Proc. Natl. Acad. Sci. USA
Vol. 76, No. 10, pp. 5402–5406, October 1979
Cell Biology

Multiple phosphorylation sites in protein I and their differential regulation by cyclic AMP and calcium

(protein phosphorylation/phosphoprotein/synaptosomes/peptide mapping)

WIELAND B. HUTTNER AND PAUL GREENGARD

Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

Contributed by Paul Greengard, July 16, 1979

ABSTRACT The phosphorylation of protein I, a specific neuronal protein, has been found to be regulated both by cyclic AMP (cAMP) and by calcium, in intact as well as in lysed synaptosome preparations from rat brain. In order to determine the phosphorylation site(s) of protein I that were regulated by cAMP and calcium, protein I was purified after it was phosphorylated under various conditions. This purified protein I was then subjected either to peptide mapping after limited proteolysis in sodium dodecyl sulfate/polyacrylamide gels or to tryptic fingerprinting. 8-Br-cAMP selectively increased the phosphorylation of the same protein I peptide fragment in both intact and lysed synaptosomes. Depolarization-induced calcium influx into intact synaptosomes, or the addition of calcium to lysed synaptosomes, caused a stimulation of the phosphorylation not only of this peptide but also of other distinct peptides. Differential regulation by cAMP and calcium of the phosphorylation of multiple sites on the same neuronal protein may provide a molecular basis for interactions between these two second-messenger systems in certain nerve terminal functions.

Proteins Ia and Ib, collectively referred to as protein I because their properties are similar, are principal substrates for cyclic AMP (cAMP)-dependent protein phosphorylation in synaptic membrane preparations (1–3). Depolarization-induced calcium influx into intact synaptosomes also stimulates the phosphorylation of proteins Ia and Ib (4, 5). Protein I appears to be a specific component of certain synapses throughout the nervous system (6, 7). Addition of cAMP or depolarizing agents to rat brain slices (8) or administration of convulsant and depressant agents to intact mice (9) affects the state of phosphorylation of protein I. In this study we describe the regulation of phosphorylation of protein I both by cAMP and by calcium and analyze the sites of phosphorylation, in intact and in lysed synaptosomes.

METHODS

Preparation of Intact and Lysed Synaptosomes. Crude synaptosome suspensions were prepared from rat brain as described (4). The P2 pellet was resuspended (1–2 mg of protein per ml) in oxygenated Krebs–Ringer buffer (KRB) (4) that contained 1.1 mM $CaCl_2$ and 0.1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. The suspension was incubated for 10 min at 37°C in an atmosphere of O_2. To one-half of the synaptosome suspension, inorganic [^{32}P]phosphate was then added (1.25 mCi/ml of KRB; 1 Ci = 3.7×10^{10} becquerels) and these intact synaptosomes were incubated at 37°C under O_2 for 30 min in order to label the intrasynaptosomal ATP. The other half of the synaptosome suspension was centrifuged at 4°C for 15 min at 12,500 × *g*. The pellet was resuspended in 10 vol of ice-cold water, homogenized by 12 strokes in a glass/Teflon homogenizer at 1200 rpm, and allowed to stand on ice for 30 min. This material is referred to as lysed synaptosomes.

Protein Phosphorylation in Intact and Lysed Synaptosomes. To study protein phosphorylation in intact synaptosomes, an 80-μl aliquot of the labeled intact synaptosome suspension was added to 20 μl of standard KRB containing various cyclic nucleotides or phosphodiesterase inhibitors, as indicated, and the samples were incubated for 10 min at 37°C; 50 μl of standard KRB containing various depolarizing agents was then added, as indicated, and the incubation was continued for another 30 sec. The reaction was terminated in either of two ways: addition of 75 μl of sodium dodecyl sulfate ($NaDodSO_4$) stop solution (2) to the sample followed by immediate boiling, or rapid freezing of the sample by placing it in a dry ice/acetone bath.

To study protein phosphorylation in lysed synaptosomes, aliquots of the synaptosomal lysate were preincubated for 10 min at 4°C in a reaction mixture that gave (final concentrations) after addition of ATP: 10 mM $MgCl_2$, 1 mM ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid, 50 mM Hepes·NaOH, pH 7.4, with or without 10 μM 8-Br-cAMP and 1.3 mM $CaCl_2$ (0.3 mM free Ca^{2+}). The phosphorylation reaction was started by the addition of 30 μl of [γ-^{32}P]ATP (final concentration, 7 μM; 2×10^7 cpm/nmol) to a final volume of 100 μl, and the reaction was carried out for 30 sec at 4°C and terminated as described above for intact synaptosomes.

