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PHOSPHORYLATION OF CARDIAC SARCOPLASMIC RETICULUM BY A CALCIUM-ACTIVATED, PHOSPHOLIPID-DEPENDENT PROTEIN KINASE.

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SUMMARY: Cardiac sarcoplasmic reticulum is phosphorylated by a cytosolic Ca^{2+}-activated, phospholipid-dependent protein kinase. This phosphorylation is independent of cyclic nucleotides and enhanced by unsaturated diacylglycerols; saturated diacylglycerols, mono- and tri-glycerides are ineffective. Diacylglycerol stimulation is due to increased Ca^{2+} sensitivity of the kinase reaction. Protein kinase catalyzed phosphorylation results in enhanced Ca^{2+}-transport ATPase activity and may be an important determinant of cardiac sarcoplasmic reticulum function.

INTRODUCTION: Calcium ion transport by cardiac sarcoplasmic reticulum can be modulated through phosphorylation by endogenous protein kinases. The best studied example is the cyclic AMP-dependent protein kinase which stimulates Ca^{2+} uptake and Ca^{2+}-ATPase activity by phosphorylating a 22,000-dalton component (phospholamban) of the sarcoplasmic reticulum (1). Phosphorylase b kinase also enhances calcium transport probably through phosphorylation of components other than phospholamban (2). Additional regulatory control is offered by calmodulin which promotes the phosphorylation of phospholamban at a site distinct from that influenced by the cyclic AMP-dependent protein kinase (3,4). It is likely that complex interactions between different protein kinases, Ca^{2+} ions, and phosphorylation sites on the sarcoplasmic reticulum regulate calcium transport by this organelle. In the present study, we report that a recently described (5-7) Ca^{2+}-activated, phospholipid-dependent protein kinase can also utilize cardiac sarcoplasmic reticulum as substrate and modify the activity of the calcium transport ATPase.

MATERIALS AND METHODS: Experiments were carried out on adult male Sprague-Dawley rats.

Isolation of sarcoplasmic reticulum: The animals were killed by cervical dislocation and their hearts were rapidly excised and chilled in crushed

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ice. The myocardial tissue was washed twice with a solution containing 10 mM $NaHCO_3$-5 mM NaN_3 and was then homogenized in the same solution with a Polytron PT-20 homogenizer three times at a rheostat setting of 3 for 5 sec with 15 sec rest intervals. The homogenates were then processed for sarcoplasmic reticulum isolation as described by Sumida et al. (8).

Ca^{2+}-activated ATPase: ATPase activity was determined at 25°C in a medium containing 40 mM histidine-HCl buffer (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 120 mM KCl, 5 mM NaN_3, calcium-EGTA buffer containg 1 µM free Ca^{2+} and 50-80 µg microsomal protein in a total volume of 0.5 ml. Reaction mixtures were pre-incubated at 25°C for 5 min. after which the reaction was started by the addition of microsomal protein. To determine "basal" (Mg^{2+}-ATPase) activity, reactions were carried out in the presence of EGTA (0.2 mM) instead of the calcium-EGTA buffer. At various intervals after the start of the reaction, 100 µl aliquots were added to tubes containing 25 ml of 25% (w/v) trichloroacetic acid and 125 µM inorganic phosphate. After centrifugation at 3,000 g for 10 min. inorganic phosphate was determined (9) in the supernatant.

Protein kinase isolation: Phospholipid-dependent protein kinase (kinase C) was isolated essentially by the method of Takai et al. (5). Rat hearts were homogenized in four volumes of 20 mM tris-HCl (pH 7.5)-50 mM 2-mercaptoethanol-2 mM EDTA (buffer A) using a Polytron PT-20 at a setting of 4 for 30 sec. The homogenate was centrifuged at 20,000 g for 40 min. and the resulting supernatant was filtered through glass wool. The extract was applied to a DEAE-cellulose column previously equilibrated with buffer B (buffer A containing 70 mM NaCl). After the column was washed with 100 ml of buffer B, the first elution was carried out with 250 ml buffer B containg 10^{-5}M cyclic AMP. The second elution was carried out with a linear gradient (70-300 mM) NaCl in buffer A containing 10^{-5}M cyclic AMP. Fractions of 10 ml were collected and assayed for protein kinase activity (Figure 1).

