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Biochemistry

Calcium/phospholipid regulates phosphorylation of a M_r "87k" substrate protein in brain synaptosomes

(phosphoprotein/protein kinase/phosphatidylserine/calcium influx/nerve terminal)

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ABSTRACT **Depolarization-induced calcium influx into rat cerebral cortex synaptosomes increased the phosphorylation of several synaptosomal proteins as examined by $^{32}P_i$ incorporation. A phosphopeptide mapping technique involving $NaDodSO_4$/polyacrylamide gels has been used to show that phosphorylation of a M_r 87,000 substrate protein is stimulated by depolarization-induced calcium influx. Phosphorylation of this M_r 87,000 substrate occurred in synaptosomal cytosol and was markedly stimulated by calcium/phosphatidylserine. Calmodulin inhibited this phosphorylation reaction. This substrate for calcium/phospholipid-dependent protein kinase is enriched in and appears to be specific to neurons.**

Calcium is recognized to play a key role in the regulation of neurotransmitter release from nerve endings (1). Increasing evidence indicates that some of the intracellular actions of calcium in neurons may be mediated through protein phosphorylation (see review in ref. 2). By using fractionated nerve endings (synaptosomes) from the brain, it was demonstrated that calcium influx induced by membrane depolarization resulted in increased phosphorylation of specific intraterminal proteins, the most prominent of which was protein I (3). Activation by calcium of the phosphorylation of protein I and of certain other synaptosomal proteins has been shown to be mediated by calmodulin-dependent protein kinases (4–6).

Recently, a second species of calcium-dependent protein kinase has been described that requires phospholipid (7) rather than calmodulin for its activity. This enzyme occurs at a relatively high level in mammalian brain (8). We report here the occurrence of a M_r 87,000 phosphoprotein (termed the 87k protein) in the nerve terminals from rat cerebral cortex; the phosphorylation of this protein is regulated by Ca^{2+} influx through activation of a Ca^{2+}/phospholipid-dependent protein kinase.

MATERIALS AND METHODS

Materials. Bovine brain L-α-phosphatidyl-1-serine (98–99% pure) and diolein (99%) were purchased from Sigma. Calmodulin was purified from rabbit brain according to the procedure of Grand *et al.* (9). Standard protein I was purified from bovine brain by a modification (unpublished results) of the original procedure (10). The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described (11).

Preparation of Synaptosomes. A crude mitochondrial fraction (P_2) containing synaptosomes was prepared as described (3). For further subfractionation, the P_2 pellet prepared from three rats was suspended in 5 ml of 0.32 M sucrose/5 mM Hepes, pH 7.4, at a protein concentration of 8–10 mg/ml. The suspension was layered on a step gradient consisting of the following sucrose solutions in 5 mM Hepes (pH 7.4): 2 ml, 1.5 M; 10 ml, 1.2 M; 6 ml, 1.0 M; 7 ml, 0.8 M. After centrifugation in a Beckman SW 25.1 rotor at 90,000 × *g* for 2 hr, 2.3-ml fractions were collected. Fractions enriched in myelin, synaptosomes, and mitochondria were identified by the use of appropriate enzyme markers (12, 13). The fractions were diluted to isotonicity with appropriate volumes of ice-cold 5 mM Hepes (pH 7.4) and centrifuged at 12,000 × *g* for 30 min. The pellets were then suspended in 1 ml of Krebs–Ringer buffer.

Preparation of Synaptosomal Cytosol. A P_2 pellet prepared from the cerebral cortices of two rats was lysed in 12 ml of ice-cold 5 mM Hepes, pH 7.4/5 mM EDTA/1 mM dithiothreitol (buffer A) and homogenized in a glass/Teflon homogenizer. The lysed preparation was separated into cytosol and particulate fractions by centrifugation in a Beckman 50 Ti rotor at 150,000 × *g* for 30 min.