Isolation of Crude Protein I. When the phosphorylation reactions had been terminated by $NaDodSO_4$ stop solution, the samples were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis in long (13 cm) 7.5% polyacrylamide slab gels as described (2). The gels were fixed, stained, destained, dried, and autoradiographed as described (2), and the radioactive bands containing phosphorylated proteins Ia and Ib were cut from the dried gel by using the autoradiograph as a guide. This material is referred to as "crude protein I." Radioactivity in the dried gel pieces was determined by liquid scintillation counting.

Isolation of Purified Protein I. When the phosphorylation reactions had been terminated by rapid freezing in dry ice/acetone, the samples were subjected to nonequilibrium pH gradient electrophoresis (NEPHGE) according to O'Farrell *et al.* (10) except that slab gels and 2% Ampholine (pH 3.5–10) were used throughout. The frozen samples were lyophilized, and the residues were solubilized by the addition of 50 μl of $NaDodSO_4$/urea buffer followed by 50 μl of lysis buffer as described (10), except that the lysis buffer contained 5% (wt/vol) Nonidet P-40 and a trace amount of pyronin Y to monitor subsequent electrophoresis. The entire amount of each sample was applied, immediately after solubilization, to a single

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; cAMP, cyclic AMP; KRB, Krebs–Ringer buffer; SAP, *Staphylococcus aureus* V8 protease; NEPHGE, nonequilibrium pH gradient electrophoresis.

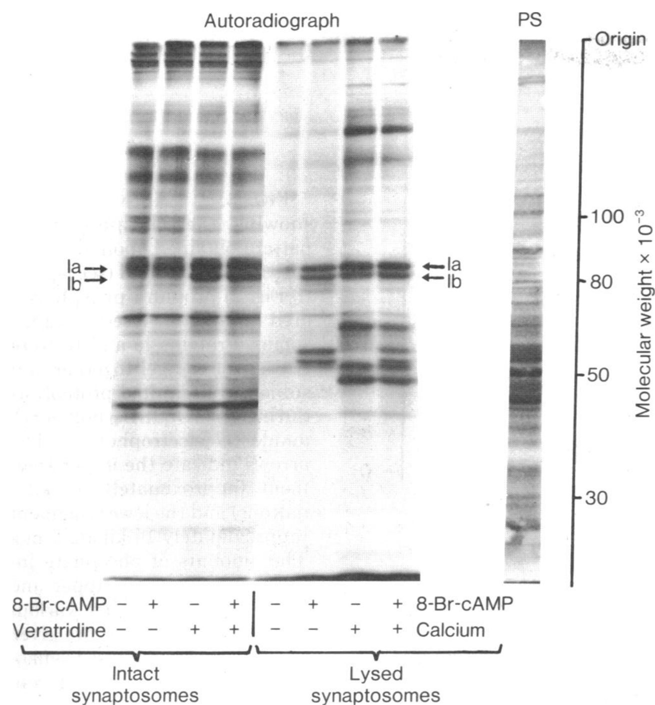

FIG. 1. Effect of cAMP and calcium on protein phosphorylation in intact and lysed synaptosomes. Intact synaptosomes, prelabeled with $^{32}P_i$, were incubated in KRB containing 1 mM free Ca^{2+}, in the absence or presence of 4 mM 8-Br-cAMP and 100 μM veratridine. Lysed synaptosomes were phosphorylated with [γ-^{32}P]ATP, in the absence or presence of 10 μM 8-Br-cAMP and 0.3 mM free Ca^{2+}. After phosphorylation, samples were subjected to one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis followed by protein staining (PS) and autoradiography.

