Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 183-192

CALCIUM DEPENDENT NEUROTRANSMITTER RELEASE AND PROTEIN PHOSPHORYLATION IN SYNAPTIC VESICLES

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Received November 30, 1977

A highly purified preparation of synaptic vesicles was prepared to study the relationship between calcium-dependent neurotransmitter release and protein phosphorylation. Calcium ions simultaneously produced significant increases in both the endogenous release of norepinephrine from the synaptic vesicles and the endogenous incorporation of [^{32}P] phosphate into specific synaptic vesicle proteins. The results are compatible with the hypothesis that the action of calcium on the phosphorylation of specific synaptic vesicle proteins is the molecular mechanism mediating some of the effects of calcium on neurotransmitter release and synaptic vesicle function.

An understanding of the molecular mechanism underlying calcium-dependent neurotransmitter release would greatly enhance our knowledge of synaptic transmission and the action of specific neuropharmacologic agents, and possibly provide new insights into human disease processes involving synaptic modulation. Previous investigations in this laboratory (1,2) suggested that the molecular mechanism mediating the effects of calcium on neurotransmitter release and synaptic vesicle function may be the action of calcium on the level of phosphorylation of specific synaptic vesicle-associated proteins. The anticonvulsant DPH* has been shown to inhibit calcium-dependent neurotransmitter release in several preparations (3-7). If synaptic vesicle protein phosphorylation mediates calcium-dependent release of neurotransmitter from the nerve terminal, DPH would be expected to inhibit this calcium-dependent protein phosphorylation. DPH in therapeutic concentrations blocked calcium-dependent phosphorylation of spe-

*Abbreviations: DPH, phenytoin, diphenylhydantoin, Dilantin; Proteins DPH-L and DPH-M, specific synaptic vesicle-associated proteins with molecular weights of approximately 62-63,000 and 51-54,000 respectively (1); SDS, sodium dodecyl sulfate.

0006-291X/78/0801-0183$01.00/0

cific synaptosomal (8-11) and synaptic vesicle (1,2) proteins DPH-L and DPH-M*. These results indicated that the inhibition of neurotransmitter release by DPH was caused by its inhibition of calcium-dependent synaptic vesicle protein phosphorylation and suggested a role for these phosphoproteins in neurotransmitter release (12). To further elucidate the role of synaptic vesicle-associated phosphoproteins in neurotransmitter release, it would be important to demonstrate that calcium ions stimulated both neurotransmitter release and protein phosphorylation in the same preparation. The current investigation was initiated to establish a more direct relationship between synaptic vesicle-associated protein phosphorylation and neurotransmitter release by simultaneously determining the effects of calcium ions on neurotransmitter release and protein phosphorylation in the same preparation of highly purified synaptic vesicles isolated from rat brain.

MATERIALS AND METHODS

Large quantities of highly purified synaptic vesicles were quickly obtained from rat brain by a modification of our previously described technique (1). Sprague-Dawley female rats (24-40) were rapidly decapitated and homogenized in 8 parts by weight of 0.32M sucrose in a 350ml glass homogenizer at 4°C with a Teflon pestle at approx. 500rpm with 12 strokes in less than 20-40 minutes. The homogenate was centrifuged at 2,000 x g for 10 min and the supernatant was removed and centrifuged at 10,000 x g for 20 min. The resultant pellet (P_2) was washed in an equal volume of 0.32M sucrose and subjected to osmotic shock by homogenization in 5.5 times its volume of distilled water with 5 strokes at 500 rpm in less than 4 minutes. Reagents were immediately added in one tenth the volume to give a solution containing 160mM KCl, 5mM NaCl, 260μM pargyline, and 10mM Tris-maleate buffer pH 6.5. The preparation was centrifuged at 20,000 x g for 25 min and the supernatant was centrifuged again at 55,000 x g for 60 min. The resultant soluble protein plus synaptic vesicle supernatant was treated with 1mM $MgCl_2$ and centrifuged at 135,000 x g for 45 min. The resultant synaptic vesicle pellet was suspended in 160mM KCl, 5mM NaCl, 260μM pargyline, 2mM EDTA, 1mM EGTA, and Tris-maleate 10mM pH 6.5 for 2 min and then 10mM $MgCl_2$ was added, giving a final $MgCl_2$ concentration of approximately 4-5mM (Iso-KCl media). This preparation was employed for the phosphorylation and neurotransmitter studies. The composition of the vesicle fraction was determined by electronmicroscopy(1,13) and enzyme markers (1,14,15), and was comparable to our previous vesicle preparation containing less than 4-8% membrane contamination. The entire preparation was performed in less than 5 hours and vesicle fractions were immediately used for phosphorylation and neurotransmitter release studies. Modifications of this standard technique were made by varying the composition of the additions to the osmotically shocked P_2 fraction as described in Table 1 and 2.

