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Involvement of electrostatic phenomena in phospholamban-induced stimulation of Ca uptake into cardiac sarcoplasmic reticulum

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The activity of the Ca-pumping ATPase of cardiac sarcoplasmic reticulum (SR) is controlled by the phosphorylation of the intrinsic regulatory protein phospholamban (PLB), which affects both the apparent K_m(Ca) and the V_{max} of the transport process. We have investigated the correlation between phosphorylation of PLB and the surface potential of the SR membrane. This latter influences the local concentration of relevant ionic species near biological membranes and thus modulates the activity of ion pumps and channels. The partitioning of the anionic probe 8-anilino-1-naphthalenesulfonate (ANS^-) into the SR membrane was found to be dependent on the phosphorylation level of PLB. Changes of the surface membrane potential up to 7 mV could be obtained by phosphorylation. The increase in the apparent affinity of the Ca pump for Ca^{2+} induced by PLB phosphorylation was clearly reduced at high ionic strength, i.e. under conditions known to reduce the surface membrane potential and all processes dependent on it. The results show that electrostatic phenomena can account, in good part, for the modulation of the Ca pump by PLB in cardiac SR.

Sarcoplasmic reticulum; Membrane potential; Phospholamban; Surface potential; Phosphorylation; (Heart)

1. INTRODUCTION

The contractile activity of cardiac muscle depends on rapid oscillations of the free Ca concentration in the cytosol, which are mainly under the control of the Ca-pumping ATPase and the Ca-releasing activities of the sarcoplasmic reticulum (SR). Regulatory mechanisms of these Ca fluxes are critical for the modulation of the mechanical performance of the heart. A mechanism known to stimulate the Ca-pumping activity of cardiac SR is based on the cAMP- and/or calmodulin-dependent phosphorylation of PLB, an intrinsic protein constituent of the cardiac SR membrane [1,2]. PLB plays a central role in mediating the action of β-agonists on the heart [3]. The protein is a complex composed of 5 identical subunits [4,5], each consisting of 52 amino acids [6]. Several positively charged amino acids render the protein rather alkaline (p*I* 10) [7]. However, a strong acidic shift is observed upon phosphorylation (p*I* 6.3–5.2) [5]. The molecular details of the mechanism by which PLB phosphorylation affects Ca-pumping ATPase activity are not known. Since PLB is present in very high amounts in cardiac SR, where it is supposed to exist in a 1 : 1 ratio with the ATPase [8], two hypotheses can be formulated: (i) phosphorylation modifies the amphiphilic properties of PLB such that it changes the hydrophobic microenvironment of the ATPase and thus the activity of the latter enzyme, and/or; (ii) phosphorylation of PLB appreciably alters the density of fixed negative charges on the surface of the SR membrane such that its electrostatic membrane potential is modified; the latter influences

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Abbreviations: PLB, phospholamban; Mops, 3-morpholinopropanesulfonic acid; ANS^-, 8-anilino-1-naphthalenesulfonate

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the local concentration of Ca^{2+} near the membrane and thus modulates the apparent affinity of the Ca pump for Ca^{2+}. This study shows that the latter mechanism does in fact play a relevant role.

2. MATERIALS AND METHODS

2.1. *Biological material*

SR membranes were isolated from dog hearts according to a modification [5] of the procedure in [9]. The light SR subfraction, enriched in the longitudinal system and practically devoid of Ca-release channels, was used in this study.

2.2. *Phosphorylation of cardiac SR*

Cardiac light SR membranes were suspended at about 5 mg/ml in a medium containing 100 mM sucrose, 2 mM $MgCl_2$, 10 mM Mops, pH 6.8 (buffer A) and, when required, 300 U/ml of the catalytic subunit of cAMP-dependent protein kinase (Sigma, St. Louis), or 3 mg/ml of protein kinase inhibitor (type II, Sigma). Phosphorylation was started by addition of 0.4 mM [γ-S]ATP and stopped after various periods of time by 100-fold dilution in ice-cold buffer A followed by centrifugation. Phosphorylated and washed membranes were suspended in buffer A at about 5 mg/ml. For quantitation of PLB phosphorylation, a trace of [γ-^{35}S]ATP was included in the reaction mixture. Membrane constituents were then separated by SDS gel electrophoresis (12% polyacrylamide gels) according to Laemmli [10]. Radioactivity associated with PLB was determined by counting the corresponding gel slices in a scintillation cocktail.

2.3. *Ca^{2+}-ATPase measurements*

The Ca-dependent ATPase activity was measured at room temperature in a coupled enzyme assay [12]. The reaction medium was composed of 10 mM Mops (pH 7), 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM Tris-ATP, 0.2 mM NADH, 3 mM phospho*enol*pyruvate, 1 U/ml each of pyruvate kinase and lactate dehydrogenase, and either 0, 100 or 400 mM KCl. Various concentrations of $CaCl_2$ were added to yield the desired free Ca concentration which was calculated as in [11]. The reaction was started by adding SR membranes (50 μg/ml). The Ca ionophore A23187 was always present in the medium to ensure linear reaction rates and to avoid complications due to the modulation of the endogenous Ca-release channels by Ca.