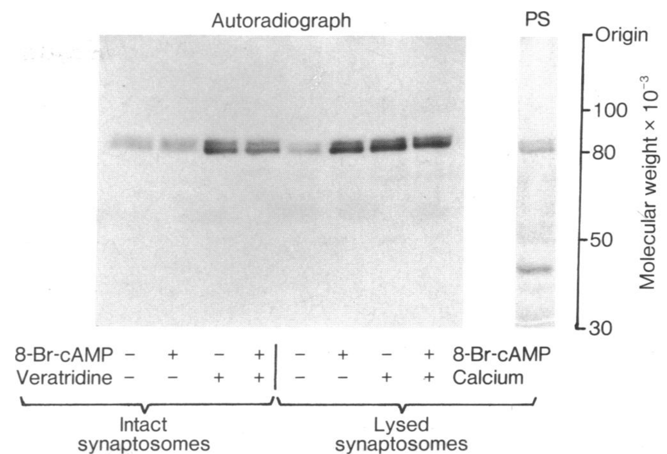

FIG. 2. Purification of protein I after its phosphorylation in intact and lysed synaptosomes. Phosphorylation was performed as described in the legend to Fig. 1. After phosphorylation, samples were subjected to NEPHGE on a polyacrylamide gel. The protein I region of the gel was cut out and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis, followed by protein staining (PS) and autoradiography of the latter gel.

slot of the gel and overlayered with 50 μl of overlay solution (10) containing 5% Nonidet P-40 and a trace amount of bromphenol blue. Electrophoresis was performed at 400 V for 4 hr as described (10). After fixing, staining, destaining, and autoradiography of the wet NEPHGE gel, the protein I region was cut out. The wet gel pieces were soaked with water to remove the destaining solution, equilibrated with O'Farrell sample buffer (11) for 30–60 min, and boiled in this buffer for 1 min, after which the buffer was immediately decanted. Each gel piece was then placed into a slot of a NaDodSO$_4$/polyacrylamide slab gel (4.5% stacking gel; 10% separating gel), and the proteins were subjected to a second electrophoresis step as described (2). After processing of this second gel as described above for the isolation of crude protein I, "purified protein I" was cut out from the protein I region of the gel and its radioactivity was determined by liquid scintillation counting. The dried gel pieces were then washed with ether to remove the scintillation fluid and processed for peptide mapping or tryptic fingerprinting as described below.

The recovery of protein I through this procedure, with pure [^{32}P]protein I (2) as standard, was 85%. No protein I could be recovered from any region of the NEPHGE gel other than the one routinely processed for the second polyacrylamide gel.

Peptide Mapping after Limited Proteolysis of Phosphorylated Protein I. Peptide mapping after limited proteolysis was performed according to Cleveland *et al.* (12) with the following modifications: gel pieces containing purified protein Ia or Ib or both were reswollen for 1 hr in 125 mM Tris·HCl, pH 6.8/0.1% NaDodSO$_4$ and were placed into the slots of a long NaDodSO$_4$/polyacrylamide gel (4.5-cm-long 3% stacking gel; 11.5-cm-long 15% separating gel). The gel pieces were overlayered with 100 μl of the same buffer containing 15% (wt/vol) glycerol, a trace amount of pyronin Y, and 10 μg of *Staphylococcus aureus* V8 protease (SAP; Miles). Electrophoresis was carried out at 60 V. After the dye had reached the bottom of the gel, the gel was dried and autoradiographed. Radioactive bands were located by using the autoradiograph, the bands were cut from the gel, and radioactivity was determined by liquid scintillation counting. In some experiments, the scintillation fluid was removed by ether, the gel pieces were reswollen and soaked in acetic acid/methanol/water, 1:5:4 (vol/vol), washed in 50% (vol/vol) methanol, and finally lyophilized. The gel pieces were then processed for tryptic fingerprinting as described below.

Tryptic Fingerprints of Phosphorylated Protein I. Dried gel pieces containing purified protein Ia or Ib or, in some experiments, lyophilized gel pieces containing fragments of protein Ia or Ib obtained after limited proteolysis, were reswollen (see ref. 13) in 700 μl of 100 mM ammonium bicarbonate, pH 8.0/1 mM dithioerythritol containing a trace amount of phenol red and 150 μg of trypsin (Sigma, type I, twice crystallized) per ml. Incubation was carried out for 24–30 hr at 37°C. Then the gel piece was removed, and the eluate, which contained 85% of the original radioactivity, was lyophilized. The residue was dissolved in 30 μl of electrophoresis buffer [10% (vol/vol) acetic acid/1% (vol/vol) pyridine, pH 3.5], and 20 μl was spotted on cellulose plates (Eastman). Separation of phosphopeptides was performed according to Axelrod (14), with electrophoresis for 90 min at 400 V in the first dimension and ascending chromatography in n-butanol/pyridine/acetic acid/water, 37.5:25:7.5:30 (vol/vol), in the second dimension. After autoradiography of the dried cellulose plate, radioactivity in the phosphopeptide spots was determined by liquid scintillation counting.

