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Stimulation of Calcium Accumulation in Cardiac Sarcolemma by Phosphorylase Kinase

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After incubation with phosphorylase kinase, calcium accumulation in cardiac sarcolemma was increased from 65 ± 2 to 95 ± 3 nmol/10 min per mg of protein. Under these conditions, phosphorylase kinase catalysed phosphorylation of membranes. This phosphorylation was hydroxylamine-insensitive, was stimulated by Ca^{2+} ions and was unaffected by 3':5'-cyclic AMP.

The regulation of membrane Ca^{2+} transport by 3':5'-cyclic AMP- and Ca^{2+} -dependent phosphorylation of membrane proteins has been intensively investigated [see Sulakhe & St. Louis (1976a) for a review]. For example, many investigators have reported stimulation of Ca^{2+} uptake in cardiac sarcoplasmic reticulum by cyclic AMP-dependent protein kinase (EC 2.7.1.37)-catalysed phosphorylation of a 22000-dalton component in sarcoplasmic reticulum (Kirchberger *et al.*, 1972, 1974; LaRaia & Morkin, 1974; Tada *et al.*, 1975; Schwartz *et al.*, 1976). The role of Ca^{2+} -dependent phosphorylase kinase (EC 2.7.1.38) system in muscle glycogenolysis is well established (Krebs, 1972). However, numerous findings suggest that this system may well serve as a link between two cyclic AMP-mediated events in muscle: excitation-contraction coupling and intermediary metabolism [see Entman *et al.* (1976) for a review]. In this context, the finding by Schwartz *et al.* (1976) that phosphorylase kinase augmented Ca^{2+} uptake in sarcoplasmic reticulum is of interest. In the present paper, we describe stimulation of calcium accumulation in cardiac sarcolemma by phosphorylase kinase. We found that phosphorylase kinase catalysed phosphorylation of cardiac sarcolemma. The phosphorylation was hydroxylamine-insensitive, stimulated by Ca^{2+} and was unaffected by cyclic AMP. Some of these observations have been published in an abstract (Sulakhe & St. Louis, 1976b).

Experimental

Materials

$^{45}\text{CaCl}_2$ (20 mCi/mg) and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (5–10 Ci/mmol) were from New England Nuclear, Montreal, Canada; phosphorylase kinase, protein kinase, ATP and cyclic AMP were from Sigma Chemical Co.,

St. Louis, MO, U.S.A. The kinase preparations were dialysed overnight at 4°C against 1000 vol. of 10 mM-Tris/HCl, pH 7.2. All other reagents were of highest purity available.

Methods

Isolation of sarcolemma. Sarcolemma from guinea-pig heart ventricles were isolated as previously described (Sulakhe *et al.*, 1976; St. Louis & Sulakhe, 1976a).

Assay of calcium accumulation. Sarcolemmal membranes (200–300 µg of protein) were pre-incubated for 5 min at 30°C in the reaction mixture (1 ml) containing 25 mM-Tris/maleate, pH 6.8, 10 mM- MgCl_2 , 100 mM-KCl, 5 mM-potassium oxalate, 5 mM- NaN_3 and 2 mM-ATP in the absence and presence of various amounts of phosphorylase kinase. The reaction was started by the addition of $^{45}\text{CaCl}_2$ (final concn. 0.1 mM), and samples (0.3 ml) were withdrawn at the desired times, and were filtered through the Millipore filters (0.45 µm pore size). The filters were washed, dried and counted for radioactivity as previously described (Sulakhe *et al.*, 1973, 1976; St. Louis & Sulakhe, 1976b).

Phosphorylation of sarcolemma and separation of membrane proteins by electrophoresis. Sarcolemmal membranes (100–200 µg) were incubated for 5 min at 24°C in the reaction mixture (0.4 ml) containing 50 mM-Tris/maleate, pH 6.8, 10 mM-mercaptoethanol, 20 mM-NaF, 10 mM- MgCl_2 , 5 mM-EGTA and 30–50 µM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence and presence of phosphorylase kinase (50–200 µg). When present, CaCl_2 was at a concentration of 50 µM (EGTA absent). The remaining procedure was essentially similar to that described by Schwartz *et al.* (1976) except that, in some instances, the pellet from trichloroacetic acid treatment was further treated with 0.8 M-hydroxylamine hydrochloride (at pH 5.0)

Table 1. Stimulatory effect of phosphorylase kinase on calcium accumulation in cardiac sarcolemma

Expt. (a): sarcolemmal membranes (270 μ g of protein) were incubated in the absence and presence of various amounts of phosphorylase kinase as indicated. Expt. (b): sarcolemmal membranes (205 μ g) were incubated in the absence and presence of two concentrations of phosphorylase kinase as indicated. Heat-denatured (boiled) phosphorylase kinase of similar amounts was present as shown. Expt. (c): sarcolemmal membranes (225 μ g) were incubated in the absence and presence of phosphorylase kinase or protein kinase with and without 5 μ M-cyclic AMP. For each experiment, a separate membrane preparation was used. For other details, see under 'Methods'. Results are averages of duplicate determinations; the duplicates agreed within 5%.

