Differential Phosphorylation of Multiple Sites in Purified Protein I by Cyclic AMP-dependent and Calcium-dependent Protein Kinases

(Received for publication, April 15, 1980, and in revised form, August 25, 1980)

Wieland B. Huttner, Louis J. DeGennaro, and Paul Greengard

From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

Protein I, a specific neuronal phosphoprotein, has previously been shown, using rat brain synaptosome preparations, to contain multiple sites of phosphorylation which were differentially regulated by cAMP and calcium. In the present study, Protein I was purified to homogeneity from rat brain and its phosphorylation was investigated using homogeneous cAMP-dependent protein kinase and a partially purified calcium-calmodulin-dependent protein kinase from rat brain. Employing various peptide mapping techniques, a minimum of three phosphorylation sites could be distinguished in Protein I; the phosphorylated amino acid of each site was serine. One phosphorylation site was located in the collagenase-resistant portion of Protein I and was the principal target for phosphorylation by the catalytic subunit of cAMP-dependent protein kinase. This site was also phosphorylated by calcium-calmodulin-dependent protein kinase. The other two phosphorylation sites were located in the collagenase-sensitive portion of Protein I. These latter sites were markedly phosphorylated by calcium-calmodulin-dependent protein kinase, but not by cAMP-dependent protein kinase in concentrations sufficient to phosphorylate maximally the site in the collagenase-resistant portion. Thus, the phosphorylation of purified Protein I by purified cAMP-dependent and calcium-calmodulin-dependent protein kinases provides an enzymological explanation for the regulation of phosphorylation of endogenous Protein I in synaptosome preparations by cAMP and by calcium observed previously. The studies suggest that certain of the synaptic actions of two distinct second messengers, cAMP and calcium, are expressed through the distinct specificities of cAMP- and calcium-dependent protein kinases for the multiple phosphorylation sites in one neuron-specific protein, Protein I.

The phosphorylation and dephosphorylation of proteins has been recognized as a principal mechanism for controlling cellular functions (see Refs. 1-4 for reviews). In the nervous system, some of the events involved in synaptic transmission, such as the regulation of ion permeability and the synthesis and release of neurotransmitters, may be controlled at the level of the phosphorylation of specific proteins (5). The state of phosphorylation of a major phosphoprotein in the nervous system, designated Protein I (6), has been shown to be altered both in vivo (7) and in vitro (6, 8-13) by various physiological manipulations known to affect neuronal function, including depolarization (8-11), as well as the neurotransmitters serotonin (12) and dopamine (13). Protein I is composed of two very similar polypeptides of 86,000 and 80,000 daltons, which have been referred to as Proteins Ia and Ib, respectively (15). Both Proteins Ia and Ib are basic proteins and contain an elongated, collagenase-sensitive portion as well as a globular, collagenase-resistant portion (15). Protein I is specifically located in synaptic regions throughout the central and peripheral nervous system, where it appears to be associated primarily with synaptic vesicles (16-19).

Using synaptosome preparations, it has recently been shown that Protein I is a major substrate for both a cAMP- and a calcium-dependent phosphorylation system, and that Protein I has multiple sites of phosphorylation which are differential targets for cAMP- and calcium-dependent phosphorylation (11). Before investigating the precise role of Protein I phosphorylation in the physiology of synaptic terminals, it seemed desirable to determine whether the specific effects of cAMP and calcium on Protein I phosphorylation observed in the complex synaptosome system could be accounted for by the activation of cAMP- and calcium-dependent protein kinases respectively, rather than by cAMP and calcium affecting phosphoprotein phosphatases and/or membrane-membrane interactions. It was also important to determine the number of phosphorylation sites in Protein I as well as their location with regard to the collagenase-sensitive and collagenase-resistant portions of the Protein I molecule. In the present study, therefore, we have used rat Protein I purified to apparent homogeneity as substrate for purified catalytic subunit of cAMP-dependent protein kinase and for a partially purified calcium-calmodulin-dependent protein kinase which phosphorylates Protein I (36), to address these questions.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats, weighing 150 to 200 g, were used for the purification of Protein I. Staphylococcus aureus V8 protease was obtained from Miles, trypsin I was from Sigma, L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin and a-chymotrypsin were from Worthington, and collagenase was from Advance Biofactures. Cellulose thin layer chromatography sheets were obtained from Eastman Kodak. Other chemicals were obtained from sources listed previously (8, 15). [γ-32P]ATP was prepared by the method of Glynn and Chappell (20).