The phosphopeptide patterns obtained after digestion with trypsin under these conditions apparently resulted not only from tryptic activity but also from contaminating chymotryptic activity: when protein I was digested with TPCK-trypsin (Worthington), trypsin that had been treated to inactivate contaminating chymotrypsin, a different phosphopeptide pattern was obtained in which the cAMP-regulated phosphopeptide was not clearly separated from one of the Ca^{2+}-regu-

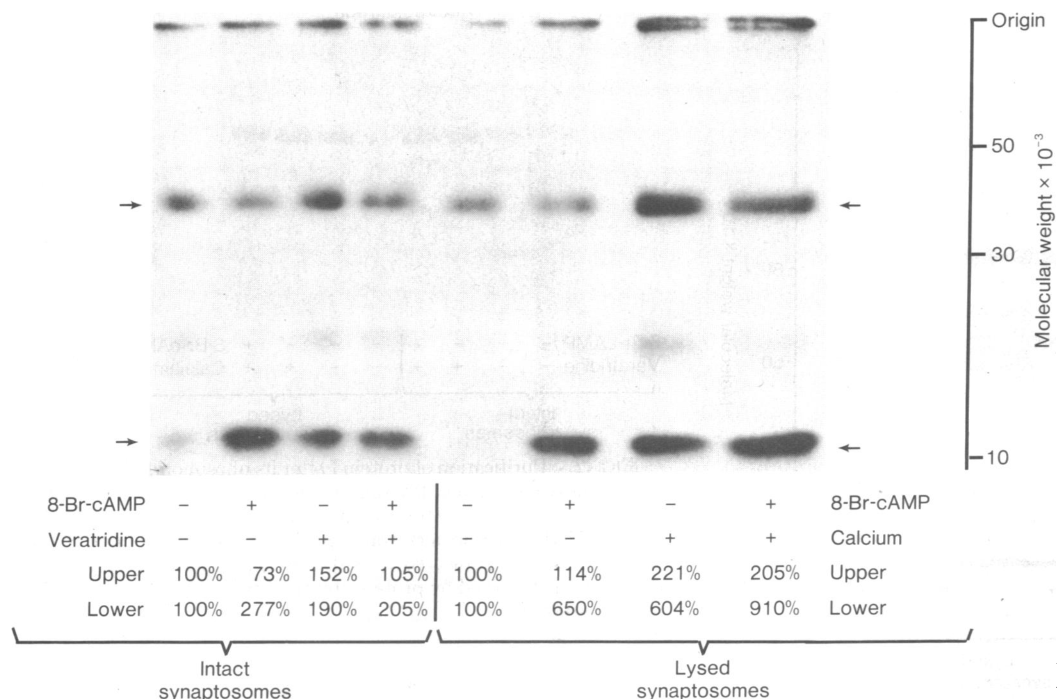

FIG. 3. Autoradiograph showing the phosphopeptide pattern obtained upon digestion with SAP of purified (see legend to Fig. 2) protein I phosphorylated in intact or lysed synaptosomes. Proteins Ia and Ib were cut out of the gel together and subjected to limited proteolysis during $NaDodSO_4$/polyacrylamide gel electrophoresis. The arrows indicate the upper fragment (approximately 35 kilodaltons) and the lower fragment (approximately 10 kilodaltons). The amounts of phosphate incorporated into the upper and lower fragments under various incubation conditions are given for both intact and lysed synaptosomes as percentage of control.

lated phosphopeptides; addition of chymotrypsin (100 μg/ml) either to trypsin (Sigma) or to TPCK-treated trypsin (Worthington) yielded the same phosphopeptide pattern as did trypsin (Sigma) to which no chymotrypsin had been added. The phosphopeptide pattern obtained with trypsin (Sigma) did not change significantly when larger amounts of trypsin or longer incubation times (up to 50 hr) were used. Also, the presence of penicillin (200 units/ml) plus streptomycin (200 μg/ml) during the incubation had no effect on the peptide pattern.

