of 21,000 and 18,000 become more prominent (Fig. 1, track 2). When calmodulin (156 nM) is added together with Ca^{++} the phosphorylation of a number of proteins with apparent M_r of 52,000, 45,000, 43,000, 37,000, 30,000, 21,000, and 18,000 is stimulated (Fig. 1, track 4). We chose two representative phosphoproteins (the 52,000-dalton doublet and the 21,000-dalton protein) to quantitate the response to calmodulin. Because our efforts using urea and larger pore gels to better resolve 52A from 52B were not successful, we treated the 52,000-dalton region as a single unit for purposes of quantitation. The phosphorylation of the 52,000-dalton doublet and 21,000-dalton band was assayed at increasing calmodulin concentrations as described under "Materials and Methods," and it was found that stimulation of their phosphorylation is dose dependent with a midpoint for activation at about 10 nM calmodulin (Fig. 2), comparable to values for calmodulin-stimulated phosphorylation in rat brain (cf. Burke and De Lorenzo, 1981). The stimulation by calmodulin is Ca^{++} dependent as it is blocked by EGTA (Fig. 1, track 5). The calmodulin-binding thio-

xanthine drug *t*-flupenthixol (Norman et al., 1979) inhibits the slight stimulation by Ca^{++} as well as the stronger stimulation by Ca^{++} /calmodulin (Fig. 1, tracks 3 and 6), suggesting that the slight stimulation by Ca^{++} in the absence of added calmodulin (Fig. 1, track 2) may be due to the presence of some residual endogenous calmodulin in these membranes. Figure 3 shows a dose-response curve for *t*-flupenthixol inhibition of calmodulin-dependent phosphorylation of the proteins with M_r 52,000 and 21,000. The inhibition is dose dependent, with an IC_{50} of about 20 μM . Table I summarizes the data showing the effects of Ca^{++} , calmodulin, EGTA, and *t*-flupenthixol on the phosphorylation of a number of membrane proteins. Whereas the phosphorylation of the 70,000-dalton protein and 52,000-dalton doublet is stimulated 50 to 70% by Ca^{++} /calmodulin, the proteins in the lower molecular weight range are stimulated more strongly, the 21,000-dalton band showing the greatest increase (160%). In every case the calmodulin stimulation is blocked by EGTA and *t*-flupenthixol.

Effect of catalytic subunit of cAMP-dependent protein kinase on membrane protein phosphorylation. Figure 4 shows the effect of cAMP and catalytic subunit of cAMP-dependent protein kinase (CS) on protein phosphorylation in supernatant and membrane fractions from *Aply-*

Figure 2. Dose-response curve for calmodulin stimulation of phosphorylation of two representative membrane proteins. The substrate was $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Assay tubes contained $3\text{ }\mu\text{M}$ free Ca^{++} together with calmodulin at the concentrations indicated. Phosphoproteins separated by SDS-gel electrophoresis and localized by autoradiography (see Fig. 1) were cut out of the dried gel, and their radioactivity was determined by liquid scintillation counting. Results (means $\pm$ SEM, $n = 3\text{--}6$) are presented for the 52,000-dalton doublet ($\bigcirc\text{--}\bigcirc$) and the 21,000-dalton protein (Δ) as percentage of maximal stimulation. Basal levels were 4083 ± 255 cpm ($n = 6$) ($\bigcirc$) and 4061 ± 227 cpm ($n = 5$) (Δ); maximally stimulated levels were 5939 ± 500 cpm ($n = 6$) ($\bigcirc$) and 5800 ± 450 cpm ($n = 5$) (Δ).

sia ganglia. In $12,000 \times g$ supernatant fractions, cAMP stimulates the phosphorylation of a large number of proteins (Fig. 4, scan 1). Membrane fractions that did not undergo EGTA treatment and washings also show cAMP-dependent phosphorylation of a number of proteins (Fig. 4, scan 3).