Purification of Protein I from Rat Brain for Use as Substrate—Rats were killed by cervical dislocation. The brains were quickly removed from the skull, and the cerebra were dissected and stored at -70°C. Protein I was purified from 150 to 200 g of rat cerebra by a modification of the original procedure (15). Briefly, after homogeni-

1. I. Walaas and P. Greengard, manuscript in preparation, as cited by Greengard (14).
2. L. J. DeGennaro and P. Greengard, manuscript in preparation.
Multiple Site Phosphorylation of a Specific Neuronal Protein

ration, purified Protein I was phosphorylated at 2°C in the presence of 50 mM Hepes (pH 7.4), 10 mM MgCl₂, 1 mM EGTA, 1.3 mM CaCl₂, 10 μg/ml of calmodulin, and 1 μM [γ-³²P]ATP (2 × 10⁶ cpm/nmol). After 30 min of phosphorylation, Protein I was repurified from the protein kinase preparation by chromatography on CM-cellulose.⁷ In some experiments, the purified phospho-Protein I was passed over a Sephadex G-100 column immediately prior to collagenase digestion in order to remove traces of low molecular weight degradation products of Protein I.

Purified phospho-Protein I (20 to 100 μg/ml) was incubated for 60 min at 30°C in 20 mM Hepes (pH 7.4), 10 mM CaCl₂, in the absence or presence of highly purified collagenase (300 units in a final reaction volume of 10 μl). The digestion was terminated by the addition of 70 μl of SDS stop solution and boiling, and Protein I and its fragments were separated by electrophoresis on 7% SDS-polyacrylamide gels. After electrophoresis, some of the gels were dried immediately and autoradiographed, whereas duplicate gels were first stained and destained, then dried and autoradiographed. Gel pieces containing Protein I and Protein I fragments were cut from stained-destained and from unstained gels, and radioactive phosphate incorporation was determined by liquid scintillation counting. The stained-destained gel pieces were then washed with ether and subjected to fingerprinting.

Tryptic-Chymotryptic Fingerprinting—Gel pieces containing phosphorylated Protein I or phosphorylated fragments of Protein I were subjected to “fingerprinting” (exhaustive digestion and two-dimensional separation of resultant peptides) as described previously (11, 28), with the following modifications. All gel pieces were swollen and soaked in acetic acid/methanol/water (11) to remove SDS and then lyophilized before they were reswollen in 1 ml of a solution containing 50 mM ammonium bicarbonate, 1 mM diisobutyryl, a trace amount of phenol red, and 75 μg/ml of trypsin I plus 75 μg/ml of a-chymotrypsin. After incubation for 24 h at 37°C, the eluate was collected, fresh trypsin I (25 μg/ml) plus a-chymotrypsin (25 μg/ml) were added, and incubation was continued for another 6 to 10 h. The resultant phosphopeptides were separated on cellulose thin layer PS Autoradiographs

- Fig. 1. Protein staining (PS) of the purified Protein I preparation, as well as autoradiographs of purified Protein I phosphorylated by purified protein kinases. Purified phospho-Protein I (3 μg/100 μl) was phosphorylated with 150 μM [γ-³²P]ATP for 60 min at 2°C in the presence of either 6 mM catalytic subunit of CAMP-dependent protein kinase (cAMP-kinase) or partially purified calcium-calmodulin-dependent protein kinase (Calcium Kinase). After phosphorylation, Protein I was subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)N,N',N'-tetraacetic acid; SDS, sodium dodecyl sulfate.
chromatography sheets as described previously (11, 28). After autoradiography of the cellulose sheet, pieces containing individual phosphopeptides were cut from the cellulose sheet using the autoradiograph as a guide, and radioactivity was determined by liquid scintillation counting. From cut pieces of duplicate cellulose sheets, individual phosphopeptides were obtained by scraping the cellulose off the plastic and eluting the phosphopeptide from the cellulose with 2 ml of 1 M formic acid followed by lyophilization. In some experiments, corresponding phosphopeptides eluted from different cellulose sheets were pooled and rerun together on one cellulose sheet using the same two-dimensional electrophoresis-chromatography system in order to verify their identity. In other experiments, certain of the eluted phosphopeptides were subjected to further incubation in the presence of trypsin followed by lyophilization and two-dimensional separation on cellulose sheets.