The standard reaction mixture for simultaneously studying neurotransmitter release and protein phosphorylation contained 5ml of the standard vesicle preparation, containing 1-2mg vesicle protein/ml and 25μM cold ATP or [γ-^{32}P] ATP from New England Nuclear (specific activity 5-10 Ci/m mole) in the presence

or absence of calcium chloride. The reactions were initiated by the addition of calcium or Iso-KCl media, following a 10 min equilibration with ATP or [γ-^{32}P] ATP, and incubated for 1 min at 37°C and immediately put in ice and centrifuged at 135,000 x g for 30 min at 4°C. The supernatant was removed and the vesicle pellets were suspended in the same volume of Iso-KCl media and studied for protein phosphorylation or neurotransmitter release. The [γ-^{32}P] ATP preparations were immediately assayed for [^{32}P] phosphate incorporation into synaptic vesicle protein by polyacrylamide gel electrophoresis and quantitation as described previously (9), except that 300mM Li Cl was added to the SDS stop solution to prevent the precipitation of potassium salts of SDS. The content of norepinephrine in the vesicles and supernatant fractions was determined spectrofluorometrically (16). Protein peaks A-M (17) and proteins DPH-L and DPH-M (9) were shown to be protein in nature as described previously.

RESULTS

Norepinephrine Content in Synaptic Vesicles

To study neurotransmitter release from synaptic vesicles, it was important to develop a vesicle preparation that was stable and as physiologically active as possible. Table 1 presents the norepinephrine content of synaptic vesicles isolated under isotonic and hypotonic conditions, at different pH values, and under different techniques of preparation. The Iso-KCl media produced the highest norepinephrine content. Isotonic sucrose was not as good a support solution. The hypotonic Tris-maleate 10mM preparation, described previously (1), caused a significant reduction in norepinephrine content of the vesicles, and the vesicles were very unstable, when suspended in hypotonic Tris-maleate in the absence of the soluble protein fraction. More vigorous homogenization during osmotic shock of the P_2 pellet, prolongation of the preparation time, and increasing the pH to 7.0 decreased the norepinephrine content of the vesicles, especially in the sucrose and hypotonic media. Calcium ions caused a marked decrease in the norepinephrine content of vesicles prepared in Iso-KCl media, but had much less of an effect on the release of neurotransmitter from vesicles prepared in sucrose or hypotonic media (Table 2). Since synaptic vesicles prepared in Iso-KCl media were the most enriched in norepinephrine, most stable, and most physiologically active, this preparation of vesicles was used to study calcium-dependent neurotransmitter release and protein phosphorylation.

Calcium-Dependent Norepinephrine Release From Synaptic Vesicles

The effect of calcium ions on the release of norepinephrine from highly

Table 1. Effects of Preparation Conditions on the Norepinephrine Content of Synaptic Vesicles.

Preparation Conditions*	Norepinephrine Concentration+
KCl 160mM, NaCl 5mM, Tris-maleate 10mM	
pH 6.5, $MgCl_2$ 5mM (Iso-KCl media)	5.36 ± 0.16
pH 6.5	5.03 ± 0.11
pH 6.5, increased preparation time	3.64 ± 0.14
pH 6.5, prolonged osmotic shock	1.72 ± 0.23
pH 7.0	4.63 ± 0.10
Sucrose 0.32 M, Tris-maleate 10mM	
pH 6.5	3.74 ± 0.14
pH 6.5, increased preparation time	1.95 ± 0.09
pH 6.5, prolonged osmotic shock	1.12 ± 0.18
pH 7.0	3.11 ± 0.13
KCl 100mM, Tris-maleate 10mM	
pH 6.5	2.98 ± 0.12
Tris-maleate 10mM, KCl 10mM	
pH 6.5	2.23 ± 0.11
pH 6.5, increased preparation time	1.01 ± 0.23
pH 6.5, prolonged osmotic shock	0.61 ± 0.31
pH 7.0	1.67 ± 0.14