RESULTS

Phosphorylation of Protein I in Intact and Lysed Synaptosomes. The pattern of protein phosphorylation in intact and lysed synaptosome preparations, as revealed by $NaDodSO_4$/polyacrylamide gel electrophoresis, is shown in Fig. 1. In intact synaptosomes incubated in the presence of calcium, addition of the depolarizing agent veratridine led to an increased phosphorylation predominantly of two protein bands at 86–80 kilodaltons in the gel; an effect of 8-Br-cAMP could not be detected by this type of analysis of protein I (however, see below). In lysed synaptosomes, the addition of either calcium or 8-Br-cAMP resulted in increased phosphorylation of a number of protein bands. The phosphorylation of two proteins migrating at 86–80 kilodaltons was markedly stimulated by each of these agents.

For the peptide mapping studies, phosphorylated protein I was purified from intact and lysed synaptosomes. For this purpose, a two-dimensional separation technique for basic proteins involving NEPHGE and $NaDodSO_4$/polyacrylamide gel electrophoresis (10) was adapted to slab gels in order to facilitate comparison of a large number of samples. Owing to the high isoelectric points of proteins Ia and Ib (2), both proteins migrated ahead of the bulk of the other proteins in the NEPHGE gel (data not shown). When the region of the NEPHGE gel that contained proteins Ia and Ib was subjected to a second electrophoresis step on $NaDodSO_4$/polyacrylamide gel (Fig. 2), proteins Ia and Ib appeared as major proteins by Coomassie blue staining and as the only ^{32}P-labeled proteins detectable except for a small amount of material migrating with the dye front. Several facts indicated that the phosphoprotein bands of 86 and 80 kilodaltons obtained after this purification were proteins Ia and Ib: both proteins comigrated with pure [^{32}P]protein I standard in both NEPHGE and subsequent $NaDodSO_4$/polyacrylamide gel electrophoresis; in addition, no radioactivity appeared in positions corresponding to the two bands when samples were incubated with highly purified collagenase (under conditions in which [^{32}P]protein I standard was degraded) prior to the two electrophoresis steps (data not shown).

When protein I was purified by NEPHGE plus $NaDodSO_4$/polyacrylamide gel electrophoresis, an effect of veratridine (Fig. 2), but not of 8-Br-cAMP (however, see the results of peptide mapping described below), was observed on protein I phosphorylation in intact synaptosomes. In contrast, both calcium and 8-Br-cAMP affected the total amount of phosphate incorporated into protein I in lysed synaptosomes when purified protein I was used for analysis.

Peptide Mapping after Limited Proteolysis of Phosphorylated Protein I. The phosphopeptide patterns of purified proteins Ia and Ib, obtained after limited proteolysis with SAP, were qualitatively similar to one another (unpublished data). When proteins Ia and Ib were combined prior to limited proteolysis with SAP, the phosphopeptide pattern shown in Fig. 3 was obtained. With the protease concentration used, autoradiography of the gel revealed undigested proteins Ia and Ib at the top of the gel, together with an upper peptide fragment of approximately 35 kilodaltons and a lower peptide fragment of approximately 10 kilodaltons. In intact as well as in lysed synaptosomes, 8-Br-cAMP selectively stimulated the phosphorylation of the lower fragment without increasing the phosphorylation of the upper fragment. Veratridine-induced calcium influx into intact synaptosomes, or the addition of calcium to lysed synaptosomes, stimulated the phosphorylation of both the upper and lower fragments. In intact synaptosomes, 8-Br-cAMP decreased the amount of radioactive phosphate incorporated into the upper fragment, both in the absence and in the presence of veratridine. No such inhibitory effect of 8-Br-cAMP was observed with lysed synaptosomes, under the conditions tested.