Phosphoamino Acid Analysis—After tryptic-chymotryptic fingerprinting and autoradiography, individual phosphopeptides were eluted from the cellulose with 1 M formic acid and, after lyophilization, were separately subjected to partial acid hydrolysis in 6 N HCl at 110°C for 90 min under vacuum (29). After drying, samples were resuspended in 2.5% (v/v) formic acid, 8.7% (v/v) acetic acid (pH 1.9) containing a trace amount of phenol red, spotted at one end of a cellulose sheet, and electrophoresed at 500 V in the same buffer until the dye migrating towards the anode had reached the other end of the cellulose sheet. Phosphoamino acids were located by autoradiography of the cellulose sheet. Phosphoserine and phosphothreonine standards, added to the samples before electrophoresis, were located by staining with ninhydrin.

RESULTS

Phosphorylation of Protein I by cAMP-dependent and Calcium-Calmodulin-dependent Protein Kinases—In the present study, Protein I was solubilized from rat brain and purified to apparent homogeneity. Purified Protein I was then used as substrate for the purified catalytic subunit of cAMP-dependent protein kinase and for a partially purified calcium-calmodulin-dependent protein kinase. Fig. 1 shows the protein staining pattern of the purified Protein I after SDS-polyacrylamide gel electrophoresis, as well as the autoradiographic pattern of purified Protein I after its phosphorylation by either protein kinase followed by SDS-polyacrylamide gel electrophoresis. Under the phosphorylation conditions used, phosphate incorporation into Protein I by either enzyme reached a plateau after 60 min (data not shown). Phosphorylation of Protein I by the calcium-calmodulin-dependent protein kinase was reduced by 90% or more if either calcium or calmodulin was omitted. The total amount of phosphate incorporated into Protein I in the presence of the calcium-calmodulin-dependent protein kinase was approximately five times the amount incorporated in the presence of the catalytic subunit of cAMP-dependent protein kinase.

Proteins Ia and Ib, phosphorylated for 60 min by the catalytic subunit of cAMP-dependent protein kinase or by calcium-calmodulin-dependent protein kinase, and then separated by SDS-polyacrylamide gel electrophoresis, were subjected to fingerprinting (Fig. 2). The phosphopeptide patterns of Proteins Ia and Ib were identical. Phosphorylation of Proteins Ia and Ib by the catalytic subunit of cAMP-dependent protein kinase occurred selectively in Peptide 1. In contrast, the calcium-calmodulin-dependent protein kinase phospho-
rylated Proteins Ia and Ib in Peptides 1 to 5, as well as in two other peptides designated 6 and 7. Evidence described below suggests that Peptides 6 and 7 are derived from Protein I rather than from a phosphoprotein present in the partially purified calcium-calmodulin-dependent protein kinase preparation. Although some phosphorylation of Peptides 6 and 7 was always observed, the extent of phosphorylation varied among experiments and was always significantly less than that of the major Peptides 1 to 5. Peptides 6 and 7 are designated by arrows only when their phosphorylation is readily apparent in the autoradiographs. (For comparison, the fingerprints of Proteins Ia and Ib phosphorylated in lysed synaptosomes in the presence of cAMP plus calcium as previously reported (11) are also presented in Fig. 2, and show the major Phosphopeptides 1 to 5.) Phosphoamino acid analysis of Peptide 1, phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, as well as of Peptides 1 to 7, phosphorylated by calcium-calmodulin-dependent protein kinase, indicated that phosphate had been incorporated in each case into serine residues.