*Synaptic vesicles were prepared under standard conditions with the addition of the above reagents or conditions following the osmotic shock of the washed P_2 pellet (Methods). Preparation time was increased from less than 5 hours to 8 hours as indicated. The P_2 pellet was subjected to prolonged osmotic shock by homogenization in 5.5 times its volume of distilled water with 20 strokes at 500 rpm for 10 minutes (Methods).

+Each value is expressed as ng (norepinephrine) /mg (protein) and represents the mean values and ranges for 4 determinations.

purified synaptic vesicles is shown in Table 3. Calcium (1mM) caused a significant decrease in the norepinephrine content of the synaptic vesicles and a corresponding increase in the amount of norepinephrine released from the vesicles. The action of calcium on neurotransmitter release was dependent upon the presence of magnesium ions. Magnesium alone caused no significant release of norepinephrine. The calcium ion concentration required to produce a half maximal increase in norepinephrine release under standard conditions was approximately 1×10^{-4}M.

Calcium-Dependent Phosphorylation of Synaptic Vesicle Proteins

The effects of calcium ions on the level of endogenous phosphorylation of

TABLE 2. Effect of Calcium on the Norepinephrine Content of Synaptic Vesicles Prepared Under Different Conditions*

Preparation Conditions	Norepinephrine Concentration	
	Control	Calcium
Iso-KCl Media	5.21 ± 0.12	3.27 ± 0.18
Sucrose 0.32M, Tris-maleate 10mM pH 6.5	3.66 ± 0.17	3.18 ± 0.09
Tris-maleate 10mM, KCl 10mM pH 6.5	2.31 ± 0.10	1.98 ± 0.21

*Synaptic vesicle plus soluble protein fractions obtained with the addition of the above reagents following osmotic shock were incubated under standard conditions in the presence or absence of calcium ions. Synaptic vesicles were isolated by centrifugation and assayed for norepinephrine (Methods). Each value is expressed as ng (norepinephrine) /mg (protein) and represents the mean values and ranges for 4 determinations.

synaptic vesicle proteins (Fig 1 and Table 3) were simultaneously studied in the same preparation employed to investigate calcium-dependent norepinephrine release. Calcium (1mM) caused a dramatic increase in the phosphorylation of proteins DPH-L and DPH-M and protein peaks K and L. (Fig 1) The concentration of calcium ions required to produce a half-maximal increase in the phosphorylation of proteins DPH-L, DPH-M, and Peak L were 2×10^{-4}M, 1×10^{-4}M, and 4×10^{-4}M, respectively. Calcium ions stimulated both the initial rate and net level of phosphorylation of synaptic vesicle-associated proteins DPH-L, DPH-M, and Peak L and caused a greater percent stimulation in the level of phosphorylation of proteins DPH-L and DPH-M in this preparation (Table 3) than in the hypotonic Tris-maleate preparation of synaptic vesicles (1). The effects of calcium ions on the phosphorylation of proteins DPH-L, DPH-M, and Peak L were dependent upon magnesium ions. Proteins DPH-L and DPH-M demonstrated only minimal magnesium-dependent phosphorylation, but protein Peak L showed approximately 55% magnesium-dependent phosphorylation (Table 3). Protein Peaks A-J also demonstrated

Table 3. Effect of Calcium on Neurotransmitter Release and Protein Phosphorylation In a Highly Purified Preparation of Synaptic Vesicles*