Protein kinase assay: The assay medium for protein kinase contained, in a total volume of 0.25 ml, 5 µmol tris-HCl (pH 7.5), 1.25 µmol $MgCl_2$, 25 nmol [γ-^{32}P]ATP (Amersham/Searle, Arlington Heights, Ill., sp. act. 6.1 Ci/mmol), 10 µg protein kinase C, 50 µg sarcoplasmic reticulum or histone H_1 (Sigma Chemical Co.) and various concentrations of $CaCl_2$ or lipids. Incubation was carried out at 37°C for variable lengths of time and the reactions were stopped by the addition of 2 ml of 25% trichloroacetic acid; precipitates were collected on Millipore filters (0.45 µm pore size) and processed for radioactivity counting.

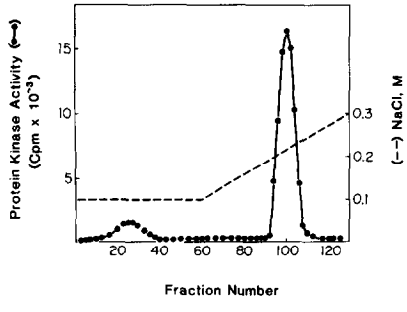


Figure 1: Isolation of protein kinase C from rat heart cytosol. Cardiac extract was passed through a DEAE-cellulose column previously equilibrated with 20 mM tris-HCl (pH 7.5)-50 mM 2-mercaptoethanol-2 mM EDTA-70 mM NaCl (buffer B). After the column was washed with buffer B, the first elution was carried out with buffer B containing 10^{-5}M cyclic AMP and then with a linear gradient of NaCl (---) in buffer. Aliquots from the eluates were assayed for protein kinase (O O) as described in the text.

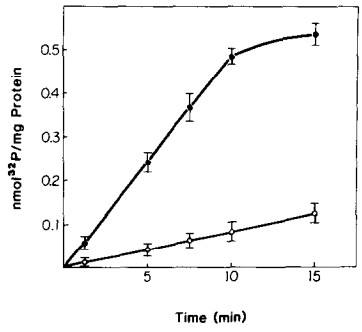


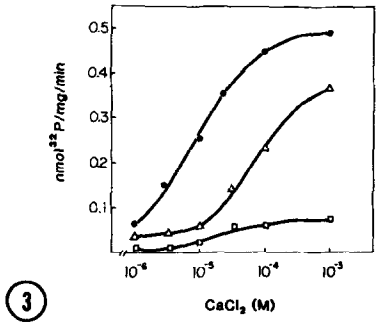
Figure 2: Effect of protein kinase C on SR phosphorylation. The assay mixture contained 20 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 µg cardiac SR, 25 nmol [γ-^{32}P]ATP, 10^{-5}M $CaCl_2$, and 50 µg protein kinase C. Reactions were carried out at 37°C for various lengths of time as described in the text. Control mixtures (○) were identical except for omission of protein kinase C. Results represent mean ± SE for six comparisons.

RESULTS AND DISCUSSION: The procedure of Takai et al. (5) was utilized to isolate protein kinase C from rat cardiac cytosol. With histone H1 as substrate, cardiac protein kinase has properties similar to those previously described for the enzyme from rat cerebrum (5-7). Specifically, it is activated by micromolar concentrations of calcium and phospholipids and is unaffected by cyclic nucleotides (data not shown). We then examined the possibility that cardiac sarcoplasmic reticulum can serve as substrate for protein kinase C. As shown in Figure 2, this kinase significantly enhances endogenous sarcoplasmic reticulum phosphorylation. The requirements for this phosphorylation are shown in Table 1: cyclic nucleotides are ineffective when either histone H_1 or SR is used as substrate. When protein kinase assay is carried out with H_1 as substrate, a definite lipid requirement is seen. Phosphatidylserine is the most potent phospholipid followed by phosphatidylinositol and phosphatidylcholine. Diacylglycerols containing unsaturated fatty acids significantly potentiate the effects of phospholipids on the protein kinase, a property not shared by saturated diacylglycerols, mono- or tri-acylglycerols. Phosphorylation of cardiac SR is significantly less stimulated by the addition of exogenous lipids. The nature of the protein kinase C enhancement by diolein is revealed by

Lipid dependency of protein kinase C. Reactions were carried out at 37°C for 10 min. with 50 g protein kinase C, 10^{-5}M $CaCl_2$, and the indicated additions. Phospholipids (16 µg) and diacyl- or triacylglycerols (6 µg) were first solubilized by sonication in the incubation buffer. Results are expressed as percentage of protein kinase activity in controls, in the absence of added lipids (4.8 nmol/mg/10 min with histone and 1.6 nmol/mg/10 min. with cardiac SR as substrate).