The following observations indicated that Peptide 2 is contained in Peptide 4 and that Peptide 3 is contained in Peptide 5. The condition of tryptic-chymotrypsin digestion of phosphorylated Protein I affected the proportion of Peptide 2 to Peptide 4, and the proportion of Peptide 3 to Peptide 5: if Protein I was digested first in the presence of chymotrypsin for 24 h and then, after the addition of trypsin, in the presence of both proteases for another 24 h, relatively more Peptide 4 than 2, and more Peptide 5 than 3, was observed; reversing the sequence of protease addition resulted in the preferential production of Peptides 2 and 3. Furthermore, Peptide 4 was converted to some extent into Peptide 2, and Peptide 5 was converted into Peptide 3, when Peptides 4 and 5 were obtained separately after fingerprinting and subjected to another incubation in the presence of trypsin followed by two-dimensional separation.

**Fingerprinting of Phosphorylated Fragments of Protein I**—The pattern of phosphorylated fragments obtained from purified phosphorylated Protein I after limited proteolysis by *S. aureus* V8 protease and the relationship of these fragments to the phosphopeptides obtained after fingerprinting of Protein I is shown in Fig. 3. Limited proteolysis by *S. aureus* V8 protease of purified phosphorylated Protein I yielded the "upper" and "lower" fragments described previously (11). The upper *S. aureus* V8 protease fragments obtained from Proteins Ia and Ib, but not the lower fragments, showed differences in their apparent molecular weights similar to undigested Proteins Ia and Ib (data not shown). In addition, in some experiments, a "middle" fragment was observed which represented a proteolytic subfragment of the upper fragment (see below). (The extent to which this middle fragment was formed appeared to depend upon the batch and age of the protease used.) Purified Protein I was phosphorylated by the purified catalytic subunit of cAMP-dependent protein kinase selectively in the lower fragment. On the other hand, the phosphorylation of purified Protein I by calcium-calmodulin-dependent protein kinase occurred markedly on the upper and middle fragments and to a smaller extent on the lower fragment of Protein I. When fragments of Protein I, obtained after limited proteolysis, were separately subjected to fingerprinting, the pattern of Protein I fragments was the same as that for the fragments obtained after digestion with trypsin followed by two-dimensional separation, as described in the legend to Fig. 2.

**Fig. 3.** Autoradiographs showing phosphorylated fragments of Protein Ib obtained upon limited proteolysis by *S. aureus* V8 protease (SAP), and their tryptic-chymotryptic fingerprints. Protein I was phosphorylated either by the catalytic subunit of cAMP-dependent or calcium-calmodulin-dependent protein kinase and subjected to SDS-polyacrylamide gel electrophoresis, as described in the legend to Fig. 1. Protein Ib was then separately subjected to limited proteolysis in SDS-polyacrylamide gels by *S. aureus* V8 protease. Autoradiographs of the resultant Protein Ib fragments are shown in the side lanes of the figure (U, upper fragment; M, middle fragment; L, lower fragment). The indicated phosphorylated fragments were then subjected separately to fingerprinting, yielding the autoradiographs indicated by the arrows. In those cases in which different limited proteolysis fragments yielded qualitatively identical fingerprints, only one representative fingerprint is shown. Separation and designation of phosphopeptides were as described in the legend to Fig. 2.