Condition	NOREPINEPHRINE RELEASE+		SYNAPTIC VESICLE PROTEIN PHOSPHORYLATION#					
			Protein DPH-L		Protein DPH-M		Protein L	
	Synaptic Vesicle Concentration	% Release	Arbitrary Units	Percent	Arbitrary Units	Percent	Arbitrary Units	Percent
Control	5.27	-	-	-	-	-	-	-
Mg	5.25	3.3	7.8	13.3	10.5	11.8	56.3	55.4
Ca	5.20	2.1	4.2	7.2	6.0	6.7	15.6	15.3
Ca + Mg	3.16	47.6	58.7	100.0	88.9	100.0	101.7	100.0

*Synaptic vesicles were incubated for 1min under standard conditions in the presence or absence of calcium (1mM) and/or magnesium (5mM) with 25 μM ATP (norepinephrine release) or [γ-^{32}P]ATP (protein phosphorylation). Vesicles were isolated by centrifugation at 4°C and assayed for norepinephrine and protein phosphorylation (Methods).

+Norepinephrine concentration is expressed as ng/mg protein. % Release is expressed as % release of norepinephrine into the supernatant as compared to control condition. The total recovery of released and bound norepinephrine was 96-98% in each condition. The data represent the mean value of four determinations and the largest ranges about the mean were 0.18 ng/mg and 1.4% and were thus omitted for clarity.

#Each arbitrary unit equals approximately 36.7cpm and percent represents the percent of the Ca + Mg condition. The data give the mean values for 6 experiments. The largest ranges about the mean were 1.98 arbitrary units and 2.4% and were omitted for clarity.

