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Structural and functional comparison of a 22 kDa protein from internal human platelet membranes with cardiac phospholamban

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We have shown that a platelet 22 kDa phosphoprotein is similar to the cardiac regulator phospholamban, in that both are phosphorylated by cAMP- and calmodulin-dependent protein kinases, and that both can be phosphorylated simultaneously by these two classes of protein kinases to yield an additive stimulation of the respective Ca^{2+} pumps (Adunyah, S.E. and Dean, W.L. (1987) Biochim. Biophys. Acta 930, 401–409). However, whereas phosphorylation of phospholamban increases the affinity of the cardiac Ca^{2+}-ATPase for Ca^{2+}, phosphorylation of the platelet 22 kDa protein increased the V_{max} of the pump. In addition, antibodies raised against canine phospholamban did not crossreact with the human platelet 22 kDa protein. Finally, it was not possible to dissociate the platelet protein into lower-molecular-weight subunits by boiling in sodium dodecylsulfate which is characteristic of cardiac phospholamban. These results show that although phosphorylation of low-molecular-weight membrane-associated regulator proteins in cardiac muscle and platelets appears to stimulate the respective Ca^{2+} pumps, these proteins have different chemical and physical properties.

Introduction

Cyclic AMP moderates the responses of platelets to agonists. Virtually all morphological and metabolic responses of platelets to activators are inhibited by elevated cAMP concentrations [1,2]. While the actual mechanism of inhibition of platelet responses by cAMP is not well understood, stimulation of the DTS Ca^{2+} pump by cAMP has been reported by several groups. Kaser-Glanzmann et al. [3–4] reported that cAMP stimulated the DTS Ca^{2+} pump through phosphorylation of a 22 kDa protein. On the other hand, Le Peuch et al. [5] observed a cAMP-dependent stimulation of Ca^{2+} efflux from internal platelet membrane vesicles. However, Enouf et al. [6] and Adunyah and Dean [7] have recently concluded that cAMP indeed stimulates the DTS Ca^{2+} pump. This stimulation may account for the inhibition of platelet activation by elevated cAMP, since activation is initiated by an increase in internal Ca^{2+} [8].

In analogy to the cardiac Ca^{2+} pump, the DTS Ca^{2+} pump is under dual regulation by cAMP-de-

Abbreviations: DTS, dense tubular system (an internal membrane system in platelets); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; Tes, 2-{[hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

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pendent and calmodulin-dependent protein kinases [7]. There appears to be a correlation between the stimulation of the Ca^{2+} pump and the phosphorylation of a 22 kDa protein in platelets by these two protein kinases. A similar correlation has been reported between the phosphorylation of phospholamban in the sarcoplasmic reticulum of cardiac cells and the stimulation of Ca^{2+} transport and Ca^{2+}-dependent ATPase activity [9–13]. Cardiac phospholamban is a pentamer of 25 kDa, which characteristically dissociates upon boiling in SDS to give identical 5 kDa monomers [11–14]. Although the detailed structure of the platelet 22 kDa protein has not yet been analyzed, there are similarities between the mode of activation of the cardiac and platelet Ca^{2+} pumps by these two low-molecular-weight membrane-associated regulators.

To draw further analogies between the platelet 22 kDa protein and cardiac phospholamban, it is necessary to investigate the chemical properties of the 22 kDa protein and its mode of activation of the Ca^{2+}-ATPase in internal platelet membranes. We have therefore studied the effect of cAMP-dependent phosphorylation on the Ca^{2+} dependency of the DTS Ca^{2+} pump, and have characterized some of the physical properties of the 22 kDa activator protein including assessment of its stoichiometry with the Ca^{2+}-ATPase in DTS membranes. We report that the 22 kDa protein from internal human platelet membranes is physically different from cardiac phospholamban, although these two proteins remain strongly implicated in the regulation of the corresponding intracellular Ca^{2+} pumps by cAMP-dependent and calmodulin-dependent protein kinases.