**Fig. 4.** Autoradiographs showing phosphorylated fragments of Protein I obtained upon collagenase digestion, and their tryptic-chymotryptic fingerprints. The top left panel shows the pattern of Protein I and Protein I fragments (cf. Ref. 10), obtained upon incubation of purified phospho-Protein I in the absence (−) or presence (+) of collagenase followed by separation by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was immediately dried and autoradiographed. (The right lane was exposed longer to show all fragments.) Phospho-Protein I and the indicated phosphorylated fragments, obtained from stained-digested gels, were subjected separately to fingerprinting, yielding the autoradiographic patterns shown. Separation and designation of phosphopeptides were as described in the legend to Fig. 2.
ing, the lower fragment yielded Peptide 1, but not Peptides 2 to 7. Conversely, the upper and middle fragments yielded Peptides 2 to 7, but not Peptide 1. When Protein I fragments were obtained after limited proteolysis by α-chymotrypsin, and then separately subjected to fingerprinting, fragments of Protein I that had been phosphorylated by either the catalytic subunit of cAMP-dependent protein kinase or calcium-calmodulin-dependent protein kinase yielded Peptide 1, whereas fragments that had been phosphorylated only by calcium-calmodulin-dependent protein kinase yielded Peptides 2 to 7 (data not shown). The finding that Peptides 6 and 7 were always obtained together with Peptides 2 to 5 upon fingerprinting of limited proteolysis fragments of Protein I suggests that Peptides 6 and 7 were in fact derived from Protein 1.

Previous studies (10, 15, 30) had shown that Protein I contains collagenase-sensitive as well as collagenase-resistant domains. The relationship of phosphorylated fragments obtained from purified phospho-Protein I after collagenase digestion to the phosphopeptides obtained after fingerprinting of Protein I is shown in Fig. 4. When Protein I was phosphorylated in all sites by calcium-calmodulin-dependent protein kinase, and then digested by collagenase, several phosphorylated fragments described previously (10, 15), including the final collagenase-resistant fragment D, were observed. Most of the radioactivity, however, migrated upon SDS-polyacrylamide gel electrophoresis with the bromphenol blue front (Fig. 4, top left), but slower than radioactive ATP or inorganic phosphate. This material appeared to be a mixture of phosphopeptides, approximately 80% of which were lost upon staining of the gel. Fingerprint analysis of fragments A, B, and D, as well as of the fragment(s) at the dye front left after fixation, showed that fragment A contained only Peptides 1, 2, and 4, and that fragments B and D contained only Peptide 1. The fragments at the dye front contained Peptides 2 to 5 but not Peptide 1. When Protein I, phosphorylated by catalytic subunit of cAMP-dependent protein kinase, was digested by collagenase, the bulk of the radioactive phosphate incorporated remained in fragments A, B, and D, all of which yielded only Peptide 1 upon fingerprinting (data not shown).

Specificity of the Catalytic Subunit of cAMP-dependent Protein Kinase for the Various Phosphorylation Sites of Protein I—Under the phosphorylation conditions described thus far, the catalytic subunit of cAMP-dependent protein kinase phosphorylated Protein I only in Peptide 1. Phosphorylation of Protein I as a function of the concentration of catalytic subunit of cAMP-dependent protein kinase is shown in Fig. 5. The effectiveness of the various peptides as phosphorylation sites in intact Protein I was Peptide 1 > Peptides 2, 4 > Peptides 3, 5. Evidence was obtained that Peptide 2 and 4, phosphorylated at higher concentrations of catalytic subunit, were identical to Peptides 2 and 4 phosphorylated by calcium-calmodulin-dependent protein kinase (see Fig. 2): after elution of each of the corresponding phosphopeptides, pooling of equal amounts, and rerunning in the same two-dimensional electrophoresis-chromatography system, a single phosphopeptide spot was observed in the typical position of Peptide 2 and Peptide 4, respectively (data not shown).

 Autoradiographs of tryptic-chymotryptic fingerprints of Protein I that had been phosphorylated to various extents by catalytic subunit of cAMP-dependent protein kinase are shown in Fig. 6. Incubation of Protein I with 0.015 μM catalytic subunit resulted in the selective phosphorylation of Peptide 1 (Fig. 6A). When Protein I was incubated with a higher concentration of catalytic subunit (1.2 μM), phosphopeptide spots were not only seen in Peptide 1, but also in Peptides 2 and 4 and, to a smaller extent, into a few other peptides (Fig. 6B). Incubation of Protein I with even higher concentration of catalytic subunit (2.5 μM), at an elevated temperature (30°C) and for a prolonged time (90 min), led to phosphorylation and incorporation into Peptides 1 to 5 as well as into a substantial number of other peptides (Fig. 6C). These other peptides were