Expt. (a)		Expt. (b)				Expt. (c)	
Amount of phosphorylase kinase (μ g)	Calcium accumulation (nmol/10 min per mg)	Calcium accumulation (nmol/mg) at:			Additions	Calcium accumulation (nmol/10 min per mg)	
		2 min	4 min	10 min			
0	65	None	34	55	72	None	68
13	72	Phosphorylase kinase (11 μ g)	44	69	83	Phosphorylase kinase (130 μ g)	87
33	92	Phosphorylase kinase (110 μ g)	47	77	95	Phosphorylase kinase (130 μ g) + cyclic AMP (5 μ M)	86
66	93	Boiled phosphorylase kinase (11 μ g)	33	53	69	Protein kinase (10 μ g)	76
132	95	Boiled phosphorylase kinase (110 μ g)	35	53	71	Protein kinase (10 μ g) + cyclic AMP (5 μ M)	93
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before solubilization with 1% sodium dodecyl sulphate. The solubilized membranes were fractionated by 1% sodium dodecyl sulphate/6% polyacrylamide-gel electrophoresis as previously described (St. Louis & Sulakhe, 1976a). The gels were stained with Coomassie Blue, destained in methanol/acetic acid and were scanned at 550 nm as described by St. Louis & Sulakhe (1976a). The gels were then cut into 1 mm sections and counted in Bray's solution (Bray, 1960). Standard reference proteins were run simultaneously.

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results and Discussion

Table 1 shows the stimulatory effect of phosphorylase kinase on calcium accumulation in cardiac sarcolemma. The stimulatory effect of the kinase was concentration-dependent and was demonstrable at the ratio (phosphorylase kinase/sarcolemmal protein) of 0.05 (Expt. a). Phosphorylase kinase increased the initial rate of as well as the total calcium accumulation (Expt. b). Heat-denatured kinase was devoid of this effect. Cyclic AMP failed to further increase phosphorylase kinase-stimulated calcium accumulation, whereas it enhanced protein kinase-stimulated calcium accumulation (Expt. c). This finding suggested that the phosphorylase kinase effect was not due to the contamination by protein kinase, which indeed enhances calcium accumulation in these membranes (Sulakhe *et al.*, 1976). Additional experiments supported this view. For example, phosphorylation of histone (100 μ g, type II-A) by either 30 μ g of protein kinase or 30 μ g of phosphorylase kinase was carried out in the presence of 5 μ M-cyclic AMP and/or 50 μ M- Ca^{2+} . Histone phosphorylation catalysed by protein kinase was stimulated (5-fold) by cyclic AMP and inhibited (40%) by Ca^{2+} . On the other hand, phosphorylase kinase-catalysed phosphorylation was unaffected by cyclic AMP and stimulated by Ca^{2+} (2-fold). Further, in accord with previous reports (Krebs, 1972), histone was a poor substrate for phosphorylase kinase compared with protein kinase (with protein kinase 190 pmol of ^{32}P was incorporated into histone, whereas with phosphorylase kinase 2 pmol of ^{32}P was incorporated). These observations suggest that the protein kinase contamination in the phosphorylase kinase preparation was negligible, if any.

Phosphorylase kinase was observed to catalyse phosphorylation of cardiac sarcolemma. The phosphorylation of sarcolemma was mainly hydroxylamine-insensitive (30 and 26 pmol of ^{32}P incorporated per mg of sarcolemmal protein before and after treatment with hydroxylamine), was due to phosphorylation of proteins rather than lipids of

membranes (chloroform/methanol treatment of phosphorylated membranes removed only up to 5% of the phosphate), and was completely unstable in 1M-NaOH at 100°C. Ca^{2+} ions (50 μM) markedly enhanced (2-fold) the kinase-catalysed phosphorylation of sarcolemma. The stimulatory effect of Ca^{2+} was observed at pH 6.8, which as a routine was the pH of the reaction mixture during phosphorylation.

Electrophoretic separation of phosphorylated membranes revealed that a number of proteins were phosphorylated. However, with Ca^{2+} present during the phosphorylation assay, phosphorylation of proteins of mol.wt. 95000, 70000, 48000 and 35000 was markedly increased (results not shown). In contrast with this, protein kinase mainly phosphorylated low-mol.wt. proteins (25000 and 14000) of these membranes (Sulakhe & St. Louis, 1976a).

Phosphorylase kinase, which was previously considered to be a more specific enzyme than protein kinase (Krebs, 1972), has been now shown to phosphorylate other proteins such as 95000-dalton component of muscle sarcoplasmic reticulum (Schwartz *et al.*, 1976) and muscle troponin (Stull *et al.*, 1972). Our study shows that phosphorylation *in vitro* of cardiac sarcolemmal proteins is catalysed by this kinase. Interestingly, such phosphorylation augmented calcium accumulation in sarcolemma, a finding in support of the view that phosphoester-type phosphorylation of membrane proteins regulates membrane Ca^{2+} fluxes (Sulakhe & St. Louis, 1976a). Further studies would be required to understand significance of membrane phosphorylation reactions in redistribution of Ca^{2+} ions in the cell, especially during hormonal effects.

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