CALCIUM-DEPENDENT PHOSPHORYLATION OF SPECIFIC SYNAPTOSOMAL FRACTION PROTEINS: POSSIBLE ROLE OF PHOSPHOPROTEINS IN MEDIATING NEUROTRANSMITTER RELEASE

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Calcium ions caused a marked increase in the level of endogenous phosphorylation of specific proteins from synaptosomal fractions prepared from rat cerebral cortex. The levels of phosphorylation of these specific proteins were dependent upon the presence of calcium and regulated by small changes in the concentration of calcium ions. The effect of calcium was independent of ATP concentration over a wide range of concentrations. The results are compatible with the hypothesis that some of the effects of calcium on synaptic transmission might be mediated by the effect of calcium on the phosphorylation of specific synaptosomal proteins.

It has recently been reported that the phosphorylation of two specific proteins, designated proteins DPH-L and DPH-M, from rat brain homogenates was dependent upon the presence of calcium ions, suggesting that the effect of calcium on phosphoproteins might play a role in mediating some of the effects of this ion on neuronal tissue (1). The calcium induced stimulation of the net level of endogenous phosphorylation of proteins DPH-L and DPH-M could be completely inhibited by the anticonvulsant diphenylhydantoin, DPH (1,2). These results indicated that the antagonistic actions of calcium and DPH on the phosphorylation of specific brain proteins might mediate the antagonistic effects of these agents on post-tetanic potentiation, PTP, (3,4,7-9). Post-tetanic potentiation (PTP) has been demonstrated to be the result of an increase in neurotransmitter release from the presynaptic neurons secondary to an increased intracellular accumulation of calcium ions during repetitive stimulation (3,4). The increased level of intracellular calcium results in an increased release of neurotransmitter from

the axon terminal (5,6). DPH has been shown to reverse PTP (7-9), and thus antagonize the action of calcium on neurotransmitter release during PTP. If phosphoproteins DPH-L and DPH-M are involved in mediating some of the effects of calcium on neurotransmitter release, they should be present in synaptosomal preparations, and calcium should regulate the level of phosphorylation of these proteins in synaptosomal fractions. The present investigation was initiated to demonstrate that the effects of calcium on the phosphorylation of proteins DPH-L and DPH-M could be observed in synaptosomal enriched fractions from cerebral cortex, and thus further suggest that these effects of calcium on protein phosphorylation might be related to some of the effects of this ion on synaptic function.

MATERIALS AND METHODS

Synaptosomal fractions were prepared from rat cerebral cortex following the methods of Whittaker, et al. (10-12). A 10% homogenate of rat cerebral cortex was prepared in 0.32 M sucrose and centrifuged at 1000 x g for 11 min. The pellet was washed in an equal volume of 0.32 M sucrose and the combined supernatants were centrifuged at 17,000 x g for 60 min. The resultant pellet (P2) was resuspended in 0.32 M sucrose and was carefully layered over a density gradient consisting of layers of 10 ml of 1.2 M sucrose and 10 ml of 0.8 M sucrose, and centrifuged for 2 hr. at 53,000 x g. The layer at the 0.8-1.2 M sucrose interface was collected by pipet, sedimented by centrifugation and suspended in 0.32 M sucrose (2mM EGTA). This fraction was the synaptosomal enriched preparation used in this study. Electron micrographic studies, following gluteraldehyde fixation and osmium staining demonstrated that the purity of this preparation was consistent with those published in the literature (10-12).

Ths standard reaction mixture for studying the effects of calcium on protein phosphorylation contained 100 µg of fresh synaptosomal fraction, 50 mM Tris-HCl (pH 7.4), 20 mM magnesium chloride, and approximately 5 µM [γ-^{32}P] ATP from New England Nuclear (specific activity greater than 10 Ci/n mole) in the presence or absence of calcium chloride in a total volume of 0.1 ml. The reaction was initiated by the addition of [γ-^{32}P] ATP and terminated at various time intervals by the addition of 50 µl of an SDS-stop solution (2). Equal aliquots from each reaction mixture were subjected to SDS-polyacrylamide slab gel electrophoresis. The gel system was modified from Lameli (13). The lower gel contained 9% acrylamide, 0.24% bis-acrylamide, 0.38 M Tris-HCl (pH 8.8), 2 mM EDTA, 0.1% SDS, and 0.15% ammonium persulfate as a catalyst. The stacking gel contained 5% acrylamide, 0.13% bis-acrylamide, 0.063 M Tris-HCl (pH 6.8), 2 mM EDTA, 0.1% SDS, and 0.15% ammonium persulfate as a catalyst. The dimensions of the total gel were 12 cm x 16 cm x 2 mm, and electrophoresis was carried out for 14 to 16 hours (overnight) at 20 ma on a vertical plate gel apparatus (14). The running buffer contained 50 mM Tris-HCl (pH 8.6), 2 mM EDTA, 0.382 M glycine, 0.1% SDS, the final pH of the buffer being adjusted to 8.6 with 10 N NaOH. The gel was stained for protein with Coomassie blue and dried under vacuum (2).