2.4. *ANS^- fluorescence measurements*

Fluorescence of ANS^- was measured basically as in [13] using a Shimadzu spectrofluorometer (RF-540). The medium was similar to that used in the Ca^{2+}-ATPase experiments. Ca and ATP, however, were omitted. Prephosphorylated SR membranes were added at 50 μg/ml to 10 mM Mops (pH 7), 1 mM EGTA, 0.5 mM $MgCl_2$. At this membrane concentration the bulk of the ANS^- remains free in solution. The concentration of ANS^- was kept low (30 μM) so that, upon binding, its charge would not affect the pre-existing charge density in the membranes [13]. The association constants of ANS^- to cardiac SR membranes were determined by double-reciprocal plot analysis of fluorescence intensity vs ANS^- concentration (5–50 μM).

3. RESULTS AND DISCUSSION

The surface of SR membranes is electrically charged. About 1 negative charge per 25–35 lipids pre-exists on the surface of skeletal muscle SR membranes [13]. As predicted by the Gouy-Chapman theory of diffuse electric double layers, these charges induce the formation of a surface potential (ψ_0):

$$\psi_0 = f(\sigma/\sqrt{J}) \quad (1)$$

where σ denotes the density of fixed charges on the surface of the membrane and J is the ionic strength of the solution. Consequently, a redistribution of electrolytes in the unstirred layer near the membrane occurs, according to the Maxwell-Boltzmann distribution equation:

$$c_0 = c_b \exp\, z\epsilon\psi_0/kT \quad (2)$$

where c_0 and c_b are the concentrations of an ion with charge z near the membrane or in the bulk solution, respectively; ϵ is the charge of an electron, k is the Boltzmann constant and T is the absolute temperature. This phenomenon could bring about changes in the apparent (measured) K_m for Ca in the active transport reaction of the Ca pump of SR. Fig.1 shows that the addition of low (non-denaturing) concentrations of the anionic detergent dodecyl sulfate increased the affinity of the ATPase for Ca. The opposite could be observed in the presence of the cationic detergent dodecylamine which caused a right shift of the Ca-dependency curve. The most likely explanation for these results is that the partitioning of the detergents in the SR membrane changes appreciably the density and quality of the fixed charges thus affecting ψ_0. Also, the extensive phosphorylation of membrane proteins was shown to affect ψ_0 and thus, to change the apparent affinity of membrane-bound enzymes for charged substrates [14]. PLB phosphorylation stimulates the V_{max} of the Ca-transport reaction in cardiac SR and also increases the apparent affinity of the pump for Ca^{2+} [15]. The latter effect, which is very important at the physiologically relevant free Ca concentrations, could be coupled to the phosphorylation-induced changes of the surface potential. To investigate this possibility, PLB was phosphorylated in the presence of [γ-S]ATP by cAMP-dependent protein kinase, as shown in

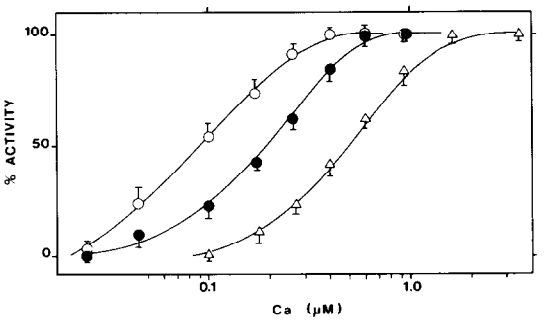

Fig.1. Effect of dodecyl sulfate and dodecylamine on the apparent K_m(Ca) of the Ca^{2+}-ATPase of cardiac SR. Experiments were carried out in the presence of 50 μg protein/ml as described in section 2. No KCl was added. The medium was supplemented with either SDS (○) or dodecylamine (△) at 50 μM. (●) No detergent added. Mean ± SDE (n = 5).

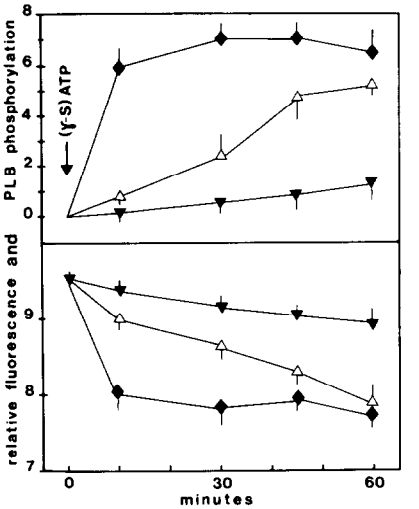

Fig.2. Correlation between PLB phosphorylation and surface membrane potential. Cardiac SR membranes were phosphorylated for various periods of time in the presence of [γ-^{35}S]ATP as described in section 2. The reaction was stopped and the radioactivity incorporated into PLB was determined after electrophoretic separation of the membrane components (upper panel). Radioactivity associated with PLB was always more than 90% of the total radioactivity incorporated. The surface membrane potential of identical samples was probed with the fluorescent dye ANS^- (lower panel), as described in section 2. (△) Control phosphorylation; (♦) in the presence of the catalytic subunit of cAMP-dependent kinase; (▼) in the presence of the protein-kinase inhibitor. Mean ± SDE (n = 3).