![Fig. 5](image-url) 
**Fig. 5.** Effect of various concentrations of catalytic subunit of cAMP-dependent protein kinase on multiple site phosphorylation of Protein I. Purified dephospho-Protein I (3 μg/100 μl) was phosphorylated for 60 min at 2°C with 7 μM [γ-32P]ATP in the presence of various concentrations of catalytic subunit of cAMP-dependent protein kinase. After phosphorylation, Protein I was subjected to SDS-polyacrylamide gel electrophoresis, and the Protein I region of the gel, containing Proteins Ia and Ib, was subjected to fingerprinting as described in the legend to Fig. 2. Phosphate incorporation into Phosphopeptides 1 to 5 was determined individually by liquid scintillation counting, with the results shown.

![Fig. 6](image-url)  
**Fig. 6.** Autoradiographs showing tryptic-chymotryptic fingerprints of Protein I phosphorylated to various extents by catalytic subunit of cAMP-dependent protein kinase. Purified dephospho-Protein I (3 μg/100 μl) was incubated with 7 μM [γ-32P]-ATP at the indicated temperature and time in the presence of the indicated concentration of catalytic subunit (CS). After phosphorylation, Protein I was subjected to SDS-polyacrylamide gel electrophoresis, and the Protein I region of the gel, containing Proteins Ia and Ib, was subjected to fingerprinting. Separation and designation of phosphopeptides were as described in the legend to Fig. 2.
Multiple Site Phosphorylation of a Specific Neuronal Protein

FIG. 7. Time course of multiple site phosphorylation of Protein I by catalytic subunit of cAMP-dependent protein kinase. Purified Protein I (5 μg/100 μl) was phosphorylated with 50 μM [γ-32P]ATP for the indicated times at 2°C in the presence of 0.7 μM catalytic subunit. After phosphorylation, Protein I was subjected to SDS-polyacrylamide gel electrophoresis, and the Protein I region of the gel, containing Proteins Ia and Ib, was subjected to limited proteolysis by S. aureus V8 protease. Phosphate incorporation into the upper, middle, and lower fragments of Protein I was determined by liquid scintillation counting. Since the upper and middle fragments contain the same phosphopeptides of Protein I (see Fig. 3), the results are given as phosphate incorporation into the lower fragment (LF) and into the upper plus middle fragments (UMF).

Also observed in tryptic fingerprints obtained from limited S. aureus V8 protease fragments of Protein I phosphorylated by catalytic subunit, indicating that they too were derived from Protein I (data not shown).

The time course of phosphorylation of various sites in Protein I was studied (Fig. 7) using a concentration of catalytic subunit (0.7 μM) which would catalyze the phosphorylation not only of Peptide 1, but also of Peptides 2 and 4 (see Fig. 5). In view of the defined relationship of these peptides to the limited S. aureus V8 protease fragments (see Fig. 3), phosphate incorporation was analyzed at the level of the limited S. aureus V8 protease fragments of Protein I. The time required for half-maximal phosphorylation of the upper plus middle fragments was at least 20-fold longer than that required for the lower fragment.

DISCUSSION

In the present study, phosphorylation of purified rat Protein I was investigated using purified cAMP-dependent protein kinase and calcium-calmodulin-dependent protein kinase which phosphorylates Protein I (39). The results indicate that cAMP-dependent protein kinase and calcium-calmodulin-dependent protein kinase have distinct specificities for the multiple phosphorylation sites in Protein I (Fig. 2). These sites can be divided into two categories, represented by Peptide 1 and by Peptides 2 to 5, respectively. The catalytic subunit of cAMP-dependent protein kinase selectively stimulated the phosphorylation of Peptide 1, as did cAMP in intact and lysed synaptosomes (31). Calcium-calmodulin-dependent protein kinase stimulated the phosphorylation not only of this peptide, but also caused a marked phosphate incorporation into Peptides 3 to 5, as did calcium entry into intact synaptosomes and calcium addition to synaptosomal lysates (11). Thus, the different specificities of cAMP-dependent protein kinase and calcium-calmodulin-dependent protein kinase observed for the multiple phosphorylation sites in purified Protein I appear to provide the enzymological basis for the differential regulation of phosphorylation of these sites in synaptosomal preparations by cAMP and calcium, respectively.