Quantitative measurements of [^{32}P] phosphate incorporation into individual protein bands was determined as described previously (2,15,16). The [^{32}P] phosphate peaks shown in Fig. 1 were shown to be associated with protein by various treatments with protease (streptomyces griseus), ribonuclease A, and deoxyribonuclease (2,17) and molecular weight determinations were performed as described previously (2,18).

RESULTS

Effect of Calcium on the Phosphorylation of Synaptosomal Fraction Proteins

The effects of calcium on the endogenous phosphorylation of synaptosomal

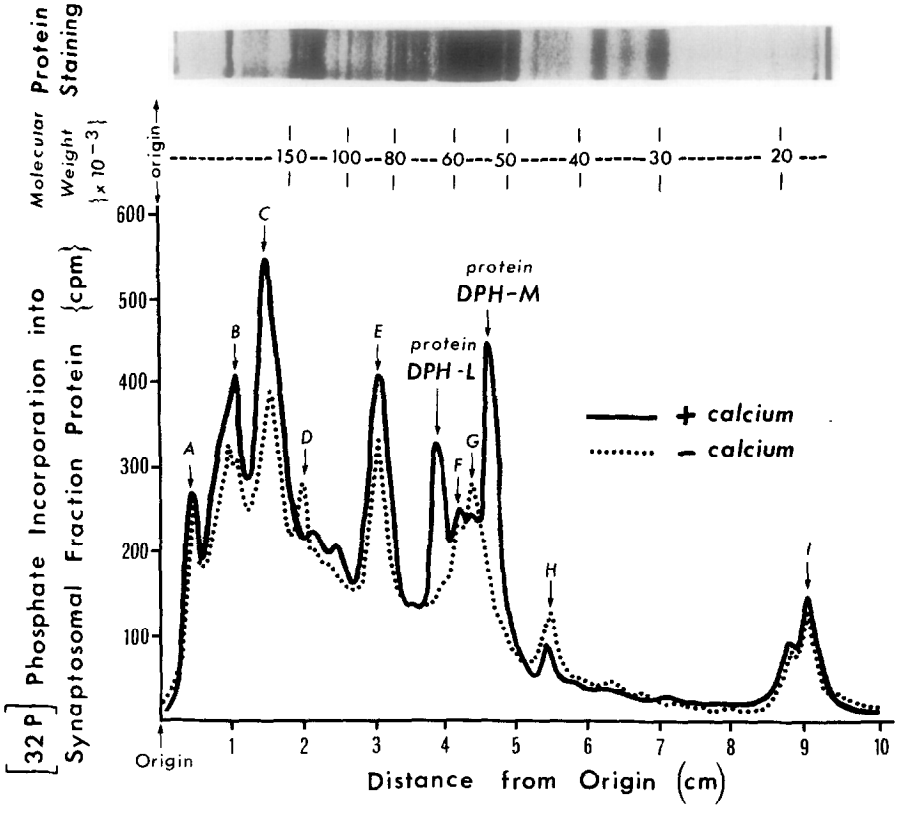

Figure 1. Effect of Calcium on the Endogenous Phosphorylation of Synaptosomal Fraction Protein.

