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CORRELATION BETWEEN PROTEIN KINASE-MEDIATED STIMULATION OF CALCIUM TRANSPORT BY CARDIAC SARCOPLASMIC RETICULUM AND PHOSPHORYLATION OF A 22 000 DALTON PROTEIN

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Summary

Increases in protein kinase-catalyzed phosphorylation of a 22 000 dalton protein correlated closely with increases in phosphate-facilitated calcium transport measured concurrently in canine cardiac sarcoplasmic reticulum under similar conditions in the presence of varying concentrations of bovine cardiac protein kinase. A correlation coefficient of 0.93 and a P value of < 0.001 were obtained. Protein kinase-catalyzed phosphorylation of the 22 000 dalton microsomal protein may mediate the abbreviation of systole seen in the mammalian heart in response to inotropic agents like catecholamines.

The rate of cardiac relaxation is generally considered to be determined by the rate of removal of calcium from the contractile proteins. This calcium is transported by an ATP-dependent calcium pump into the sarcoplasmic reticulum [1]. We have previously suggested that the relaxation-promoting effects of catecholamines on the mammalian myocardium [2] can be attributed to a cyclic AMP-mediated increase in the rate of calcium transport into the sarcoplasmic reticulum [3—5]. The increased calcium transport seen in the presence of oxalate after brief treatment of cardiac sarcoplasmic reticulum with cyclic AMP and protein kinase appears to be related to increased phosphorylation of serine and possibly threonine residues of these membranes [4]. Fractionation of the cardiac sarcoplasmic reticulum by sodium dodecyl sulfate-polyacrylamide gel electrophoresis has led to the

Abbreviation: EGTA, ethyleneglycol bis(α-aminoethylether)-*N,N'*-tetraacetic acid.

identification of a 22 000 dalton protein whose phosphorylation is catalyzed by cyclic AMP-dependent protein kinase [6, 7]. In the present communication we correlate protein kinase-catalyzed phosphorylation of this 22 000 dalton protein with the protein kinase-mediated increase in calcium transport, measured under similar conditions.

Cardiac microsomes, consisting mainly of fragmented sarcoplasmic reticulum, were prepared by the method of Harigaya and Schwartz [8] modified slightly [4]. Bovine protein kinase was prepared and assayed with histone as substrate, as described previously [4]. Its specific activity ranged from 1.4 to 2.6 nmol P transferred /mg protein per min.

For measurements of phosphorylation, microsomes (0.5 mg/ml final concentration) were incubated at 25°C in 40 mM histidine-HCl buffer (pH 6.8), 120 mM KCl, 50 mM KH_2PO_4, 5 mM Mg[γ-^{32}P]ATP, 1 μM cyclic AMP, and soluble protein kinase at concentrations ranging from 0.025 to 0.500 mg/ml. All solutions used in studies on phosphorylation and calcium uptake were adjusted to pH 6.8 prior to addition to the reaction mixture. Reactions were started by addition of microsomes. After a 10 min incubation, reactions were stopped by addition of 2 ml of an ice-cold solution of 10% trichloroacetic acid containing 0.1 mM KH_2PO_4. Samples were prepared for gel electrophoresis as described in a previous report under Procedure II [6]. Sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis was carried out according to the method of Weber and Osborn [10]. Gels were sliced into 1 mm thick sections, which were transferred to liquid scintillation vials for counting [6]. Calculations of microsomal phosphorylation are based on counts in the peak of radioactivity on the gels corresponding to a 22 000 dalton protein and in a minor peak corresponding to a protein less than 22 000 daltons.

For concurrent measurements of protein kinase-stimulated microsomal calcium uptake, except where indicated, microsomes were phosphorylated under the same conditions described above except that unlabelled ATP was used. After a 10 min preincubation, the calcium uptake reaction was started by transferring an aliquot of the reaction mixture to a medium containing 40 mM histidine-HCl buffer, pH 6.8 at 25°C, 120 mM KCl, 50 mM KH_2PO_4, 5 mM NaN_3, 5 mM MgATP, and a calcium-EGTA buffer [11] that gave an ionized calcium concentration of 1 μM (25 μM $CaCl_2$ and 63.8 μM EGTA) when calculated using an association constant of $4.4 \cdot 10^5$ M^{-1} for the calcium-EGTA complex [12]. The final microsome concentration was 28 μg/ml. Samples were obtained by the Millipore filtration method and calcium uptake was calculated from the decrease in radioactive calcium in the filtrate.