The classification of phosphorylation sites into Peptide 1 and Peptides 2 to 5 is supported by the results of limited proteolysis of Protein I by S. aureus V8 protease (Fig. 3) or chymotrypsin. Each limited proteolysis fragment of Protein I yielded, upon separate tryptic fingerprinting, either Peptide 1 or Peptides 2 to 5. These results suggested that Peptide 1 is derived from a region of the Protein I molecule that is distinct from the region which yields Peptides 2 to 5. Previously, it was described (10, 15) that Proteins Ia and Ib each consisted of two principal domains, a globular collagenase-resistant portion (referred to as fragment D), and an elongated collagenase-sensitive portion. The present results (Fig. 4) indicate that Peptide 1 is located in the collagenase-resistant portion of Protein I, whereas Peptides 2 to 5 originate from the collagenase-sensitive portion of the molecule. This location of Peptides 2 to 5 is of interest, in view of recent findings (31) that the collagenase-sensitive portion of Protein I is involved in the binding of Protein I to membranes.

Peptides 1 to 5 apparently represent a minimum of three sites of phosphorylation. One site appears to be represented by Peptide 1. However, since both the catalytic subunit of cAMP-dependent protein kinase and calcium-calmodulin-dependent protein kinase phosphorylate this peptide, it is possible that different serine residues are phosphorylated by the two enzymes. Several lines of evidence indicate that Peptides 2 to 5 represent two, not four, distinct sites, and that these peptides originate from two distinct regions located at the end of the collagenase-sensitive portion of Protein I. First, fragment A of Protein I, produced by collagenase digestion, no longer contained Peptides 3 and 5, but still contained Peptides 2 and 4, and fragment B no longer contained Peptides 2 and 4 (Fig. 4). Second, phosphate incorporation into Peptides 2 and 4 was very similar when Protein I was phosphorylated by various concentrations of the catalytic subunit of cAMP-dependent protein kinase, and was considerably different from that of Peptides 3 and 5 (Fig. 5). Third, the proportion of Peptide 2 to Peptide 4, and of Peptide 3 to Peptide 5, were affected by the condition of tryptic-chymotryptic digestion. Furthermore, Peptides 4 and 5 were convertible to some extent into Peptides 2 and 3, respectively, by further incubation in the presence of trypsin. Thus, it appears that Peptides 2 and 4 represent one phosphorylation site, and that Peptides 3 and 5 represent a second, distinct phosphorylation site.

Several observations suggest that phosphate incorporation into Peptides 2 to 5 catalyzed by cAMP-dependent protein kinase (in contrast to that catalyzed by calcium-calmodulin-dependent protein kinase) has little physiological relevance: 1) neither in intact nor in lysed synaptosomes did cAMP significantly stimulate the phosphorylation of Peptides 2 to 5 (11); 2) when purified Protein I as substrate, the effectiveness of Peptides 2 to 5 as phosphorylation sites for the catalytic subunit was considerably less than that of Peptide 1 (Figs. 5 and 7); and 3) the concentration of catalytic subunit required for phosphorylation of Peptides 2 to 5 was high and led to phosphorylation of other peptides (Fig. 6). The phosphorylation of which was not observed in intact synaptosomes (11).

The findings described in the present study and in our previous report (11) demonstrate that two distinct second messengers, cAMP and calcium, acting via stimulation of cAMP- and calcium-dependent protein kinase, respectively, differentially regulate the phosphorylation of multiple sites in

W. B. Huttner, P. De Camilli, and P. Greengard, manuscript in preparation, as cited by Greengard (14).
multiple Site Phosphorylation of a Specific Neuronal Protein

the same protein, Protein I. Several proteins in addition to Protein I have been found to be phosphorylated in multiple sites by cAMP- and calcium-dependent protein kinases (29, 32-35). In the case of Protein I, intriguing observations pointing to an important role of this protein in the functioning of synaptic terminals are its specific association with synaptic vesicles (17, 18), its interesting molecular properties (15), and the biological regulation of its phosphorylation as evidenced by a variety of physiological experiments (7-13). The present results should make it possible to study the effects of phosphorylation of specific sites of Protein I on its structure and on its interaction with synaptic vesicles, and therefore should help in understanding the precise role of Protein I in synaptic transmission.