Synaptosomal fraction protein was incubated under standard conditions for 20 seconds with 5μM [γ-^{32}P] ATP in the presence or absence of calcium chloride (10mM), and subjected to polyacrylamide gel electrophoresis, protein staining, and quantitation, as described previously (2,15,16). The radioactivity in the gel in the presence or absence of calcium is plotted as a function of distance from the origin. The molecular weight scale was determined using known molecular weight standards (2,18). The results of the experiment shown are representative of 10 individual experiments.

fraction proteins incubated under standard conditions are shown in Fig. 1. Calcium chloride (10 mM) caused a dramatic increase in the level of endogenous phosphorylation of two specific proteins labelled DPH-L and DPH-M with molecular weights of 60-63,000 and 49-52,000,respectively. These two proteins migrated and behaved identically to proteins DPH-L and DPH-M from homogenate preparations. In the absence of calcium ions no significant phosphorylation of proteins DPH-L or DPH-M was observed. Magnesium ions were present in all incubation mixtures, and were required to observe the effects of calcium on the phosphorylation of proteins DPH-L and DPH-M. The phosphorylation of peaks A,B,C,E,F and I were partially stimulated by calcium ions; however, in the absence of calcium ions, significant phosphorylation of these bands was still observed. The phosphorylation of three minor peaks (D,G and H) were slightly inhibited by the presence of calcium ions. Under standard conditions, only the levels of phosphorylation of proteins DPH-L and DPH-M were completely dependent upon the presence of calcium ions (Fig. 1).

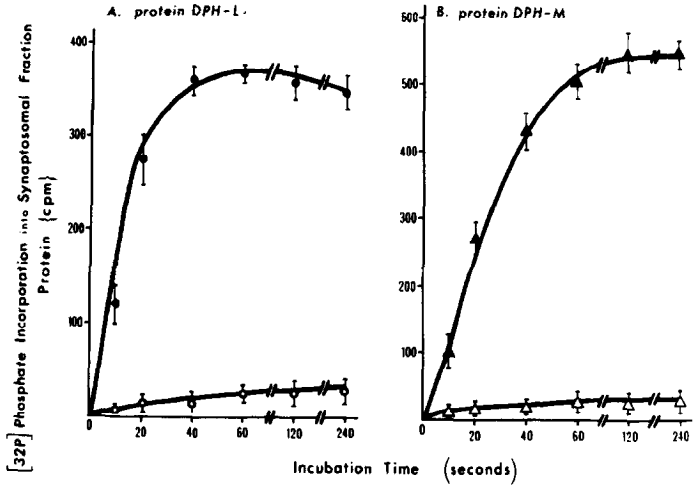

Figure 2. Time Course of Phosphorylation

Time course of [^{32}P] phosphate incorporation into synaptosomal fraction proteins DPH-L (A) and DPH-M (B) in the presence (● - ● ; ▲ - ▲) or absence (O - O ; Δ - Δ) of calcium ions (10 mM). Reactions were conducted under standard conditions. The data give mean values and ranges for 7 experiments.

Time Course of Phosphorylation of Proteins DPH-L and DPH-M.

The time course of phosphorylation of proteins DPH-L and DPH-M in the presence and absence of calcium is shown in Fig. 2. Calcium stimulated both the initial rate and the net level of phosphorylation of these synaptosomal fraction proteins, as it did in homogenate preparations (1,2). The peak levels of phosphorylation of both proteins were reached within 60 seconds.

Effect of Varying the Concentration of ATP

The effects of calcium on the net level of phosphorylation of proteins DPH-L and DPH-M were independent of the concentration of ATP over a range of concentrations from 0.5-50 μM (Fig.3). This result suggests that the effect of calcium on the phosphorylation of these proteins was not due to a competitive effect between calcium and ATP.

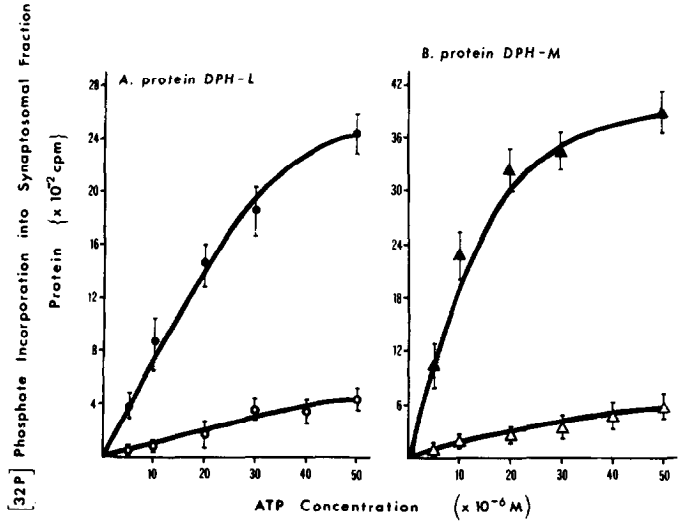

Figure 3. ATP Concentration Curves for Proteins DPH-L and DPH-M.

