CALCIUM-DEPENDENT PHOSPHORYLATION OF SYNAPTIC VESICLE PROTEINS AND ITS POSSIBLE ROLE IN MEDIATING NEUROTRANSMITTER RELEASE AND VESICLE FUNCTION

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Calcium ions caused a marked increase in the level of endogenous phosphorylation of specific proteins from highly purified synaptic vesicle fractions. The calcium-dependent phosphorylation of two synaptic vesicle proteins was inhibited by phenytoin. The effects of calcium and phenytoin were independent of ATP concentration. The results are compatible with the hypothesis that the effects of calcium ions on the level of phosphorylation of specific synaptic vesicle-associated proteins may be the underlying molecular mechanism mediating some of the effects of calcium on neurotransmitter release and synaptic vesicle function.

Although convincing evidence has been presented to demonstrate the calcium-dependent release of neurotransmitter from the presynaptic nerve terminal (1-3), the mechanism of action of calcium in triggering neurotransmitter release is still unknown. Calcium ions have been shown to stimulate protein phosphorylation in both broken (4-7) and intact (8) synaptosome preparations, suggesting a possible role of calcium-dependent protein phosphorylation in mediating some of the effects of calcium ions on synaptic function. The anticonvulsant DPH* has been demonstrated in several systems to inhibit the calcium-dependent release of neurotransmitter (9-13). Studies of the effects of DPH on the calcium-dependent phosphorylation of rat brain homogenate (14,15) and synaptosome (4-7) preparations have demonstrated that DPH in therapeutic concentrations significantly inhibits the calcium-dependent phosphorylation of synaptosomal proteins DPH-L* and DPH-M*, and developed the hypothesis that the antagonistic actions of calcium and DPH on the phosphorylation of specific synaptosomal proteins may mediate the opposing action of these agents on neurotransmitter release.

*Abbreviations: DPH, phenytoin, diphenylhydantoin; Proteins DPH-L and DPH-M, specific synaptosomal fraction proteins with molecular weights of approximately 63,000 and 53,000, respectively (4); SDS, sodium dodecyl sulfate.

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Since synaptosome preparations have been shown to be very heterogeneous in composition (16), it is important to demonstrate that the calcium-dependent phosphorylation of proteins DPH-L and DPH-M occurs within the presynaptic nerve endings, and not in the membrane, glial cell fragment, and mitochondrial contaminations of the synaptosome preparation. Preliminary results from this laboratory (17) have demonstrated that the calcium-dependent phosphorylation of synaptosomal proteins DPH-L and DPH-M is enriched in synaptic vesicle fractions isolated from synaptosomes. The current investigation was initiated to determine if the antagonistic actions of calcium and DPH on the phosphorylation of synaptosomal proteins DPH-L and DPH-M could be observed in highly purified preparations of synaptic vesicles, and thus attempt to provide evidence for the localization of these proteins within the presynaptic nerve terminal and further suggest a role of calcium-dependent protein phosphorylation in mediating neurotransmitter release and synaptic vesicle function.