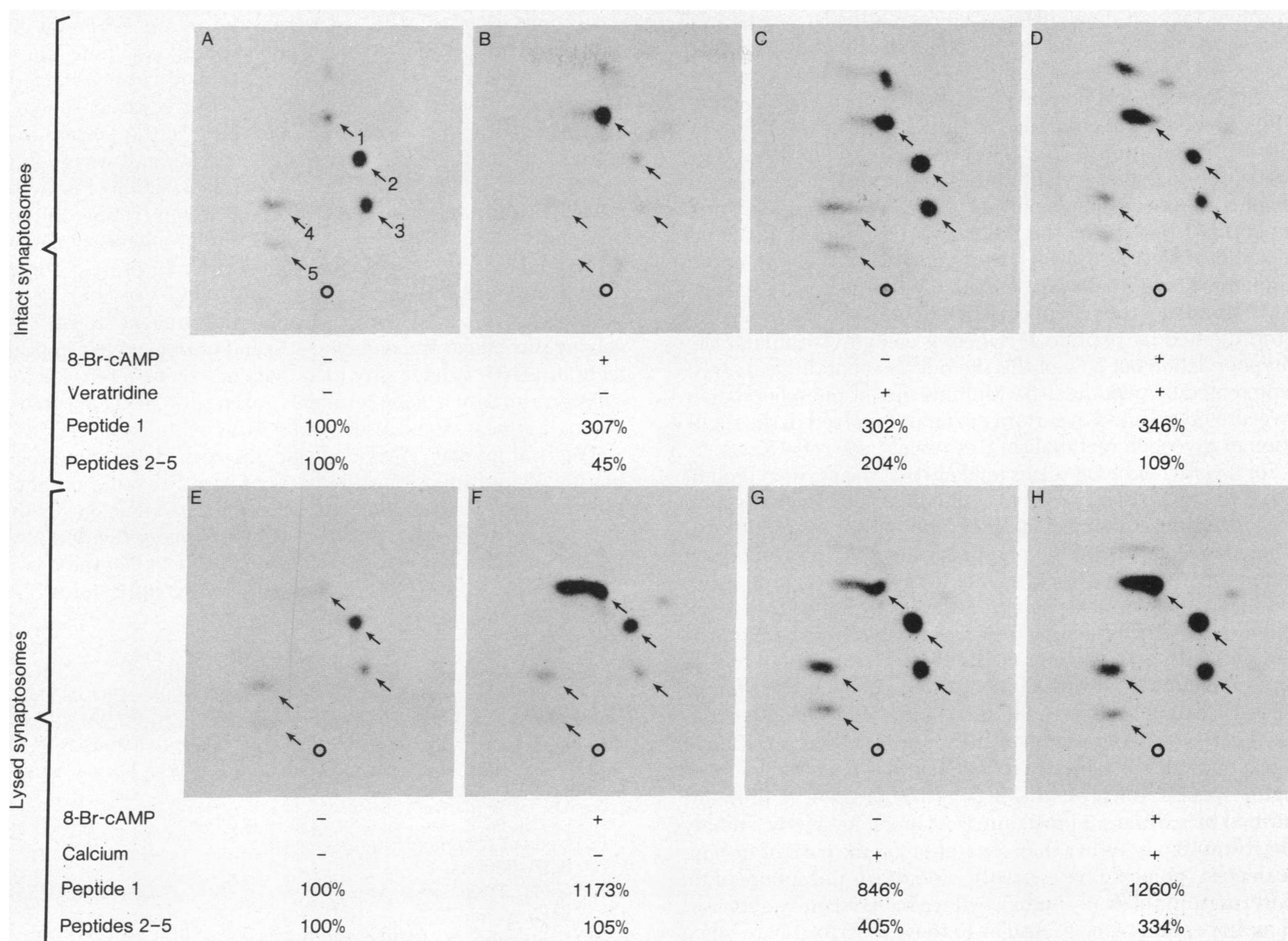

FIG. 4. Autoradiographs showing tryptic fingerprints of purified phosphorylated protein Ib. Protein I was phosphorylated, in intact or lysed synaptosomes, and purified as described in the legend to Fig. 2. Proteins Ia and Ib were cut out of the gel separately and subjected to exhaustive digestion by trypsin. Phosphopeptides were spotted on cellulose plates (O designates origin) and were separated in two dimensions, first by electrophoresis in the horizontal dimension (negative pole left; positive pole right) and then by ascending chromatography in the vertical dimension. In each autoradiograph, the five arrows indicate peptides 1–5, as designated in *A*. The amounts of phosphate incorporation into peptide 1 and into peptides 2–5 under various incubation conditions are given for both intact and lysed synaptosomes as percentage of control.