fig.2. The thiophosphoproteins thus formed are resistant to the action of phosphatases. In the absence of exogenous cAMP-dependent kinase, maximal levels of PLB phosphorylation were slowly reached (fig.2). This could be prevented by the addition of protein kinase inhibitor. In parallel experiments, the surface potential of the prephosphorylated cardiac SR was determined using ANS^- fluorescence. The probe is an obligatory anion and its binding to the SR membrane is directly influenced by the surface potential [16]. The results show an excellent correlation between the distribution of ANS^- in the membrane and the level of PLB phosphorylation. The association constant (K_a) of ANS^- with cardiac SR membranes was determined from double-reciprocal plots of the intensity of fluorescence vs the ANS^- concentration. Table 1 shows that phosphorylation of PLB induces a substantial increase in the K_a of ANS^-. Changes in the surface potential can be deduced from changes in the association constants of ANS^- [16]:

$$K_a'/K_a'' = \exp z(\psi_0' - \psi_0'')/kT \quad (3)$$

Applying eqn 3 to the data of table 1, it can be calculated that PLB phosphorylation changes the surface potential by about 7 mV.

To test whether the change in surface potential observed after PLB phosphorylation could be responsible for the increase in the apparent affinity of the pump for Ca, the effect of ionic strength was investigated. High KCl concentrations in the medium screen the negative charges, resulting in lower absolute values of ψ_0 (see eqn 1), and thus, of c_0. This effect explains in part the reduction of the affinity of the ATPase for Ca at high ionic strength (see fig.3). High ionic strength is also ex-

Table 1

Association constants of ANS^- to cardiac SR membranes

	K_a (μM)
Control SR	25.3 ± 0.5 (n = 5)
Phosphorylated SR	33.3 ± 1.9 (n = 7)

SR vesicles were phosphorylated for 10 min in the presence of [γ-S]ATP, in the presence of kinase inhibitor (control SR) or of the catalytic subunit of cAMP-dependent kinase (phosphorylated SR). The association constants (K_a) of ANS^- were determined as described in section 2 in a medium containing 10 mM Mops (pH 7), 1 mM EGTA, 0.5 mM $MgCl_2$, 20 μg SR protein/ml and various concentrations of ANS^-

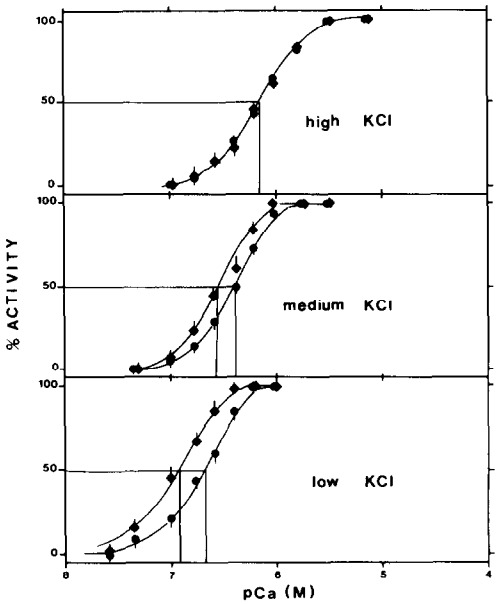

Fig.3. Effect of the ionic strength on the shift in the apparent K_m(Ca) of the Ca^{2+}-ATPase induced by PLB phosphorylation. Cardiac SR vesicles were phosphorylated for 10 min in the presence of 0.4 mM [γ-S]ATP and either protein kinase inhibitor (●) or the catalytic subunit of cAMP-dependent kinase (◆). The Ca-dependency of the Ca^{2+}-ATPase reaction was then determined as described in section 2. The medium was supplemented with either 400 mM KCl (upper panel), 100 mM KCl (medium panel) or no KCl (lower panel). Mean ± SDE (n = 4).

pected to reduce the effect of phosphorylation on the redistribution of Ca^{2+} near the membrane. Fig.3 shows that the ability of PLB phosphorylation to induce a left shift of the Ca-dependency curve of the ATPase reaction is indeed greatly reduced at high ionic strength. On the other hand, the 30–50% stimulation of V_{max} was not dependent on the ionic strength (not shown).

In conclusion, the data indicate that purely electrostatic phenomena play a crucial role in the stimulation of the Ca-pumping activity of cardiac SR by agents that affect the phosphorylation state of the regulatory protein PLB.

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