Effect of ATP concentration on the level of phosphorylation of synaptosomal fraction proteins DPH-L and DPH-M in the presence (● - ● ; ▲ - ▲) or absence (O - O ; △ - △) of calcium ions (10 mM). Reactions were performed under standard conditions for 20 sec. The data give the mean values and ranges for 6 experiments.

Variations in the Concentration of Calcium

The concentrations of calcium required to produce a half-maximal increase in the net levels of phosphorylation of synaptosomal fraction proteins DPH-L and DPH-M were 3 x 10^{-5} and 8 x 10^{-5}M, respectively (Fig. 4). Small increases or decreases in the concentration of calcium caused corresponding increases or decreases in the net level of phosphorylation of these specific proteins. Concentrations of calcium above 2 x 10^{-3}M caused an inhibition of phosphorylation. The half-maximal concentrations of calcium cannot be strictly viewed as K_M values, since the endogenous level of calcium in the synaptosomal preparations is not known, and since EGTA was used in the preparation of the synaptosomes.

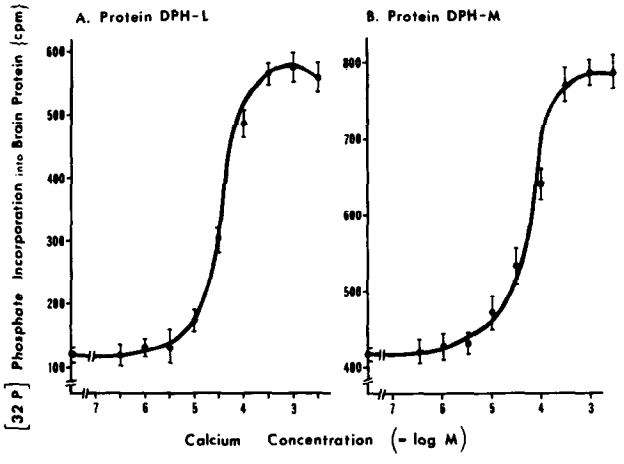

Figure 4. Calcium Concentration Curves for Proteins DPH-L and DPH-M

Effect of calcium concentration on the level of phosphorylation of synaptosomal fraction proteins DPH-L (A) and DPH-M (B). Reactions were performed with synaptosomal fraction protein under standard conditions. The data give mean values and ranges for 8 experiments.

DISCUSSION

The results demonstrate that the net level of endogenous phosphorylation of two specific proteins from synaptosomal fractions was dependent

upon the presence of calcium ions. These proteins were shown to be identical to proteins DPH-L and DPH-M from homogenate preparations in both electrophoretic mobility and properties of phosphorylation. The levels of phosphorylation of these specific proteins could be regulated by small changes in the concentration of calcium ions. The evidence presented in this paper further suggests that the effects of calcium on the phosphorylation of specific synaptosomal proteins may mediate some of the actions of calcium on synaptic transmission.

The calcium-induced stimulation of the net level of phosphorylation of proteins DPH-L and DPH-M has been demonstrated to be almost completely inhibited by DPH in homogenate (1-2) and synaptosomal (DeLorenzo, in preparation) preparations. DPH has also been demonstrated to reverse post-tetanic potentiation (PTP) (7-9) which has been attributed to an increased intracellular accumulation of calcium ions in the presynaptic terminal during the tetanus (3,4). This increase in intracellular calcium ions during PTP has been shown to cause an increased release of neurotransmitter from the axon terminal (5,6). It has also been demonstrated that DPH and calcium act antagonistically on the release of norepinephrine from brain slices (19). This data is compatible with the hypothesis that the antagonistic effects of calcium and DPH on neurotransmitter release during PTP and from brain slices may be mediated by the opposing actions of these agents on the level of phosphorylation of specific synaptosomal proteins.

These results present the first suggestion of a possible role of phosphoproteins in mediating some of the effects of calcium on the release of neurotransmitter from presynaptic nerve terminals. Calcium has been shown to play a major role in initiating the release of neurotransmitter from nerve terminals (5,6,20,22). The possible role of phosphoproteins in mediating this effect of calcium on synaptic transmission is an important area for further research.

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