However, in membranes that were EGTA washed, cAMP has little or no effect (not shown), although the addition of CS stimulates the phosphorylation of the same proteins that are stimulated by cAMP in the more crude fraction (Fig. 4, scan 5). In contrast to the membrane-bound Ca^{++} /calmodulin-dependent protein kinase, the cAMP-dependent protein kinase seems to be only loosely attached to the membranes. Figure 5 illustrates differential effects of the catalytic subunit of cAMP-dependent protein kinase and the Ca^{++} /calmodulin-dependent protein kinase in terms of their reaction rates. Catalytic subunit either in low amounts (10 nM , 5-min reactions) or for a short time (100 nM , 30-sec reaction) preferentially stimulates phosphorylation of the 105,000- and 70,000-dalton proteins and of the upper band of the 52,000-dalton doublet (Fig. 5, tracks 3 and 4). Tracks 5 to 9 of Figure 5 show a time course of protein phosphorylation by the endogenous Ca^{++} /calmodulin-dependent protein kinase. In contrast to the effect of catalytic subunit, the phosphoprotein labeled most rapidly (track 5, 30-sec reaction) is the 21,000-dalton protein: After 2 min the 21,000-dalton protein has reached maximal phosphorylation, whereas the 52,000-dalton doublet has reached 58% and the 70,000-dalton protein 38% of their maximally phosphorylated levels.

Thiophosphorylation with $\text{ATP}_{\gamma}\text{S}$. $\text{ATP}_{\gamma}\text{S}$ has been

used to thiophosphorylate proteins irreversibly (Cassidy et al., 1979). It is known to be a relatively poor substrate for cAMP-dependent protein kinase (Kerrick and Hoar, 1981) and is thought to preferentially label phosphoproteins that are substrates for Ca^{++} /calmodulin-dependent protein kinases. We found that the effects of catalytic subunit and Ca^{++} /calmodulin-dependent protein kinase on the phosphorylation of the 70,000-dalton protein and the 52,000-dalton doublet could be distinguished more readily using $\text{ATP}_{\gamma}\text{S}$ as substrate. The autoradiograph in Figure 6 shows a comparison of the effect of Ca^{++} /calmodulin and of catalytic subunit on membrane protein phosphorylation. Expressed on a molar basis, overall incorporation of ^{35}S from $\text{ATP}_{\gamma}\text{S}$ is less than incorporation of ^{32}P from $\text{ATP}_{\gamma}\text{P}$. Nevertheless, a number of thiophosphorylated proteins are visible. In the presence of EGTA, a protein of 52,000 daltons (52A) as well as phosphoproteins of 45,000 and 42,000 daltons can be seen (Fig. 6, track 1). Addition of $3\text{ }\mu\text{M}$ free Ca^{++} stimulates the thiophosphorylation of the 52A protein slightly (Fig. 6, track 2) and calmodulin added together with Ca^{++} (Fig. 6, track 4) leads to increased incorporation of ^{35}S into this protein. This stimulation is blocked by *t*-flupenthixol as well as EGTA (Fig. 6, tracks 5 and 6). When catalytic subunit is added in the presence of EGTA (Fig. 6, track 7) a phosphoprotein of 70,000 dalton appears and a second band closely above 52A is visible (52B). Addition of both Ca^{++} /calmodulin and catalytic subunit together (Fig. 6, track 8) demonstrates that 52B is preferentially stimulated by catalytic subunit, whereas 52A is stimulated by Ca^{++} /calmodulin.

When catalytic subunit and Ca^{++} /calmodulin are added in the presence of EGTA (Fig. 6, track 9), the Ca^{++} /calmodulin-dependent effect is eliminated (as in

Figure 3. Dose-response curve for *t*-flupenthixol inhibition of the stimulation of phosphorylation by Ca^{++} /calmodulin. The substrate was $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Assay tubes contained $3\text{ }\mu\text{M}$ free Ca^{++} and 156 nM calmodulin, together with *t*-flupenthixol at the concentrations indicated. Results (means $\pm$ SEM, $n = 3$) are presented for the 52,000-dalton doublet ($\bigcirc\text{--}\bigcirc$) and the 21,000-dalton protein (Δ) as percentage maximal stimulation. Maximally stimulated levels were 4376 ± 542 cpm ($\bigcirc$) and 2478 ± 342 cpm (Δ); maximally inhibited levels were 3157 ± 181 cpm ($\bigcirc$) and 1327 ± 131 cpm (Δ).