Materials and Methods

Out-dated human platelet concentrates were supplied by the Louisville Chapter of American Red Cross. [γ-^{32}P]ATP and $^{45}Ca^{2+}$ were purchased from ICN Radiochemicals, Irvine CA. The Ca^{2+} ionophore A23187, was from Calbiochemical-Behring, San Diego, CA, and rabbit muscle catalytic subunit of cAMP-dependent protein kinase (C subunit) was from Sigma, St. Louis, MO.

The glycerol gradient purification of crude membranes and the evaluation of membrane purity was performed as described in our earlier reports [15,16]. Ca^{2+}-ATPase activity was determined by a coupled enzyme assay as previously described [15–17]. Protein concentrations were determined by the procedure of Lowry et al. [18] using bovine serum albumin as the standard.

Protein phosphorylation. Purified internal platelet membranes were phosphorylated by a slight modification of the method of Le Peuch et al. [19] as described by Adunyah and Dean [7]. The phosphorylation medium consisted of 10 mM Tes buffer (pH 7.5) containing 100 mM KCl, 10 mM NaF, 50 mM KPO_4, 5 mM EGTA, 40–120 μg membranes and 4–10 μg C subunit in 1 ml total volume. The reaction was initiated by the addition of 10 mM $MgCl_2$ and 100 μM ATP (final concentration) containing 20 μCi [γ-^{32}P]ATP. After 12 min incubation at 30 °C, the reaction was terminated by the addition of SDS sample buffer [20] to a final concentration of 1.7 to 3% SDS, 8.5% glycerol and 0.012% Bromophenol blue. In some experiments, the sample buffer contained 0.1% Triton X-100 or 2–5 M urea in addition to SDS. The samples were either boiled for various times or kept at room temperature for 30 min prior to analysis on 12% polyacrylamide gels according to Laemmli [20]. Phosphorylated bands were identified by autoradiography using Kodak X-Omat AR film. Film was kept at −70 °C for several hours during exposure. For quantitation of phosphorylation of the 22 kDa protein, the radiolabelled band corresponding to the 22 kDa protein was excised from a wet, destained gel and the gel piece was digested in 30% hydrogen peroxide at 60 °C for 12 h followed by liquid scintillation counting.

Phosphorylation of the Ca^{2+}-ATPase

The technique used for detection of Ca^{2+}-ATPase phosphoenzyme was similar to that described by Sarkardi et al. [21]. Phosphorylation of purified internal membranes (60–150 μg protein) was performed on ice in 10 mM Tes buffer (pH 7.0) containing 100 mM KCl and 50 mM KPO_4. The inclusion of 1 mM EGTA in the above buffer defined the control condition under which Ca^{2+} pumps were not phosphorylated. Other samples had either $MgCl_2$ (1 mM) alone or $MgCl_2$ plus $CaCl_2$ (60 μM) added. Phosphorylation reac-

tions were started by adding 100 μM ATP containing 20–30 μCi of [γ-^{32}P]ATP and were terminated after 60 s by the addition of stop solution consisting of ice-cold trichloroacetic acid (6%) containing 1 mM nonradioactive ATP and 10 mM inorganic phosphate. The samples were pelleted by centrifugation at 30 p.s.i. in a Beckman Airfuge and the pellets were washed twice with the stop solution. After the final wash, the pellets were resuspended in electrophoresis sample buffer containing 0.15% Tris-HCl (pH 6.8), 10 mM EDTA, 60% sucrose, 1% lithium laurylsulfate and 0.014% Bromophenol blue, and aliquots were used for SDS-polyacrylamide gel electrophoresis. Heavy sarcoplasmic reticulum fractions from rabbit skeletal muscle [155] enriched in Ca^{2+}-ATPase were phosphorylated and processed in an identical fashion.