MATERIALS AND METHODS

Synaptosome preparations were prepared from rat brains as described previously (4). Synaptic vesicles were isolated from synaptosome fractions by a modification of the well established technique of Kodota and Kodota (18). The washed synaptosome pellet was suspended in 6 volumes of distilled water and homogenized at 300 rpm for 45 sec in a glass homogenizer with a Teflon pestle to release the synaptic vesicles from the synaptosomes by osmotic shock. The preparation was immediately diluted with 0.25 volumes of buffer, giving a final concentration in the mixture of 10mM KCl and 10mM Tris-maleate buffer (pH 6.5), and centrifuged at 20,000 x g for 30 min. The pellet, containing mainly mitochondria, unbroken synaptosomes, and large membrane fragments, was discarded. The resultant supernatant was centrifuged at 55,000 x g for 60 min. The pellet containing membrane fragments, coated synaptic vesicles, and other cellular debris was discarded. The supernatant was again centrifuged at 80,000 x g for 75 min and the resultant synaptic vesicle pellet was suspended in a buffered solution containing 10mM KCl, 10mM Tris-maleate (pH 6.5) and 1mM EGTA. The composition of the synaptic vesicle fraction was determined by electronmicroscopy (18) (Fig 1), and contained approximately 60% plain synaptic vesicles, 30% coated synaptic vesicles, 5% vesicle shell fragments, and less than 5% membrane contamination. Employing enzyme markers (19,20) for mitochondria (succinate dehydrogenase) and membrane (Na-K-ATPase, Cholinesterase) confirmed that this synaptic vesicle fraction contained less than 4-8% membrane and virtually no mitochondria. The exact percentage of membrane contamination varied slightly from preparation to preparation, but only synaptic vesicle-fractions with low membrane contamination, as determined by electronmicroscopy and enzyme markers, were employed. Almost complete removal of small membrane fragments by sephadex column chromatography (21) had no significant effect on the phosphorylation results obtained with the vesicle preparation, indicating that the calcium-dependent phosphorylation was associated with the synaptic vesicles. The slightly acidic pH (6.5) and KCl were employed in the isolation procedure to avoid disso-

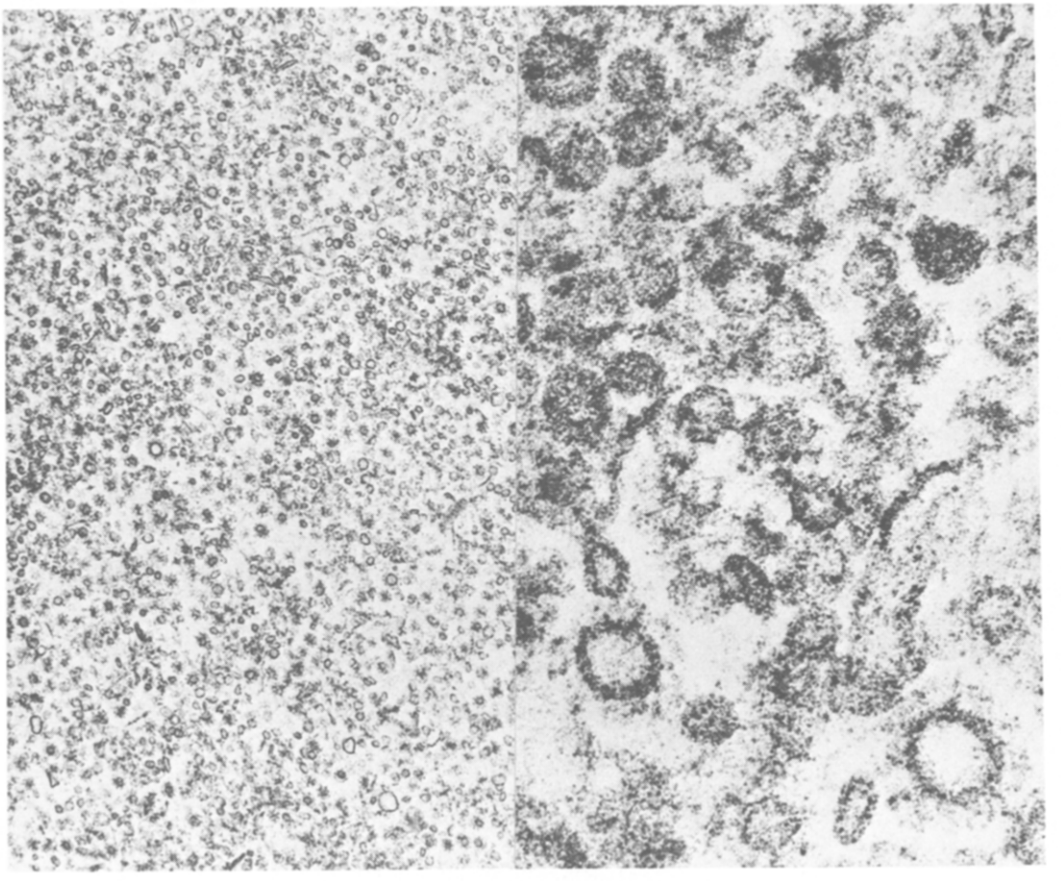

Figure 1. Electronmicrograph of the Synaptic Vesicle Preparation

The synaptic vesicle fraction was fixed and stained as described previously (18). The vesicle fraction at low (x 28,500, left) and high (x 200,000, right) magnification was found to contain plain and coated synaptic vesicles, vesicle shell fragments, and very little contamination by membrane or other organelles. The composition of this fraction was further confirmed by enzyme markers (Methods).