In some experiments, the cytosol was depleted of calmodulin by adding $(NH_4)_2SO_4$ to 55% saturation (0.35 g/ml). After being stirred on ice for 20 min, the suspension was centrifuged at 27,000 × *g* for 15 min. The calmodulin-free pellet was suspended in buffer A to half the original volume. The suspension was dialyzed at 4°C against 2 liters of buffer A for 2 hr and diluted with an equal volume of buffer A to yield calmodulin-free cytosol.

Protein Phosphorylation in Synaptosomes. To examine endogenous protein phosphorylation in intact synaptosomes and in myelin- and mitochondria-enriched fractions, preparations were suspended in Krebs–Ringer buffer containing 20 mM Hepes, pH 7.4, 132 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 0.1 mM EGTA, and 10 mM glucose, at a protein concentration of 3–5 mg/ml. The suspension was incubated with $^{32}P_i$ at 37°C for 30 min to label endogenous ATP pools as described (3). Aliquots were then transferred to an equal volume of Krebs–Ringer buffer, with or without $CaCl_2$ and an appropriate depolarizing agent (veratridine or a high concentration of KCl) at 37°C and incubated for 30 sec. The reaction was terminated by the addition of $NaDodSO_4$ stop solution (10).

Protein Phosphorylation in Synaptosomal Cytosol. Protein phosphorylation in cytosolic preparations was carried out at 30°C in a 100-μl volume. The final assay mixture was 20 mM Hepes, pH 7.4/6 mM $MgSO_4$/1 mM dithiothreitol/1 mM EGTA/2.5 μM [γ-^{32}P]ATP/other additions as indicated. The phosphorylation reaction was initiated by the addition of 40 μl of the cytosol preparation, and the reaction was terminated after 10 sec by the addition of 50 μl of $NaDodSO_4$ stop solution.

Comparison of Phosphoproteins in Brain and in Non-Neuronal Tissue. Total particulate and cytosol fractions from rat cerebral cortex, kidney, liver, lung, heart, skeletal muscle, and spleen were prepared as described above. Proteins were phosphorylated as described above for synaptosomal cytosol.

Electrophoretic Analysis of Phosphoproteins. Phosphorylated proteins in the samples were separated by one-dimensional

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$NaDodSO_4$/polyacrylamide gel electrophoresis according to the method of Laemmli (14). The gels were stained, destained, dried, and subjected to autoradiography as described (10). Gel pieces containing phosphorylated protein bands were subjected to limited proteolysis using *Staphylococcus aureus* V8 protease as described (5). In some experiments, phosphoproteins were separated by two-dimensional gel electrophoresis according to O'Farrell *et al.* (15), using nonequilibrium pH gradient electrophoresis in the first dimension.

RESULTS

Depolarization-Induced Phosphorylation of a M_r 87k Protein in Intact Synaptosomes. The effect of membrane depolarization on protein phosphorylation in crude synaptosomes from rat cerebral cortex is shown in Fig. 1. Depolarization, induced either by veratridine or by 60 mM KCl, resulted in an increase in the phosphorylation of a protein doublet (protein I) that migrated at M_r 86,000 (protein Ia) and M_r 80,000 (protein Ib) as described (3). This effect was completely dependent on the presence of Ca^{2+} in the medium (Fig. 1) and probably resulted from Ca^{2+} influx into the synaptosomes (3).

Protein I is a synaptic vesicle-associated protein that is a substrate for both Ca^{2+}/calmodulin-dependent and cAMP-dependent protein kinases (5). The regions of the protein I molecule that are phosphorylated under various conditions have been analyzed by limited proteolysis of phosphorylated protein I with V8 protease followed by phosphopeptide mapping (5). Two regions of the molecule, yielding M_r 35,000 and 10,000 fragments, have been shown to contain phosphorylated sites. Ca^{2+}/calmodulin-dependent protein kinases phosphorylate both regions of the molecule, but cAMP-dependent protein kinase phosphorylates only the region yielding the M_r 10,000 phosphopeptide fragment. Peptide mapping, following limited proteolysis with V8 protease, of the protein I that had been phosphorylated in response to depolarization-induced Ca^{2+} influx as described above, and subsequently purified, also yielded both the M_r 35,000 and 10,000 phosphopeptide fragments (5). Similar results were obtained when phosphorylated protein Ia and phosphorylated protein Ib were analyzed separately (16).