SDS-polyacrylamide gel electrophoresis of the Ca^{2+}-ATPase phosphoenzyme. The samples were analyzed on 7 or 10% vertical discontinuous slab gels as described by Sarkardi et al. [21]. In most cases, the trichloroacetic acid-precipitated samples solubilized in sample buffer were adjusted to pH 7.0 by the addition of Tris-base to restore the blue color of the tracking dye. The samples (25–50 μl) were placed in the wells of a stacking gel (3.75% acrylamide) with a 40:1 ratio of acrylamide/bisacrylamide, 65 mM Tris-phosphate (pH 5.5), 0.1% SDS, 2% saturated potassium persulfate and 0.08% TEMED. The running gel was 7 or 10% acrylamide (40:1 ratio of acrylamide/bisacrylamide) containing 65 mM Tris-phosphate (pH 6.5), 0.1% SDS, 0.4% potassium persulfate and 0.05% TEMED. The electrophoresis reservoir buffer was 0.17% Mops (pH 6.0) and 0.1% SDS. After polymerization, the gels were kept at 4°C for 16 h prior to electrophoresis at 30–50 mA for 2.5–4 h. The radiolabelled bands were identified by autoradiography as described above. The band corresponding to the E ~ P intermediate of the ATPase was excised and digested in 30% hydrogen peroxide at 60°C for 10 h followed by scintillation analysis in 10 ml of scintillation cocktail. Low-molecular-weight protein standards (Bio-Rad) were run in adjacent gel lanes for the estimation of molecular weights.

Antibody preparations. Polyclonal antibodies against canine phospholamban were raised in rabbits as described by Jones et al. [14]. This antibody preparation has been shown to crossreact with phospholamban in a variety of tissues and species including cardiac muscle from guinea pig and rat, smooth muscle isolated from dog and pig stomach and endoplasmic reticulum from rat liver. The monoclonal antibody was obtained after immunization of Balb C mice with canine phospholamban [14] followed by fusion of spleen cells with SP-2 myeloma cells using poly(ethylene glycol). The antibody was obtained from mouse ascites fluid after pristane-treated mice were injected with the hybridomas. The antibodies were shown to be of the IgM class. Studies to determine crossreactivity of this antibody with tissues and species other than canine heart had not been undertaken prior to this work.

Results

The results in Fig. 1A show that stimulation of DTS Ca^{2+}-ATPase activity by C subunit (catalytic subunit of cAMP-dependent protein kinase) occurs over the same range of free Ca^{2+} concentrations as for stimulation of the ATPase by Ca^{2+} alone. Under our experimental conditions, maximal stimulation of the ATPase was observed at free Ca^{2+} concentrations of about 3–10 μM. The same result was observed when the membranes were stripped of endogenous calmodulin by extensive washing with a calmodulin antagonist (chlorpromazine) and EGTA as seen in Fig. 1B. A similar observation was made for the stimulation of the cardiac Ca^{2+} pump by cAMP [9,10], although the effect of the C subunit was predominantly on the affinity of the Ca^{2+}-ATPase for Ca^{2+}. In contrast to the effect of the C subunit on the cardiac sarcoplasmic reticulum ATPase, phosphorylation increased the V_{max} and did not alter the apparent affinity for Ca^{2+} in platelet membranes (Fig. 1). At higher Ca^{2+} levels, the platelet ATPase was inhibited, as we reported earlier [16].

In some experiments, membranes were washed with the Ca^{2+} ionophore A23187 in addition to chlorpromazine and EGTA prior to analysis of the effect of the C subunit on the Ca^{2+}-ATPase as in Fig. 1. In this case, no stimulation by the C subunit was observed (data not shown). Furthermore, the data in Fig. 2 show that there was no