In experiments with intact synaptosomes, the stimulation by 8-Br-cAMP of the phosphorylation of the lower fragment could be mimicked by 4 mM monobutyryl cAMP or 1 mM 3-isobutyl-1-methylxanthine but not by 4 mM 8-Br-cyclic GMP (data not shown). Omission of calcium from the KRB did not affect the stimulation of phosphorylation of the lower fragment by any of these agents. Confirming and extending previous observations with intact synaptosomes (4), the effect of veratridine on the phosphorylation of both the upper and lower fragments could be mimicked by high potassium concentration. The action of both veratridine and potassium was dependent on the presence of external Ca^{2+} (data not shown).

Tryptic Fingerprints of Phosphorylated Protein I. Tryptic fingerprints of purified proteins Ia and Ib were qualitatively similar to one another. Fig. 4 shows the two-dimensional separation of phosphopeptides derived from purified protein Ib after exhaustive digestion with trypsin. Five phosphopeptides, designated peptides 1–5, were observed both in intact and in lysed synaptosomes. 8-Br-cAMP selectively stimulated the phosphorylation of peptide 1, both in intact and in lysed synaptosomes. Veratridine-induced calcium influx into intact synaptosomes or the addition of calcium to lysed synaptosomes stimulated the phosphorylation of peptide 1, as did 8-Br-cAMP, but in addition stimulated the phosphorylation of peptides 2–5. In intact synaptosomes, 8-Br-cAMP decreased the amount of radioactive phosphate incorporated into peptides 2–5, both in the absence and in the presence of veratridine. No significant inhibitory effect of 8-Br-cAMP was observed with lysed synaptosomes, under the conditions tested.

In order to relate the phosphopeptide pattern obtained after limited proteolysis with SAP to peptides 1–5 of the tryptic fingerprint, the upper and lower fragments obtained upon limited proteolysis with SAP (Fig. 3) were separately subjected to exhaustive digestion with trypsin followed by two-dimensional separation of the phosphopeptides. The SAP lower fragment yielded peptide 1 and the SAP upper fragment yielded peptides 2–5.

DISCUSSION

The results of the present study indicate that (*i*) in intact synaptosomes, cAMP analogs or agents causing depolarization-induced calcium influx stimulate the phosphorylation of protein I, (*ii*) these two types of stimulation of phosphorylation can be mimicked in lysed synaptosomes by activation of the respective protein phosphorylation system upon additon of 8-Br-cAMP or calcium, and (*iii*) there are multiple phosphorylation sites

in protein I which show differential regulation by cAMP and by calcium. Stimulation of the phosphorylation of protein I by 8-Br-cAMP, both in intact and in lysed synaptosomes, occurred selectively on the SAP lower fragment (Fig. 3), and on peptide 1 (Fig. 4) which was contained in the SAP lower fragment. In contrast, stimulation of the phosphorylation of protein I by veratridine in intact synaptosomes and by calcium in lysed synaptosomes occurred not only on the SAP lower fragment and on peptide 1 but also on the SAP upper fragment (Fig. 3) and on peptides 2–5 (Fig. 4) which were contained in the SAP upper fragment. These results are consistent with the possibility that cAMP stimulates the phosphorylation of only one site in protein I (represented by peptide 1), whereas calcium stimulates the phosphorylation not only of this site but also of additional site(s) (represented by peptides 2–5). Multiple sites of phosphorylation have also been found in certain enzymes involved in the regulation of glycogen metabolism (for review, see ref. 15).

For several reasons, it seems unlikely that the purified protein Ia and Ib bands (Fig. 2) used for limited proteolysis and tryptic fingerprinting contained another phosphoprotein that contributed one or more of the phosphorylated peptides, observed upon proteolysis, that we attribute to protein I: (*i*) proteins Ia and Ib gave similar phosphopeptide patterns, and therefore any contaminating phosphoprotein would have to have comigrated similarly with both proteins Ia and Ib; (*ii*) treatment of the synaptosomal material with highly purified collagenase prior to the purification of protein I indicated that all radioactive material in the protein I region of the gel (Fig. 2) was sensitive to digestion with collagenase [collagenase has been shown to be highly specific for protein I (2, 5)]; (*iii*) digestion of protein I purified by a different procedure (i.e., precipitation by a highly specific antibody rather than separation on the basis of its high isoelectric point) gave virtually identical phosphopeptide patterns (unpublished results); (*iv*) we have recently observed phosphopeptide patterns similar to those described here when exogenous protein I, purified to homogeneity as previously described (2) was used as substrate (unpublished results).