In order to minimize the dephosphorylation of microsomes that is catalyzed by phosphoprotein phosphatase associated with the microsomes [9], KH_2PO_4, an inhibitor or phosphatase, was included in the incubation medium during the phosphorylation and calcium uptake reactions. However, since in preliminary experiments calcium uptake rates measured under these conditions were somewhat lower than those measured in experiments reported previously for oxalate-supported calcium uptake [4, 5], a comparison between the two types of measurements was made (Table I). Although phosphate-supported calcium uptake by microsomes preincubated in the absence (control) and presence of cyclic AMP and protein kinase was less than in oxalate, the

TABLE I

COMPARISON OF OXALATE- AND PHOSPHATE-FACILITATED CALCIUM UPTAKE BY CARDIAC MICROSOMES PREINCUBATED IN THE PRESENCE AND ABSENCE (CONTROL) OF 1 μM CYCLIC AMP AND 0.1 mg/ml BOVINE CARDIAC PROTEIN KINASE

In measurements of phosphate-facilitated calcium uptake, 50 mM KH_2PO_4 was present during the phosphorylation reaction as well as during the uptake reaction. In measurements of oxalate-facilitated calcium uptake, 2.5 mM oxalate was present only during the uptake reaction. Values represent averages of 3 independent experiments ± S.E. Statistical significance was determined by Student's *t*-test for paired variates.

Conditions	Calcium uptake	
	Oxalate ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)	Phosphate ($\mu mol \cdot mg^{-1} \cdot min^{-1}$)
Control	0.066 ± 0.005	0.041 ± 0.005
Cyclic AMP + protein kinase	0.118 ± 0.012	0.084 ± 0.009
% Stimulation	79	105
P	< 0.01	< 0.01

percent increase resulting from pretreatment with protein kinase remained approximately the same.

The stimulation of phosphate-facilitated calcium transport, measured as a function of protein kinase concentration, was compared with the extent of phosphorylation of the 22 000 dalton protein* of cardiac microsomes. This protein is evident as a single major peak of radioactivity on sodium dodecyl sulfate-polyacrylamide gels, as reported previously [6, 7]. Both phosphorylation and calcium uptake showed parallel increases over control incubates when microsomes were incubated at concentrations of protein kinase ranging from 0.025 to 0.500 mg/ml. A close correlation between phosphorylation of the 22 000 dalton protein and an increase in calcium uptake was found ($r = 0.93$, $P < 0.001$) in studies on 4 different microsomal preparations (Fig. 1A).

In addition to the single major peak of radioactivity found on gels on which phosphorylated microsomes had been fractionated, a minor peak corresponding to a lower molecular weight protein was also seen [6, 7]. In the 4 microsomal preparations studied, the extent of phosphorylation of this protein ranged from 0.02 to 0.03 nmol P/mg microsomal protein or approximately five percent of the values obtained for the 22 000 dalton protein. When the increase in phosphorylation of this protein was correlated as before with the increased calcium transport that resulted from treatment of cardiac microsomes with cyclic AMP and protein kinase (Fig. 1B), no relationship could be detected ($r = 0.07$, $P > 0.1$).

The present findings are not compatible with the view that the minor peak of phosphorylation seen in the gels is directly related to the protein kinase-induced increase in calcium transport. Instead, it is more likely that the minor peak is either a contaminant or a degradation product of the 22 000 dalton protein because its presence was both highly variable and did not correlate with an increase in calcium uptake. In contrast, the present study provides further evidence that the increase in calcium transport that is seen

*We have tentatively named this protein "phospholamban".

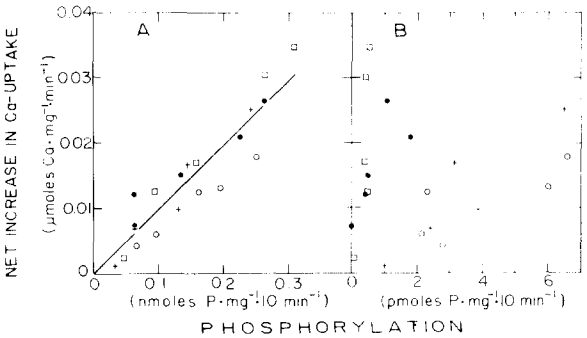

Fig. 1. Correlation of protein kinase-induced increase in oxalate-facilitated calcium transport by cardiac sarcoplasmic reticulum with net increase in phosphorylation of (A) 22 000 dalton protein and (B) a minor peak associated with a smaller protein, obtained upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis of phosphorylated microsomes. Microsomes were incubated in the presence of cyclic AMP, varying concentrations of protein kinase, and either [γ-^{32}P]ATP or unlabelled ATP for measurements of phosphorylation or calcium uptake, respectively. Plotted are increases in calcium uptake versus increases in phosphorylation over control values. See text for experimental details. Symbols (●, ○, □, +) denote data from each of 4 independent experiments.

when cardiac sarcoplasmic reticulum is incubated in the presence of cyclic AMP-dependent protein kinase [6, 7] is mediated by a 22 000 dalton phosphoprotein associated with these membranes.

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