TABLE I

Effects of Ca^{++} /calmodulin and catalytic subunit of cAMP-dependent protein kinase (CS) on endogenous phosphorylation in membranes from the *Aplysia* nervous system

[^{32}P]Phosphate was incorporated from [$\gamma\text{-}^{32}\text{P}$]ATP into EGTA-washed membrane preparations from *Aplysia* ganglia. Phosphoproteins were separated by SDS-PAGE, and ^{32}P incorporation into the individual polypeptides was quantitated by liquid scintillation spectroscopy. The protein designated as M_r 52,000 consists of two closely spaced bands, 52A and 52B (see the text). Data are means $\pm$ SEM

Condition	Phosphoproteins (M_r)						
	70,000	52,000	42,000	37,000	30,000	21,000	18,000
Phosphate incorporated expressed as fmol/ μg of total membrane protein							
EGTA (2.5 mM) basal level	12.0	10.8	7.0	5.0	6.5	9.0	9.0
Phosphate incorporated expressed as % of basal levels (basal level = 100%)							
Ca^{++} (3 μM)	133 $\pm$ 15	127 $\pm$ 13	124 $\pm$ 14	117 $\pm$ 25	154 $\pm$ 13	175 $\pm$ 11	168 $\pm$ 11
Ca^{++} + $\pm$ -flupenthixol (500 μM)	90 $\pm$ 9	116 $\pm$ 16	113 $\pm$ 13	143 $\pm$ 7	133 $\pm$ 23	131 $\pm$ 7	87 $\pm$ 8
Ca^{++} + calmodulin (156 nM)	149 $\pm$ 19	172 $\pm$ 14	197 $\pm$ 12	233 $\pm$ 19	233 $\pm$ 44	258 $\pm$ 21	228 $\pm$ 22
EGTA + calmodulin	84 $\pm$ 4	95 $\pm$ 12	89 $\pm$ 8	108 $\pm$ 6	96 $\pm$ 12	112 $\pm$ 12	91 $\pm$ 6
$\pm$ -Flupenthixol + calmodulin	76 $\pm$ 6	115 $\pm$ 19	105 $\pm$ 15	138 $\pm$ 9	121 $\pm$ 15	142 $\pm$ 17	83 $\pm$ 7
EGTA + CS (100 nM)	818 $\pm$ 90	1101 $\pm$ 122	323 $\pm$ 27	478 $\pm$ 7	401 $\pm$ 24	467 $\pm$ 32	403 $\pm$ 99

Figure 4. Densitometric scans of autoradiographs showing the effect of cAMP on endogenous protein phosphorylation in *Aplysia* nervous system. The subcellular fractions were 12,000 g supernatant (SN), 12,000 g membrane fraction (P), and EGTA-washed membrane fraction (PW). Fractions were dialyzed overnight against 20 mM MOPS, pH 7.0, 0.1% β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride and then phosphorylated with [$\gamma\text{-}^{32}\text{P}$]ATP in the presence of 2.5 mM EGTA. (1) SN, 10 μM cAMP; (2) SN, control; (3) P, 10 μM cAMP; (4) P, control; (5) PW, 100 nM catalytic subunit; (6) PW, control.

Fig. 6, tracks 1 and 6), whereas the effect of catalytic subunit is not diminished. The lower M_r phosphoproteins from 42,000 daltons to 18,000 daltons present in membranes phosphorylated by $\text{ATP}\gamma\text{P}$ are barely visible when $\text{ATP}\gamma\text{S}$ is used. The group of proteins from 70,000 to 45,000 daltons, however, is labeled in a specific way: the 70,000-dalton protein appears to be a specific substrate for catalytic subunit, the 52,000-dalton doublet consists of one band stimulated by catalytic subunit and the other stimulated by Ca^{++} /calmodulin, and the 45,000-dalton protein seems unaffected by either of the two protein kinases.

Limited proteolysis of specific membranes phosphoproteins. The 70,000-dalton protein as well as 52B are the major membrane protein-substrates for cAMP-dependent protein kinase: in crude membrane fractions their phosphorylation is stimulated by endogenous cAMP-dependent protein kinase (Fig. 4); in EGTA-

washed membranes their phosphorylation is rapidly stimulated by low concentrations of catalytic subunit (Fig. 5). With $\text{ATP}\gamma\text{S}$ as substrate they are specifically labeled in the presence of catalytic subunit (fig. 6). The phosphorylation of both the 70,000-dalton protein and of 52A is stimulated by Ca^{++} /calmodulin. The 70,000-dalton protein as well as the 52,000-dalton doublet 52A,B (which were taken together because they cannot be resolved from one another) were subjected to limited proteolysis, and their phosphopeptide patterns were compared. The proteins were phosphorylated either by the endogenous Ca^{++} /calmodulin-dependent protein kinase or by low concentrations of catalytic subunit (in order to phosphorylate only sites with a high affinity for this enzyme). The protein bands were cut out from the gel and subjected to digestion with *Staphylococcus aureus* V8 (SV8) protease or with chymotrypsin as described under "Materials and Methods." The resulting phosphopep-