ciation of the synaptic vesicles (18). The entire fractionation procedure was carried out at 4°C and conducted in less than 7 hours. Vesicle fractions were immediately used for the phosphorylation experiments. Prolonged handling, further purification steps, multiple washes, or increases in temperature, markedly decreased the calcium-dependent protein phosphorylation of the fraction.

The standard reaction mixture for studying protein phosphorylation contained 50µg of fresh synaptic vesicle protein, 50mM Tris-HCl (pH 7.4), 10mM magnesium chloride, and 10µM [γ-^{32}P] ATP from New England Nuclear (specific activity 5-10 Ci/m mole) in the presence or absence of calcium chloride and DPH and incubated for 1 min at 37° C. DPH was dissolved in NaOH as described previously (6). Control tubes received the same amount of NaOH and both control and DPH solutions were allowed to stand with the protein preparation for 15 min prior to incuba-

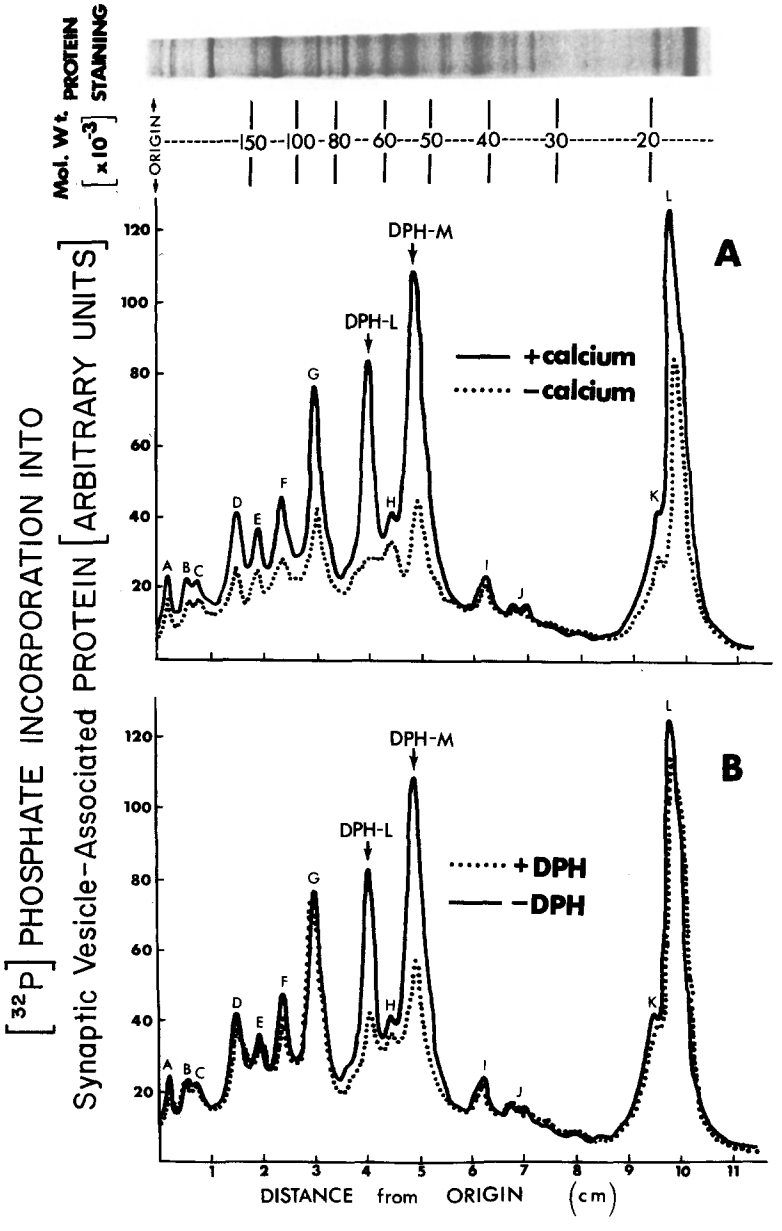

Figure 2. Effects of Calcium and DPH on the Endogenous Phosphorylation of Synaptic Vesicle Fraction Proteins

Synaptic vesicle protein was incubated under standard conditions for 1 min in the presence or absence of calcium ions, 1mM (A), or in the presence of calcium ions with or without DPH, 0.1mM (B), and subjected to polyacrylamide gel electrophoresis, protein staining, and quantitation (Methods). The results shown are representative of 15 individual experiments. Each arbitrary unit equals approximately 11.6 cpm.

tion. The reaction was initiated with the addition of [γ-^{32}P] ATP. The reactions were stopped, and samples were subjected to SDS-gel electrophoresis, protein staining, autoradiography, and quantitation for [^{32}P] phosphate incorporation as described previously (6). Protein peaks A-K (15) and proteins DPH-L and DPH-M (6) were shown to be protein in nature as described previously. Molecular weight determinations were conducted as described previously (6).

RESULTS

Effects of Calcium on the Phosphorylation of Synaptic Vesicle Proteins