ADDITIONS	PROTEIN KINASE ACTIVITY	
	With Histone H_1 (50 µg)	With SR (50 µg)
None (Control)	100	100
Phosphatidylinositol	346	127
Phosphatidylserine	359	136
Phosphatidylcholine	117	108
Phosphatidylethanolamine	108	104
Phosphatidylserine + diolein	489	146
" + dilinolein	426	138
" + diarachidonin	501	151
" + dipalmitin	352	130
" + distearin	361	126
" + triolein	348	128

a study of the Ca^{2+} dependency of the reaction (Figure 3). Addition of diolein significantly increases the sensitivity of the kinase to calcium so that lower concentrations are needed to maximally activate the enzyme.



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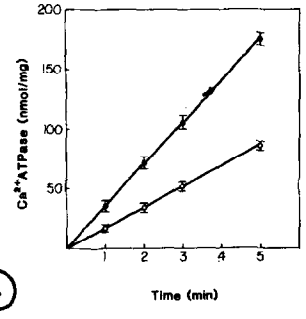

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Figure 3: Calcium dependency of protein kinase C activity. Reactions were carried out at 37°C for 10 min. with 50 µg histone H_1, 50 µg protein kinase C, various concentrations of $CaCl_2$, and 6 µg diolein (□), 10 µg phosphatidylserine (△), or 6 µg diolein plus 10 µg phosphatidylserine (●). Results represent mean ± SE for six experiments.

Figure 4: Effect of protein kinase C on the calcium transport ATPase of cardiac sarcoplasmic reticulum. Reactions were carried out at 25°C in the presence of 50 µg SR alone (○), or 50 µg protein kinase C plus 10 µg phosphatidylserine and 6 µg diolein (●). Results represent mean ± SE for six experiments.

Experiments on the Ca^{2+} transport ATPase of the cardiac sarcoplasmic reticulum strongly suggest that phosphorylation by protein kinase C has functional implications. Protein kinase stimulates ATPase activity (Figure 4) in the presence of both phospholipid and diolein. In preliminary studies, we have also found a stimulatory effect of protein kinase C on Ca^{2+} uptake by cardiac sarcoplasmic reticulum.

These results indicate that protein kinase C catalyzed phosphorylation may represent a novel and important mechanism for regulating the function of cardiac sarcoplasmic reticulum. The site(s) of phosphorylation and the possible interactions with other protein kinases are currently under investigation in our laboratory. Protein kinase C is clearly not related to the cyclic AMP-dependent kinase and differs from calmodulin-dependent enzyme which, although calcuim-dependent, does not stimulate the Ca^{2+}-ATPase (4). Since phosphorylase b kinase is stimulated by protein kinase C (6), it is possible that it participates in the enhancement of calcium transport by the latter enzyme; this possibility needs to be further explored.

Regardless of mechanisms involved, protein kinase C catalyzed phosphorylation of cardiac sarcoplasmic reticulum has two attractive features. First, in view of the calcium dependency of the enzyme it can be envisaged as a sensor of cytoplasmic calcium concentration and a link to the calcium pump of the sarcoplasmic reticulum. Secondly, its stimulation by membrane phospholipids and diglycerides relates calcium transport to glycerolipid metabolism. This may be particularly relevant to phosphatidylinositol turnover which has been associated with calcium fluxes across cell membranes in response to metabolic, hormonal, or neural stimuli (10). Phosphatidylinositol breakdown leads to the formation of diacylglycerol which activates protein kinase C. Such activation would not require major increases in intracellular calcium levels since it depends on a shift in the Ca^{2+} sensitivity of the enzyme. The enhancement of calcium sensitivity by unsaturated diglycerides is especially intriguing because it provides a mechanism for amplifying the effects of protein kinase C on the sarcoplasmic reticulum. This mechanism would

be primarily important in disease states, such as cardiac hypertrophy and failure, in which lower intracellular calcium levels are associated with decreased capacity of the sarcoplasmic reticulum to accumulate calcium. Diacylglycerol mediated stimulation of protein kinase C may be, under these circumstances, an important adaptation for maintaining cardiac sarcoplasmic reticulum function.

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