REFERENCES
1. Rubin, C. S., and Rosen, O. M. (1975) Annu. Rev. Biochem. 44, 831-887
2. Nimmo, H. G., and Cohen, P. (1977) Adv. Cyclic Nucleotide Res. 8, 145-206
3. Greengard, P. (1978) Science 199, 146-152
4. Krebs, E. G., and Beavo, J. A. (1979) Annu. Rev. Biochem. 48, 923-959
5. Greengard, P. (1978) Cyclic Nucleotides, Phosphorylated Proteins, and Neuronal Function, Raven Press, New York
6. Ueda, T., Maeno, H., and Greengard, P. (1973) J. Biol. Chem. 248, 8295-8305
7. Strombom, U., Forn, J., Dolphin, A. C., and Greengard, P. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4687-4690
8. Krueger, B. K., Forn, J., and Greengard, P. (1977) J. Biol. Chem. 252, 2764-2773
9. Forn, J., and Greengard, P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5195-5199
10. Sieghart, W., Forn, J., and Greengard, P. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2475-2479
11. Hutner, W. B., and Greengard, P. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5402-5406
12. Dolphin, A. C., and Greengard, P. (1980) Nature, in press
13. Neuder, E. J., and Greengard, P. (1980) Proc. Natl. Acad. Sci. U. S. A., in press
14. Greengard, P. (1980) Harvey Lect., in press
15. Ueda, T., and Greengard, P. (1977) J. Biol. Chem. 252, 5155-5163
16. De Camilli, P., Ueda, T., Bloom, F. E., Battenberg, E., and Greengard, P. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5977-5981
17. Bloom, F. E., Ueda, T., Battenberg, E., and Greengard, P. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5982-5986
18. Ueda, T., Greengard, P., Berzins, K., Cohen, R. S., Blomberg, F., Grab, D. J., and Siekert, V. (1979) J. Cell Biol. 83, 308-319
19. De Camilli, P., Cameron, R., and Greengard, P. (1980) J. Cell Biol. 87, 72a
20. Glynn, I. M., and Chappell, J. B. (1964) Biochem. J. 93, 147-149
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 285-295
22. Chou, C.-K., Alfano, J., and Rosen, O. M. (1977) J. Biol. Chem. 252, 2858-2869
23. Beavo, J. A., Bechtle, P. J., and Krebs, E. G. (1974) Methods Enzymol. 48C, 299-309
24. Schulman, H., and Greengard, P. (1978) Nature 271, 478-479
25. Schulman, H., and Greengard, P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5432-5436
26. Watterson, D. M., Harrelson, W. G., Keller, P. M., Sharief, F., and Vanaman, T. C. (1976) J. Biol. Chem. 251, 4501-4513
27. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1192-1196
28. Axelrod, N. (1978) Virology 87, 396-393
29. Alper, S. L., Palfrey, H. C., DeRiemer, S. A., and Greengard, P. (1980) J. Biol. Chem. 255, 11029-11039
30. Lohmann, S. M., Ueda, T., and Greengard, P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4037-4041
31. Ueda, T. (1980) J. Neurochem., in press
32. Roach, P. J., DePaoli-Roach, A. A., and Larner, J. (1978) J. Cyclic Nucleotide Res. 4, 245-257
33. Rylatt, D. B., Embi, N., and Cohen, P. (1979) FEBS Lett. 98, 76-81
34. Srivastava, A. K., Waisman, D. M., Brostrom, C. O., and Soderling, T. R. (1979) J. Biol. Chem. 254, 583-596
35. LePesch, C. L., Haiech, J., and Demaille, J. G. (1979) Biochemistry 18, 5150-5157
36. Kennedy, M. B., and Greengard, P. (1981) Proc. Natl. Acad. Sci. U. S. A., in press