The effects of calcium ions on the level of endogenous phosphorylation of synaptic vesicle fraction proteins is shown in Fig. 2A. Calcium (1mM) caused a significant increase in the phosphorylation of two synaptic vesicle fraction proteins that migrated in identical positions on SDS-gel polyacrylamide electrophoresis to proteins DPH-L and DPH-M from synaptosome preparations and were designated synaptic vesicle-associated proteins DPH-L and DPH-M (Fig. 2). The concentrations of added calcium ions required to produce a half-maximal increase in the phosphorylation of proteins DPH-L and DPH-M were 7×10^{-4}M and 8×10^{-4}M, respectively. The level of phosphorylation of proteins D, E, F, G, K, and L also showed significant calcium-dependent phosphorylation, while peaks, A, B, C, H, I, and J showed only minimal calcium dependence. The synaptic vesicle preparation demonstrated more calcium dependent phosphorylation per mg protein than the synaptosome preparation. The effects of calcium on vesicle protein phosphorylation were independent of ATP concentration (0.5-50μM ATP). Calcium ions stimulated both the initial rate and the net level of phosphorylation of synaptic vesicle-associated proteins DPH-L and DPH-M.

Effects of DPH on the Calcium-Dependent Phosphorylation of Vesicle Proteins.

The effects of DPH in therapeutic concentrations on the calcium-dependent phosphorylation of synaptic-vesicle fraction proteins is shown in Fig. 2B. DPH caused a marked inhibition of the calcium-dependent phosphorylation of synaptic vesicle-associated proteins DPH-L and DPH-M, without producing any significant decrease in the levels of calcium-dependent phosphorylation of protein peaks A-L. The concentration of DPH required to produce half-maximal decreases in the levels of calcium-dependent phosphorylation of proteins DPH-L and DPH-M were 1×10^{-4}M and 3×10^{-4}M respectively. The DPH inhibition of calcium-dependent pro-

TABLE 1. Effects of Therapeutic and Toxic Concentrations of DPH on the Calcium-Dependent Phosphorylation of Synaptic Vesicle-Associated Proteins*

Synaptic Vesicle Associated Protein	DPH Concentration		
	8×10^{-5} M	5×10^{-4} M	1×10^{-3} M
Protein DPH-L	56.4	36.1	18.3
Protein DPH-M	66.2	45.9	29.6
Protein Peak A	98.3	96.4	91.3
B	98.4	96.9	90.5
C	98.0	97.1	91.6
D	97.4	91.3	83.1
E	99.5	98.1	96.6
F	98.1	97.6	95.4
G	98.9	90.6	83.8
H	97.4	91.5	81.9
I	99.8	97.9	96.7
J	99.7	98.6	97.3
K	99.6	97.4	93.2
L	97.3	91.2	83.4