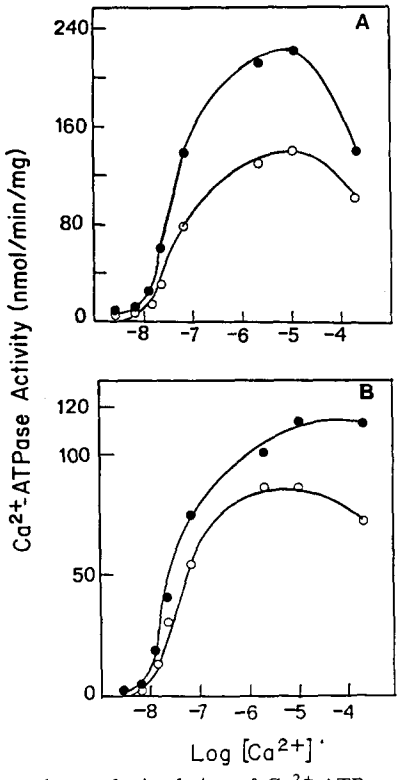

Fig. 1. Dependence of stimulation of Ca^{2+}-ATPase by the C subunit on external Ca^{2+} concentration. Purified internal platelet membranes (20 μg washed or 26.6 μg unwashed) were prephosphorylated in a coupled enzyme assay mixture [15] in the absence or presence of the C subunit (3 μg/ml) for 2 min. Different amounts of Ca^{2+} were added to start the ATPase reaction at 30 °C and pH 7.5. The coupled enzyme assay system was not affected by the C subunit. Mg-ATP was present at a concentration of 5 mM and 500 μM EGTA was included in the assay mixture. Free Ca^{2+} levels were calculated using the appropriate affinity constants [26]. Data points are averages of two determinations. (A) Unwashed membranes; (B) membranes prewashed in 50 μM chlorpromazine and 1 mM EGTA. Control (○) plus C subunit (●).

additional stimulation by the C subunit when A23187 was added to membranes that were not washed. The behavior of the ATPase in the presence of A23187 was similar to that observed with the C subunit in the absence of A23187 (Fig. 1). Thus, A23187 mimics the effect of C subunit-dependent phosphorylation, although the mechanism must be different, since A23187 completely eliminates Ca^{2+} uptake, whereas the C subunit stimulates uptake.

Jones et al. [11,12,14] and Gasser et al. [13] demonstrated that cardiac phospholamban is a 25 kDa oligomer which dissociates into 5 kDa monomers upon boiling in 3% (w/v) SDS. To investigate whether the 22 kDa platelet internal membrane protein shares similar or identical properties with cardiac phospholamban, phosphorylated membranes were subjected to several harsh treatments in an attempt to induce subunit dissociation prior to electrophoretic analysis. The autoradiograph in Fig. 3A shows C subunit-dependent phosphorylation of the 22 kDa protein (indicated by the arrow). The results in Fig. 3B show that boiling in 3% SDS alone, SDS plus 0.1% Triton X-100, or SDS plus 5 M urea did not alter the electrophoretic mobility of the phosphorylated 22 kDa protein. When this band was extracted from gels into sample buffer which contained either 3% SDS alone or SDS plus 0.1% Triton X-100 or 2 M urea, and the samples were re-electrophoresed on 12% gels, the protein did not dissociate into lower-molecular-weight species (data not shown). This observation shows a significant difference in physical properties between 22 kDa platelet phosphoprotein and cardiac phospholamban.

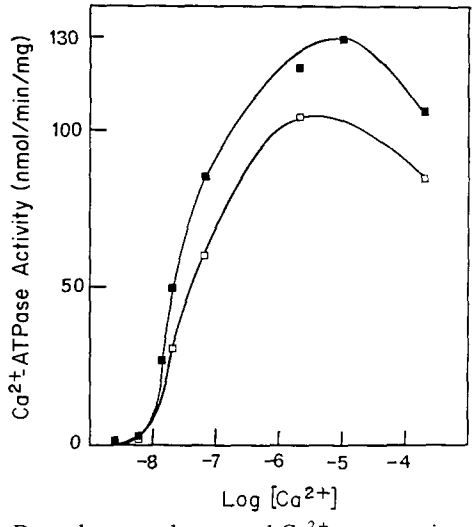

Fig. 2. Dependence on the external Ca^{2+} concentration of the stimulation of Ca^{2+}-ATPase by A23187. Purified membranes (61.5 μg) were assayed for Ca^{2+}-ATPase activity in the absence (□) or presence (■) of A23187 (5 μM). The assay conditions were identical to those described in Fig. 1, except that the C subunit was omitted. Data points represent averages of two determinations.