In the present study, endogenous proteins phosphorylated in response to synaptosomal depolarization as described above (Fig. 1) were analyzed by phosphopeptide mapping using V8 protease. Peptide mapping of the M_r 86,000/80,000 doublet region excised directly from a one-dimensional $NaDodSO_4$/polyacrylamide gel showed the presence of a phosphopeptide fragment of M_r 13,000 in addition to the M_r 35,000 and 10,000 phosphopeptide fragments characteristic of purified protein I. Peptide maps of the individual M_r 86,000 and 80,000 phosphoprotein bands, excised separately from a 6% polyacrylamide gel (which allowed optimal separation of the M_r 86,000 and 80,000 bands), showed that the M_r 86,000 band yielded M_r 35,000, 13,000, and 10,000 phosphopeptides while the M_r 80,000 band yielded only the M_r 35,000 and 10,000 phosphopeptide fragments characteristic of protein I. One-dimensional peptide mapping studies using V8 protease on depolarized crude synaptosomal samples showed that Ca^{2+} influx into synaptosomes, induced by either veratridine or KCl, consistently increased phosphorylation of the M_r 13,000 fragment as well as of the M_r 35,000 and 10,000 fragments known to be derived from protein I (Fig. 2). These results indicated that the M_r 13,000 phosphopeptide fragment originated from a phosphoprotein that migrated near the M_r 86,000 band of protein I. That this was the case was confirmed by two-dimensional gel analysis described below, which showed that the M_r 13,000 fragment was derived from an acidic phosphoprotein with a M_r of 87k.

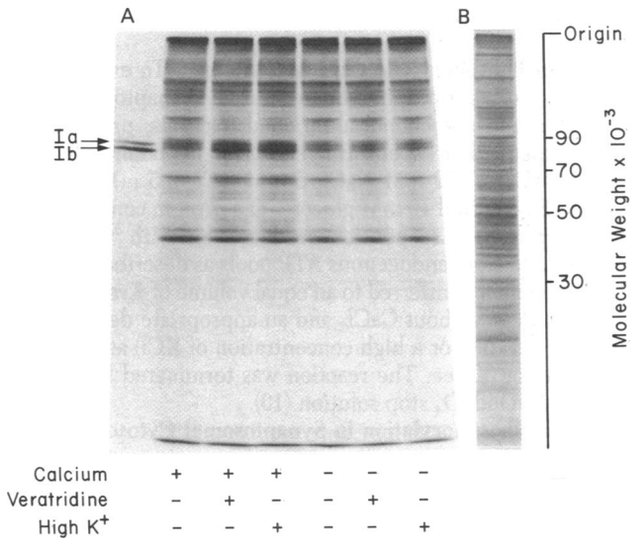

FIG. 1. Effect of depolarizing agents on endogenous phosphorylation of proteins in a crude brain synaptosomal preparation (P_2). Synaptosomes were labeled with $^{32}P_i$ and then incubated in the presence of a depolarizing agent (100 μM veratridine or 60 mM K^+) for 30 sec in the presence or absence of 1.0 mM free Ca^{2+}. After termination of the phosphorylation reaction, the samples were subjected to one-dimensional $NaDodSO_4$/10% polyacrylamide gel electrophoresis, followed by protein staining (*B*) and autoradiography (*A*). The left lane shows standard protein I purified from bovine brain and phosphorylated by using the purified catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP.