*Reactions were conducted in the presence of calcium ions (1mM) under standard conditions in the presence or absence of DPH in therapeutic (8×10^{-5}M) or toxic (5×10^{-4}M, 1×10^{-3}M) concentrations. Each value is expressed as percentage of the control condition containing no DPH, and represents the mean of the results obtained from 8 reactions. The largest range about the mean was 4.2 and thus the ranges were omitted for clarity.

tein phosphorylation was independent of ATP concentration over a wide range of concentrations (0.5-50μM ATP). DPH inhibited both the initial rate and net level of calcium-dependent incorporation of [^{32}P] phosphate into synaptic vesicle-associated proteins DPH-L and DPH-M. The effects of DPH in therapeutic (8×10^{-5}M) and toxic (5×10^{-4} and 1×10^{-3}M) concentrations on the calcium-dependent phosphorylation of synaptic vesicle proteins is shown in Table 1. The results demonstrate that the calcium-dependent levels of phosphorylation of proteins DPH-L and DPH-M were significantly inhibited at therapeutic concentrations of DPH and reduced to almost background levels at toxic concentrations. The phosphorylation of peaks A-L were not significantly inhibited by DPH in therapeutic concentrations. Toxic concentrations of DPH produced only minimal effects on the level of calcium-dependent phosphorylation of A-C, E, F, I, J, and K and had small, but significant effects on peaks D, G, H, and L.

DISCUSSION

The results demonstrate that the levels of phosphorylation of several synaptic vesicle-associated proteins were dependent upon the presence of calcium ions. DPH in therapeutic concentrations was demonstrated to inhibit the calcium-dependent phosphorylation of synaptic vesicle fraction proteins DPH-L and DPH-M, while not significantly inhibiting the calcium-dependent phosphorylation of the other synaptic vesicle proteins. Since synaptic vesicles originate only from within the presynaptic terminals in the synaptosome preparation (16), the results strongly suggest that the antagonistic actions of calcium and DPH on the phosphorylation of proteins DPH-L and DPH-M occur within the presynaptic nerve terminal in association with the synaptic vesicles. These results confirm and expand our initial hypothesis suggesting that the antagonistic actions of calcium and DPH on the level of phosphorylation of specific proteins within the presynaptic terminal in close association with the synaptic vesicles may be the underlying molecular mechanism mediating the opposing action of calcium and DPH on the release of neurotransmitter from the presynaptic nerve terminal.

Synaptic vesicles have been shown to bind high concentrations of ATP in association with protein and neurotransmitter substances, implying a possible role of ATP in mediating vesicle function and neurotransmitter release (22). The demonstration that the phosphorylation of several synaptic vesicle proteins was dependent upon calcium provides a possible biochemical mechanism utilizing vesicle bound ATP in mediating the effects of calcium on the function of synaptic vesicles. Since DPH has been shown to inhibit calcium-dependent neurotransmitter release in several preparations (9-13), the demonstration in this investigation that DPH inhibited the calcium-dependent phosphorylation of synaptic vesicle proteins DPH-L and DPH-M without significantly affecting the phosphorylation of the other major vesicle phosphoproteins, suggests that the calcium-dependent phosphorylation of these specific proteins may mediate the antagonistic actions of DPH and calcium on neurotransmitter release.

Although the functional significance of calcium-dependent protein phos-

phorylation in mediating synaptic vesicle function and neurotransmitter release must still be determined, the observations provided in this paper present evidence that the calcium-dependent phosphorylation of specific synaptic vesicle-associated proteins may play a role in mediating synaptic vesicle function and neurotransmitter release. It is hoped that these results will stimulate further investigation of the possible role of calcium-dependent synaptic vesicle protein phosphorylation in synaptic transmission.

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