It was important for the present study that the state of phosphorylation of protein I existing at the end of the incubation period not be altered during its purification. That this was in fact the case can be concluded from the following observations. Three different procedures were used to terminate the reaction and to obtain protein I after its phosphorylation in intact or lysed synaptosomes: (*i*) immediate boiling of the sample in $NaDodSO_4$ stop solution followed by $NaDodSO_4$/polyacrylamide gel electrophoresis; (*ii*) rapid freezing with subsequent solubilization in Nonidet P-40/9.5 M urea followed by NEPHGE and $NaDodSO_4$/polyacrylamide gel electrophoresis; and (*iii*) addition of 5 mM Zn^{2+} [which has been used to preserve the state of phosphorylation of protein I (8)] followed by immunoprecipitation and $NaDodSO_4$/polyacrylamide gel electrophoresis. Digestion of protein I obtained by these three methods and analysis of the phosphorylation sites led to identical conclusions concerning regulation of phosphorylation by cAMP and by calcium (unpublished results).

In a previous study (4), it was found that depolarizing agents, acting via calcium influx, increased the phosphorylation of protein I in subcellular fractions from rat brain containing intact synaptosomes. It was concluded that phosphorylation occurred in the intact isolated axon terminals themselves rather than in intact dendritic or non-neuronal particles. In the present investigation, using the same type of preparation, it was found that 8-Br-cAMP also stimulated the phosphorylation of protein I and, in addition, modified the effect of veratridine on the phosphorylation of protein I. These results suggest that the action of 8-Br-cAMP on the phosphorylation of protein I, like that of depolarizing agents, occurred in intact isolated axon terminals. Consistent with this suggestion, recent studies involving subcellular fractionation (16) and immunocytochemical techniques (6, 7) have provided evidence that protein I is localized primarily in axon terminals at certain types of synapses, where it appears to be associated with synaptic vesicles.

The finding that cAMP and calcium cause differential regulation of multiple-site phosphorylation of the same protein, protein I, is of interest. Interactions between cAMP and calcium at the level of phosphorylation of protein I may provide a molecular basis for physiological interactions between these two intracellular second messengers in those synaptic terminals containing this protein.

We thank Drs. Seth Alper and Dana Aswad for their helpful advice on some of the separation techniques used in this study. This work was supported by U.S. Public Health Service Grants DA-01627, MH-17387, and NS-08440 and a grant from the McKnight Foundation. W.B.H. was the recipient of a fellowship from the Deutsche Forschungsgemeinschaft.

1. Ueda, T., Maeno, H. & Greengard, P. (1973) *J. Biol. Chem.* **248**, 8295–8305.
2. Ueda, T. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 5155–5163.
3. Greengard, P. (1978) *Cyclic Nucleotides, Phosphorylated Proteins and Neuronal Function* (Raven, New York).
4. Krueger, B. K., Forn, J. & Greengard, P. (1977) *J. Biol. Chem.* **252**, 2764–2773.
5. Sieghart, W., Forn, J. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2475–2479.
6. Bloom, F. E., Ueda, T., Battenberg, E. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, in press.
7. DeCamilli, P., Ueda, T., Bloom, F. E., Battenberg, E. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, in press.
8. Forn, J. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **75**, 5195–5199.
9. Strombom, U., Forn, J., Dolphin, A. C. & Greengard, P. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4687–4690.
10. O'Farrell, P. Z., Goodman, H. W. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142.
11. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
12. Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106.
13. Kelly, P. T. & Cotman, C. W. (1978) *J. Cell Biol.* **79**, 173–183.
14. Axelrod, N. (1978) *Virology* **87**, 366–383.
15. Cohen, P. (1978) *Curr. Top. Cell. Regul.* **14**, 117–196.
16. Ueda, T., Greengard, P., Berzins, K., Cohen, R. S., Blomberg, F., Grab, D. J. & Siekevitz, P. (1979) *J. Cell Biol.*, in press.