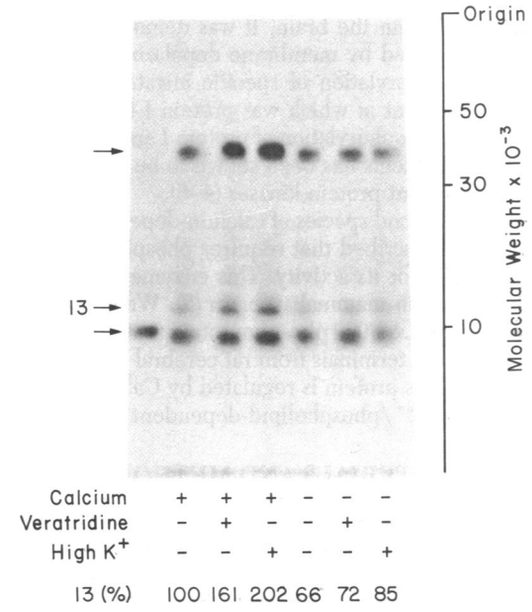

FIG. 2. Autoradiogram showing phosphopeptide maps made after limited proteolysis with V8 protease of phosphoproteins isolated from a crude synaptosomal preparation. Synaptosomal proteins phosphorylated endogenously as described in Fig. 1 were electrophoresed in a $NaDodSO_4$/6% polyacrylamide gel. The gel was not stained for protein. Phosphoproteins were visualized by autoradiography and the bands corresponding to the M_r 87,000/86,000 region (which included protein Ia) were excised from the gel and subjected to limited proteolysis during $NaDodSO_4$/15% polyacrylamide gel electrophoresis. →, Major phosphopeptide fragments generated. The left lane shows the M_r 10,000 phosphopeptide fragment of standard protein Ia, which had been phosphorylated by purified catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP. 13 (%), Relative amounts of ^{32}P found in the M_r 13,000 fragment for the various conditions used.

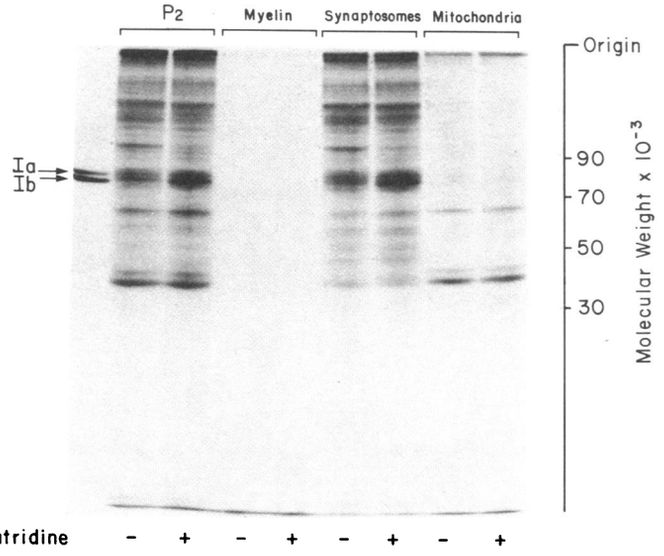

FIG. 3. Endogenous protein phosphorylation and its regulation by veratridine in subfractions of a crude synaptosomal preparation. The crude synaptosomal preparation (P_2) was fractionated by centrifugation in a sucrose step gradient. The fractions were labeled with $^{32}P_i$. Protein phosphorylation in the crude synaptosomal fraction and in each of the subfractions was carried out in the presence of 1 mM Ca^{2+} and in the absence or presence of 100 μM veratridine. Other experimental details were as described in Fig. 1. Ia and Ib, proteins Ia and Ib, respectively.