Figure 5. Effects of Ca^{++} /calmodulin-dependent protein kinase and catalytic subunit on membrane protein phosphorylation: influence of reaction time. Autoradiograph of a gel containing proteins phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Conditions were: (1) 2.5 mM EGTA, 5-min reaction; (2) 3 μM free Ca^{++} + 156 nM calmodulin, 5 min; (3) EGTA + 10 nM catalytic subunit, 5 min; (4) EGTA + 100 nM catalytic subunit, 30 sec; (5) Ca^{++} + calmodulin, 30 sec; (6) Ca^{++} + calmodulin, 1 min; (7) Ca^{++} + calmodulin, 2 min; (8) Ca^{++} + calmodulin, 5 min; (9) Ca^{++} + calmodulin, 10 min. The 52,000-dalton region contains a closely spaced doublet.

Figure 6. Effect of Ca^{++} /calmodulin and catalytic subunit on incorporation of $[\text{S}^{35}]$ from $[\gamma\text{-}^{35}\text{S}]\text{ATP}$ into membrane proteins. Incubations were as described under "Materials and Methods." Additions to the assay tubes were: (1) 2.5 mM EGTA; (2) 3 μM free Ca^{++} (100 μM EGTA, 89 μM CaCl_2); (3) Ca^{++} + 500 μM *t*-flupenthixol; (4) Ca^{++} + 156 nM calmodulin; (5) Ca^{++} + calmodulin + *t*-flupenthixol; (6) Ca^{++} + calmodulin + EGTA; (7) EGTA + 640 nM catalytic subunit; (8) Ca^{++} + calmodulin + catalytic subunit; (9) EGTA + calmodulin + catalytic subunit. The 52,000-dalton region consists of two closely spaced bands.

tides were separated on gels according to their M_r and were localized by autoradiography. Figure 7 shows scans of autoradiographs with phosphopeptides originating from the 70,000-dalton protein and the 52,000-dalton doublet. Using *Staphylococcus aureus* V8 protease it can

be seen that in the 70,000-dalton protein, endogenous Ca^{++} /calmodulin-stimulated protein kinase (Fig. 7, scan 1) as well as catalytic subunit (Fig. 7, scan 2) increase phosphorylation of the same peptides (with some quantitative differences). When 52A,B is phosphorylated with

Figure 7. Partial proteolysis of the 70,000-dalton protein and 52,000-dalton-doublet with 0.1 μg per track of *Staphylococcus aureus* V8 protease (scans 1 to 4) or 5 μg per track of chymotrypsin (scans 5 to 10). Densitometric scanings of autoradiographs of phosphopeptides originating either from proteins phosphorylated with Ca^{++} /calmodulin-dependent protein kinase (scans 1, 3, 5, 8), 10 nM catalytic subunit (scans 2, 4, 6, 9), and 100 nM catalytic subunit (scans 7, 10).

Ca^{++} /calmodulin-dependent protein kinase (Fig. 7, scan 3), some additional phosphopeptides appear which are not visible in the case of phosphorylation by catalytic subunit (Fig. 7, scan 4). These additional phosphopeptides probably arise from 52A. Partial digestion using chymotrypsin gives similar results (Fig. 7, scans 5, 6, 8, and 9). Comparison of the phosphopeptide patterns arising from the 70,000-dalton protein and from the 52,000-dalton doublet phosphorylated by catalytic subunit show that they are very similar either using SV8 protease (compare scans 2 and 4, Fig. 7) or chymotrypsin (compare scans 7 and 10, Fig. 7). This result suggests that the 70,000-dalton protein and 52B may be structurally related.

Discussion

There is only limited information available concerning protein phosphorylation in the *Aplysia* nervous system (Levitan and Barondes, 1974; Levitan et al., 1974; Jennings et al., 1979; Paris et al., 1980). In contrast, Ca^{++} -dependent and cAMP-dependent protein phosphorylation have been studied thoroughly by a number of investigators in membranes from vertebrate brain (cf. Schulman and Greengard, 1978; Burke and De Lorenzo, 1981; Grab et al., 1981; Walter et al., 1979; Callaghan et al., 1980). In brain membranes an endogenous Ca^{++} /calmodulin-dependent protein kinase stimulates phosphorylation of proteins with apparent M_r 50,000, 60,000, 80,000 as well as of several proteins with M_r between 90,000 and 240,000. A cAMP-dependent membrane-bound protein kinase stimulates the phosphorylation of proteins with M_r of 300,000, 86,000 (protein I_a), 80,000 (protein I_b), and

two proteins with M_r around 50,000 (regulatory subunits of type I and type II cAMP-dependent protein kinase). Phosphorylation of proteins I_a and I_b (cf. Huttner and Greengard, 1979), as well as of α and β tubulin (M_r 51,000 and 64,000) (cf. Burke and De Lorenzo, 1981), is affected by both Ca^{++} /calmodulin and cAMP.