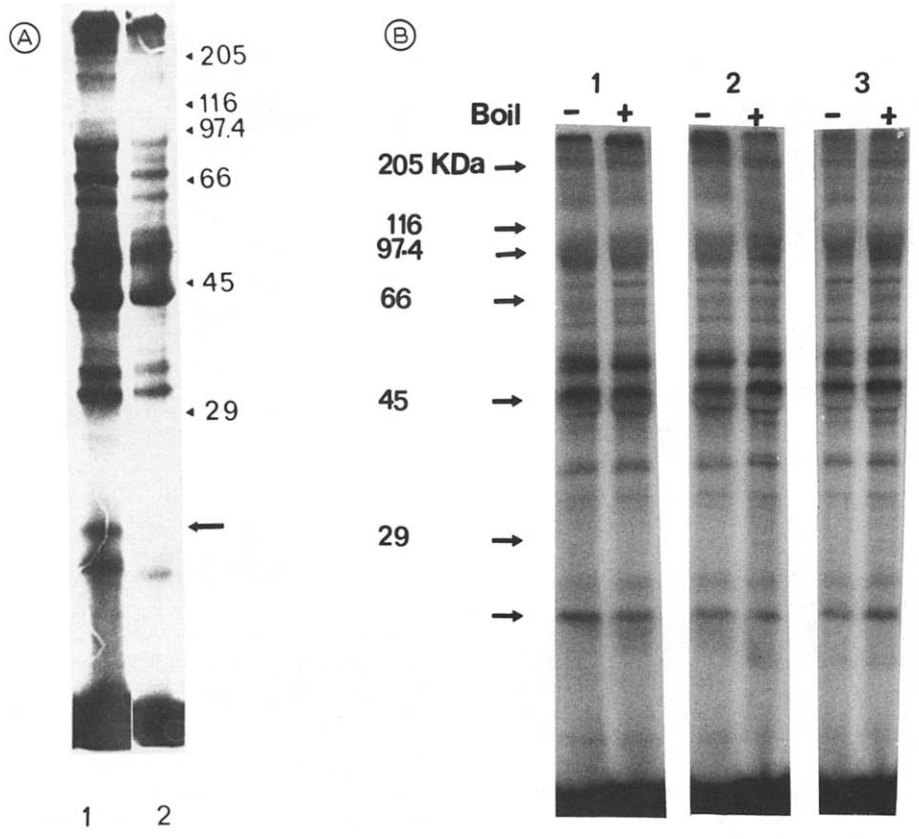

Fig. 3. Attempted subunit dissociation of platelet 22 kDa phosphoprotein after phosphorylation by the C subunit. (A) Purified platelet membranes (50 μg) were phosphorylated by the C subunit as described in the Materials and Methods. Samples were kept at room temperature in 3% SDS sample buffer for 30 min prior to analysis on 12% polyacrylamide gels followed by autoradiography. Lanes: 1, plus C subunit (3 μg/ml); 2, plus 5 mM EGTA (control). (B) Purified membranes (60 μg) which had been phosphorylated by the C subunit, as in A, were subjected to various treatments to induce subunit dissociation prior to electrophoretic and autoradiographic analysis as described in Materials and Methods. Samples were either kept at room temperature for 30 min or boiled for 4 min. Lanes: 1, 3% SDS; 2, 3% SDS plus 0.1% Triton X-100; 3, 3% SDS plus 5 M urea. The 22 kDa protein is indicated by the unnumbered arrow.