Phosphorylation in Purified Synaptosomes. Since the crude P_2 preparation is contaminated with myelin and mitochondria, an additional fractionation step was carried out in some experiments to verify the localization of the M_r 87k protein to synaptosomes. A P_2 preparation was fractionated on a sucrose gradient into major fractions enriched in myelin, in synaptosomes, or in mitochondria. Samples from all three preparations were labeled with $^{32}P_i$, and proteins were phosphorylated with or without depolarization-induced Ca^{2+} influx. Depolarization stimulated phosphorylation of the M_r 13,000 region of the M_r 87k protein, as well as of the M_r 35,000 and 10,000 regions of protein I, in the purified synaptosomal fraction (Figs. 3 and 4). No such effect was seen in the myelin or mitochondrial fractions. Thus, the M_r 87k phosphoprotein, as protein I, appears to be localized in the synaptosomal interior and to undergo phosphorylation in response to depolarization-induced Ca^{2+} influx.

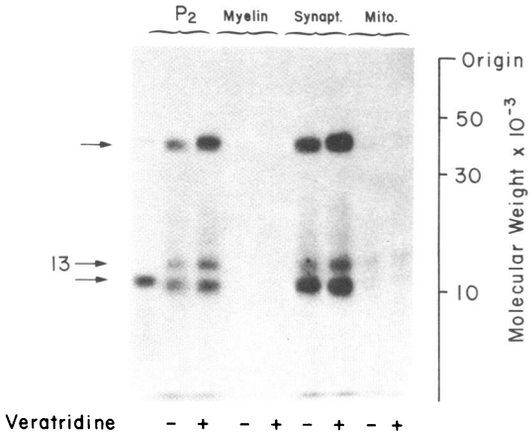

FIG. 4. Autoradiogram showing phosphopeptide maps made after limited proteolysis with V8 protease of phosphoproteins isolated from subfractions of a crude synaptosomal preparation (P_2). Proteins in subfractions were phosphorylated endogenously as described in Fig. 3, and subsequent experimental steps were analogous to those described in Fig. 2.

Ca^{2+}/Phospholipid-Dependent Phosphorylation of the M_r 87k Protein. The finding that the M_r 87k protein is present in the synaptosomal fraction and that it appears to undergo Ca^{2+}-dependent phosphorylation prompted us to search for a Ca^{2+}-stimulated protein kinase that might be responsible. Phosphorylation of endogenous substrates in a total synaptosomal lysate, followed by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis analysis showed that addition of Ca^{2+} greatly stimulated the phosphorylation of protein bands of M_r 87,000/86,000, M_r 80,000, M_r 62,000, and M_r 51,000 (not shown). When a two-dimensional nonequilibrium pH gradient/$NaDodSO_4$/polyacrylamide gel electrophoresis system was used, an acidic protein of M_r 87k whose phosphorylation was stimulated by Ca^{2+} was observed. After limited proteolysis with V8 protease, this protein yielded phosphopeptide fragments of M_r 13,000 and 9,000, the larger of which was the predominant product (not shown). This phosphoprotein is clearly distinct from protein Ia, which is a very basic molecule with an isoelectric point greater than 10 (10). Moreover, addition of cAMP to a synaptosomal lysate under conditions that stimulated the phosphorylation of protein I and other substrates for cAMP-dependent protein kinase had no effect on phosphorylation of the M_r 87k protein (not shown).

Experiments comparing protein phosphorylation in particulate and soluble fractions of the synaptosomal lysate, prepared in Ca^{2+}-free medium, indicated that Ca^{2+}-dependent phosphorylation of the M_r 87k protein occurred predominantly in the synaptosomal cytosol (not shown). In contrast, protein I does not occur in the synaptosomal cytosol fraction in significant amounts and thus does not contaminate the M_r 87k protein in $NaDodSO_4$/polyacrylamide gel analysis of phosphoproteins in this fraction. Therefore, in subsequent experiments, synaptosomal cytosol, rather than the total synaptosomal lysate, was used for studies on the regulation of phosphorylation of the M_r 87k protein.