Our results show the presence of a Ca^{++} /calmodulin-stimulated endogenous protein kinase in membranes from *Aplysia* nervous system. In addition to phosphorylating a number of membrane proteins ranging in M_r from 200,000 to 50,000, this kinase also phosphorylates some lower molecular weight proteins between 47,000 and 18,000 daltons. One criterion for calmodulin-dependent reactions is that, in calmodulin-depleted preparations, the effect of Ca^{++} should be completely dependent on exogenously added calmodulin. Although *Aplysia* membranes were prepared in the presence of EGTA and underwent two further EGTA washes, we always observed a small effect of Ca^{++} alone on protein phosphorylation. This result indicates that either endogenous calmodulin was not completely removed or that there is additionally a Ca^{++} -dependent, calmodulin-independent protein kinase present. A phospholipid requiring Ca^{++} -dependent protein kinase (stimulated by phosphatidylserine) has been described recently by Takai et al. (1979). We did not find an effect of phosphatidylserine on membrane protein phosphorylation, suggesting that the stimulation by Ca^{++} alone may indeed be due to endogenous calmodulin. Another criterion for calmodulin-dependent reactions is that they be inhibited by calmodulin-binding neuroleptic drugs. We find that the stimulations by Ca^{++} alone as well as by Ca^{++} /calmodulin are inhibited by *t*-

flupenthixol, again indicating that the effect of Ca^{++} alone is probably due to residual endogenous calmodulin.

In crude membrane fractions cAMP stimulates the phosphorylation of a number of proteins (105,000, 70,000, 52,000, 30,000, 18,000 daltons). In contrast, EGTA-washed membrane fractions exhibit little cAMP-dependent phosphorylation. Addition of catalytic subunit of cAMP-dependent protein kinase to these EGTA-washed membrane fractions leads to the phosphorylation of the same proteins, as are affected by cAMP in the crude membranes. This suggests that membrane protein phosphorylation may also be regulated by a cAMP-dependent protein kinase, which is either a soluble enzyme or is loosely attached to the membranes. The major substrates for cAMP-dependent phosphorylation in membranes are proteins with apparent M_r 70,000 and 52,000. Limited proteolysis with two different proteases shows remarkably similar phosphopeptide patterns for these proteins, suggesting that they are related. A comparison of the phosphopeptides of the proteins phosphorylated by either Ca^{++} /calmodulin-dependent protein kinase or catalytic subunit indicates that in the 70,000-dalton protein both enzymes stimulate phosphorylation of the same phosphopeptide fragments, although quantitative differences are evident. In the 52,000-dalton doublet phosphorylation by the endogenous Ca^{++} /calmodulin-dependent protein kinase leads to the appearance of new phosphopeptides that are not observed when catalytic subunit is used. This preliminary phosphopeptide analysis probably underestimates the differences in the phosphopeptide patterns, since a two-dimensional separation would be required to fully resolve all the phosphopeptides produced.

The selective effects of Ca^{++} /calmodulin on membrane protein phosphorylation are also clearly visible in experiments with $\text{ATP}\gamma\text{S}$, which is a relatively inefficient substrate for cAMP-dependent protein kinase (cf. Kerrick and Hoar, 1981). In the case of the 52,000-dalton doublet, the lower of the two closely spaced double bands that comprise this region can be seen to be specifically stimulated by Ca^{++} /calmodulin, whereas the upper band is dependent on catalytic subunit.

The results obtained by phosphorylation of proteins in cell homogenates or subcellular fractions using endogenous or exogenous protein kinases do not necessarily reflect the *in vivo* situation. The spatial relationships between the enzymes and their substrates are likely to be changed by breaking the cells. In *Aplysia* we have been able to study protein phosphorylation in individual nerve cells *in vivo* by intracellularly injecting $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Lemos et al., 1982). Preliminary results suggest that at least some of the proteins phosphorylated *in vivo* are among those phosphorylated by Ca^{++} /calmodulin-dependent and/or cAMP-dependent protein kinase in the present *in vitro* experiments. Thus it should prove possible to use this *in vitro* system for detailed characterization of phosphoproteins which have been shown to be physiologically relevant *in vivo*.

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