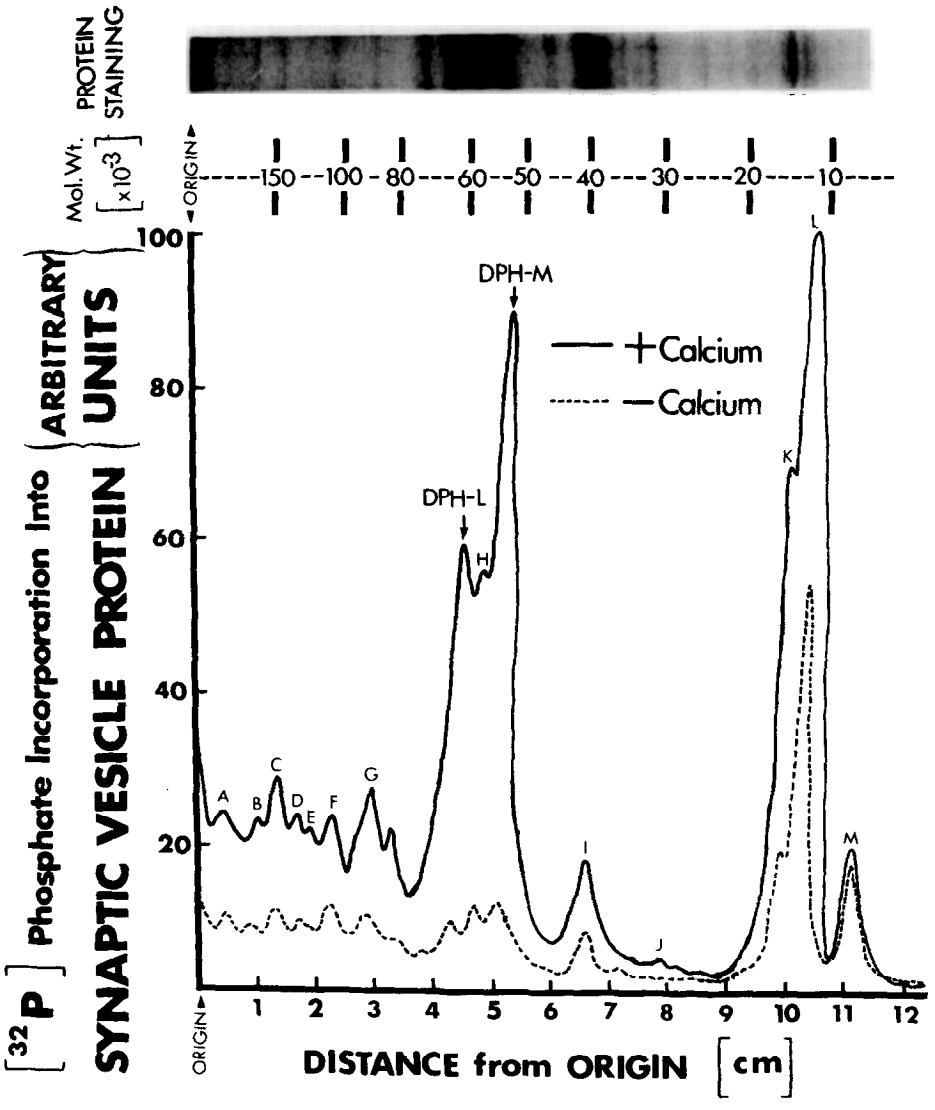

Figure 1. Effect of Calcium on the Endogenous Phosphorylation of Synaptic Vesicle Proteins

Synaptic vesicles were incubated for 1 min. under standard conditions in the presence or absence of calcium ions (1mM), isolated by centrifugation at 4°C, and subjected to poly acrylamide gel electrophoresis, protein staining and quantitation (Methods). The results shown are representative of 10 individual experiments. Each arbitrary unit equals approximately 32.5 cpm.

calcium-dependent phosphorylation, but these phosphoproteins represented a small proportion of the total [^{32}P] phosphate incorporation into protein.

DISCUSSION

The results demonstrate that calcium ions simultaneously stimulate the endogenous release of norepinephrine from synaptic vesicles and the endogenous incorporation of [^{32}P] phosphate into specific synaptic vesicle proteins in the same preparation of synaptic vesicles treated under identical conditions. The concentrations of calcium ions required to produce a half-maximal release of neurotransmitter and incorporation of [^{32}P]phosphate were almost identical. Calcium ions caused a greater increase in the level of phosphorylation of specific synaptic vesicle proteins in vesicle preparations that showed the greatest calcium-dependent norepinephrine release and contained the highest concentration of norepinephrine. These results indicate a more direct relationship between calcium-dependent phosphorylation of specific synaptic vesicle proteins and release of neurotransmitter from the vesicles, and are consistent with our initial hypothesis (1, 12) that calcium's effect on synaptic vesicle-associated protein phosphorylation is the molecular mechanism mediating calcium's physiologic action on neurotransmitter release from the presynaptic nerve terminal.

Synaptic vesicles contain high concentrations of endogenous ATP in association with several neurotransmitter substances (18-21). Synaptic vesicle bound ATP has been shown to decrease during neurotransmitter release in both intact and broken preparations (22). Our results suggest that calcium-dependent phosphorylation of specific vesicle proteins may be the endogenous mechanism utilizing this vesicle bound ATP to initiate calcium-dependent neurotransmitter release.

The synaptic vesicle preparation from whole brain represents a mixture of several types of synaptic vesicles, containing different neurotransmitter substances. To further establish the role of calcium-dependent synaptic vesicle-associated protein phosphorylation in mediating neurotransmitter release, it is important to demonstrate that the release of other neurotransmitters contained

in this mixed vesicle population from brain behave like norepinephrine. Preliminary results in this laboratory (DeLorenzo, in preparation) have demonstrated that the calcium-dependent release of dopamine, acetylcholine, and γ-aminobutyric acid give similar results to norepinephrine. It has also been recently shown (DeLorenzo and Freedman, in preparation) that DPH inhibits both the calcium-dependent release of norepinephrine and the calcium-dependent phosphorylation of proteins DPH-L and DPH-M in this preparation and that a protein fraction can be removed or added to the vesicle preparation that controls both the calcium-dependent release of neurotransmitter and the calcium-dependent phosphorylation of specific vesicle-associated proteins, indicating a direct relationship between neurotransmitter release and protein phosphorylation.

The synaptic vesicle preparation utilized in this investigation provides a useful model system for studying the biochemistry of neurotransmitter release and synaptic vesicle function. Further investigation of the molecular mechanism of calcium-dependent synaptic vesicle-associated protein phosphorylation may provide a clearer understanding of the effects of calcium on neurotransmitter release and synaptic vesicle function.

ACKNOWLEDGEMENTS

This research was supported by Research Career Development Award NSI-EA 1 K04 NS 245, USPHS grant NS 13632, and the Esther A. and Joseph Klingenstein Fund.

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