Addition of Ca^{2+} and phosphatidylserine together to synaptosomal cytosol greatly stimulated phosphorylation of the M_r 87k protein; ^{32}P incorporation into this protein was increased about 9 fold over that found under basal conditions (Fig. 5A, lanes 1 and 3). A prominent M_r 47,000 phosphoprotein was also observed in the presence of Ca^{2+}/phosphatidylserine. In the

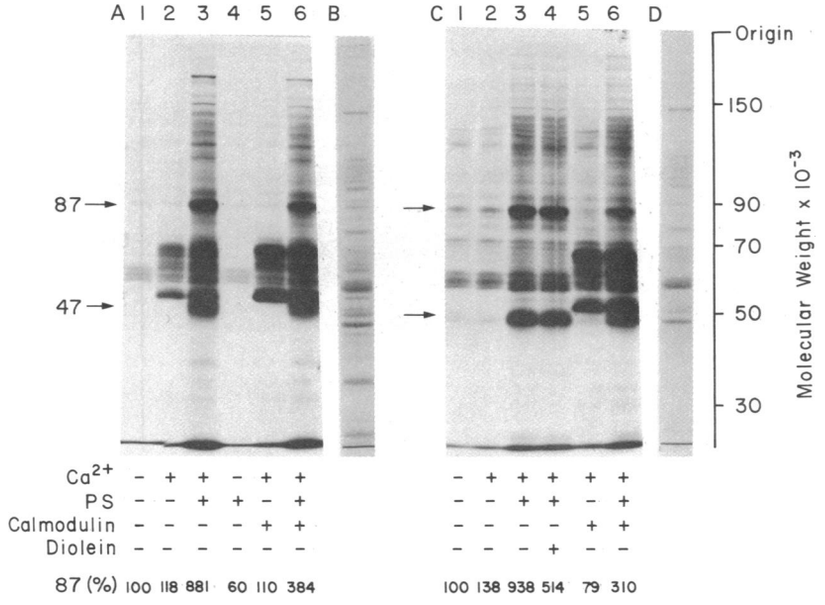

FIG. 5. Phosphorylation in synaptosomal (*A* and *B*) and calmodulin-depleted synaptosomal (*C* and *D*) cytosols. The indicated additions were present in the following final concentrations: free Ca^{2+}, 0.5 mM; phosphatidylserine (PS), 5 μg/100 μl; calmodulin, 1 μg/100 μl; diolein, 1 μg/100 μl. After termination of the phosphorylation reaction, samples were subjected to one-dimensional $NaDodSO_4$/8% polyacrylamide gel electrophoresis, followed by protein staining (*B* and *D*) and autoradiography (*A* and *C*). →, M_r 87k and 47,000 bands, the phosphorylation of which were most prominently stimulated by Ca^{2+}/phosphatidylserine. 87(%), Relative amounts of ^{32}P found in the M_r 87k bands for the various conditions used.

presence of either Ca^{2+} or phosphatidylserine alone, little effect was observed on the phosphorylation of either the M_r 87k or the M_r 47,000 phosphoproteins. In contrast, addition of Ca^{2+} alone greatly stimulated phosphorylation of proteins of M_r 62,000 and 51,000 that have been identified as substrates for synaptosomal Ca^{2+}/calmodulin-dependent protein kinase(s) (Fig. 5*A* and ref. 4). Purified exogenous calmodulin in the presence of Ca^{2+} increased phosphorylation of the M_r 62,000 and 51,000 proteins but not of the M_r 87k protein over that in the presence of Ca^{2+} alone. In fact, addition of calmodulin inhibited the Ca^{2+}/phosphatidylserine-stimulated phosphorylation of the M_r 87k protein in the synaptosomal cytosol (Fig. 5*A*, lanes 3 and 6).

Protein phosphorylation was also studied using a cytosol preparation that had been depleted of calmodulin. The characteristic Ca^{2+}-stimulated phosphorylation of the M_r 62,000 and 51,000 proteins was no longer observed in this preparation in the absence of added calmodulin (Fig. 5*C*, lanes 1 and 2), confirming the effectiveness of the procedure used for removal of calmodulin. The stimulating effect of Ca^{2+}/phosphatidylserine on phosphorylation of the M_r 87k protein seen in untreated

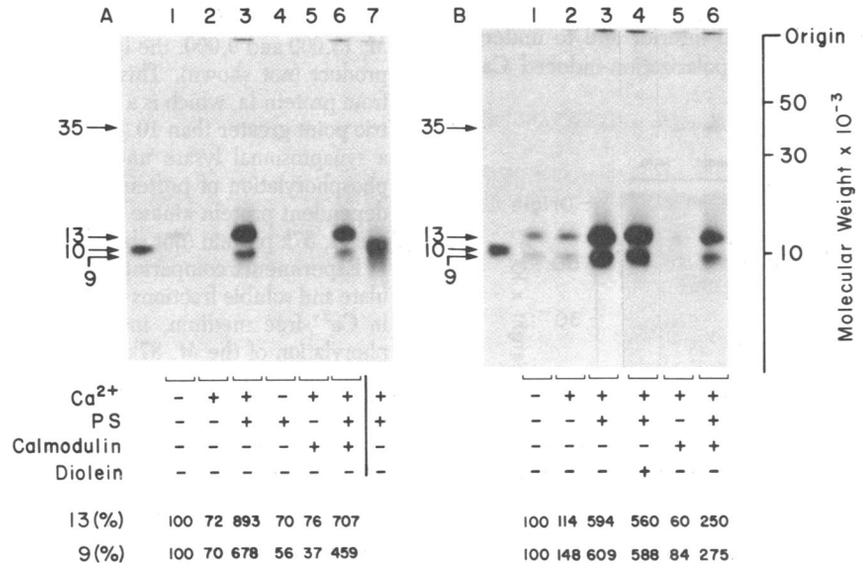

FIG. 6. Autoradiogram showing phosphopeptide maps made after limited proteolysis with V8 protease of the M_r 87k phosphoprotein isolated from synaptosomal (*A*) and calmodulin-depleted synaptosomal (*B*) cytosols. M_r 87k bands of $NaDodSO_4$/polyacrylamide gels containing phosphorylated cytosolic proteins (see Fig. 5) were excised and subjected to limited proteolysis during $NaDodSO_4$/15% polyacrylamide gel electrophoresis. Lanes: 1–6, maps of M_r 87k bands of the corresponding lanes in Fig. 5 *A* and *C*; unmarked left lanes, M_r 10,000 phosphopeptide fragment of standard protein Ia, which had been phosphorylated by purified catalytic subunit of the cAMP-dependent protein kinase and [γ-^{32}P]ATP; 7, phosphopeptide band (M_r, approximately 11,000) generated from the M_r 47,000 substrate protein shown in Fig. 5*A*, lane 6. 13(%) and 9(%), Relative amounts of ^{32}P found in the M_r 13,000 and 9,000 fragments, respectively, derived from the M_r 87k protein for the various conditions used.

synaptosomal cytosol was also seen in calmodulin-deficient cytosol (Fig. 5*C*, lane 3). Addition of diolein, which stimulates purified Ca^{2+}/phospholipid-dependent protein kinase (17), did not stimulate phosphorylation of the M_r 87k protein beyond the level seen in the presence of Ca^{2+}/phosphatidylserine (Fig. 5*C*, lanes 3 and 4). It is possible that diacylglycerol or a suitable substitute was present in the cytosol preparation at a level sufficient to support maximal phosphorylation in the presence of Ca^{2+}/phosphatidylserine. Exogenous calmodulin inhibited phosphorylation of the M_r 87k protein in the presence of Ca^{2+} alone as well as in the presence of Ca^{2+}/phosphatidylserine. It is not clear whether this inhibition reflects a direct or an indirect action of calmodulin on the protein kinase.

Peptide mapping after limited proteolysis with V8 protease of the M_r 87k phosphoprotein from either cytosol preparation yielded phosphopeptide fragments of M_r 13,000 and 9,000 (Fig. 6) similar to those found in experiments using intact synaptosomes. In contrast, negligible amounts of the M_r 35,000 fragment characteristic of protein I (5) were observed, confirming that protein I is not a significant contaminant in the synaptosomal cytosol.

Similar peptide mapping of the M_r 47,000 phosphoprotein showed one broad phosphopeptide band of M_r about 11,000 (Fig. 6*A*, lane 7). Thus, the M_r 47,000 phosphoprotein does not appear to be derived from the M_r 87k protein.

Protein Phosphorylation in Non-Neuronal Tissues. By using total cytosol from rat cerebral cortex, it was found that Ca^{2+}/phosphatidylserine stimulated phosphorylation of the M_r 87k protein (not shown). In contrast, phosphorylation of proteins in cytosol fractions from liver, heart, kidney, lung, skeletal muscle, and spleen in the presence of Ca^{2+}/phosphatidylserine gave no evidence of a phosphoprotein in the M_r 87k region of the gel either by autoradiography or by scintillation counting (not shown). However, Ca^{2+}/phospholipid-dependent phosphorylation of several other proteins was observed in the cytosol prepared from spleen, lung, and liver, confirming previous reports of the presence of Ca^{2+}/phospholipid-dependent protein kinase activity in these tissues (8).

Phosphorylation experiments in which cerebral cortex and lung cytosols were combined showed that addition of the lung preparation had no effect on the Ca^{2+}/phosphatidylserine-stimulated phosphorylation of the M_r 87k substrate in the cortex cytosol (not shown). Thus, the apparent lack of a M_r 87k phosphoprotein in non-neuronal tissue cannot be explained by the absence of an appropriate protein kinase or by the presence of inhibitory factors. The M_r 87k phosphoprotein, therefore, appears to be specific to neuronal tissue.

DISCUSSION

Experiments reported here show the presence in synaptosomes of a M_r 87k phosphoprotein, the phosphorylation of which occurs on depolarization-induced Ca^{2+} influx. Moreover, experiments using synaptosomal cytosol show that the M_r 87k protein is a substrate for Ca^{2+}/phospholipid-dependent protein kinase and that it is not phosphorylated under similar conditions by Ca^{2+}/calmodulin-dependent protein kinase. This M_r 87k phosphoprotein migrates on $NaDodSO_4$/polyacrylamide gels in the immediate vicinity of protein Ia, which has a M_r of 86,000. However, the M_r 87k protein is distinct from protein Ia as shown by several criteria, including (*i*) subcellular distribution, (*ii*) isoelectric point, (*iii*) peptide maps, and (*iv*) effectiveness as substrate for various classes of protein kinase. The M_r 87k protein described here also appears to be different from several other phosphoproteins observed previously that migrate in this region of $NaDodSO_4$/polyacrylamide gels (18–20). These other phosphoproteins include a M_r 85,000 substrate in synaptic membranes that undergoes Ca^{2+}-independent phosphorylation (18), a M_r 85,000 substrate in cytosol that undergoes Ca^{2+}/calmodulin-dependent phosphorylation (19), and a M_r 82,000 substrate in purified plasma membranes that undergoes Ca^{2+}-independent phosphorylation (20).

The findings described here indicate that influx of Ca^{2+} into the nerve terminal, resulting from depolarization of the plasma membrane, activates a Ca^{2+}/phospholipid-dependent protein kinase and that this protein kinase phosphorylates an endogenous M_r 87k substrate protein. The results suggest the possibility that this Ca^{2+}/phospholipid-dependent protein phosphorylation system serves an important regulatory role in the nerve terminal.

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