To obtain further information about the identity of the 22 kDa protein, cardiac phospholamban and purified internal platelet membranes were immunoblotted with monoclonal and polyclonal antibodies against cardiac phospholamban. The results in Fig. 4 indicate no crossreactivity between the platelet membranes and phospholamban antibodies. Boiling caused dissociation of cardiac phospholamban but did not prevent binding of the polyclonal antibodies. These results show that the platelet 22 kDa protein and canine cardiac phospholamban are immunochemically distinct.

The autoradiograph in Fig. 5 shows the phosphoenzyme intermediate of the Ca^{2+}-ATPase. The radiolabelled band indicated by an arrow had the same mobility as the 97.4 kDa protein standard and occurred at a position slightly below the skeletal muscle SR Ca^{2+}-ATPase which was used as a marker (data not shown). The data in Table I indicate that under optimal experimental conditions, the ratios of phosphate to protein for the phosphoprotein intermediate of the Ca^{2+}-ATPase and the 22 kDa protein phosphorylated by C subunit were 13 and 12 pmol phosphate/mg protein, respectively. This implies a 1 : 1 stoichiometry

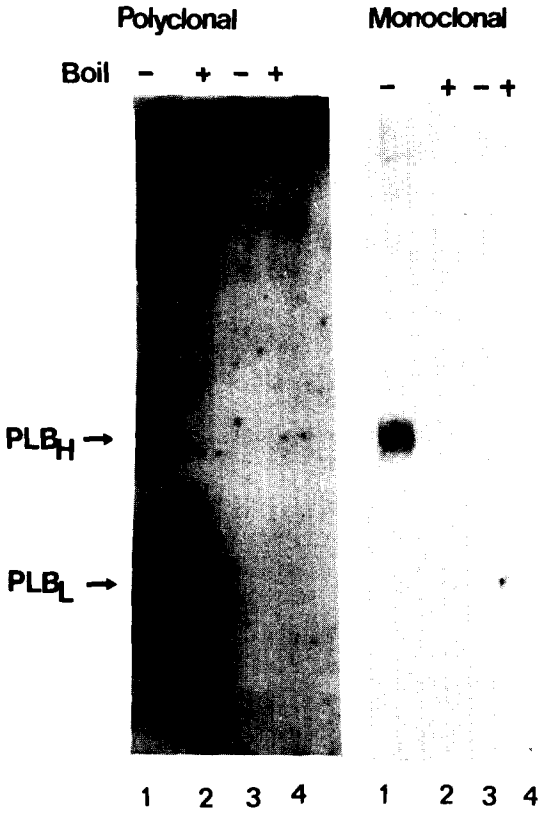

Fig. 4. Immunoblots of cardiac and internal platelet membranes with antibodies against canine phospholamban. Canine cardiac sarcoplasmic reticulum membranes (40 μg) or internal human platelet membranes (40 μg) were electrophoresed on 8% gels according to Porzio and Pearson [27]. They were transferred to nitrocellulose paper and reacted with canine phospholamban polyclonal or monoclonal antibodies (1 : 500 dilution) and visualized with ^{125}I-labelled protein A as described by Towbin et al. [28]. In the indicated lanes, the samples were boiled for 3 min in SDS sample buffer. Immunolabelled bands (indicated by arrows) were identified by exposing the gels to X-ray film on ice overnight. Lanes 1 and 2 contained cardiac membranes and lanes 3 and 4 contained platelet membranes. PLB$_H$ and PLB$_L$ indicate the high- (pentameric) and low- (monomeric) molecular-weight forms of phospholamban.

between Ca^{2+}-ATPase and 22 kDa polypeptide assuming one phosphate per polypeptide. It is not possible to estimate the actual number of phosphates per 22 kDa polypeptide, since we cannot determine the percent of the total membrane protein that is 22 kDa due to the very low concentration of this protein in platelet membranes. The ratio of ATPase phosphoenzyme to 22 kDa phosphoprotein has been determined for the cardiac

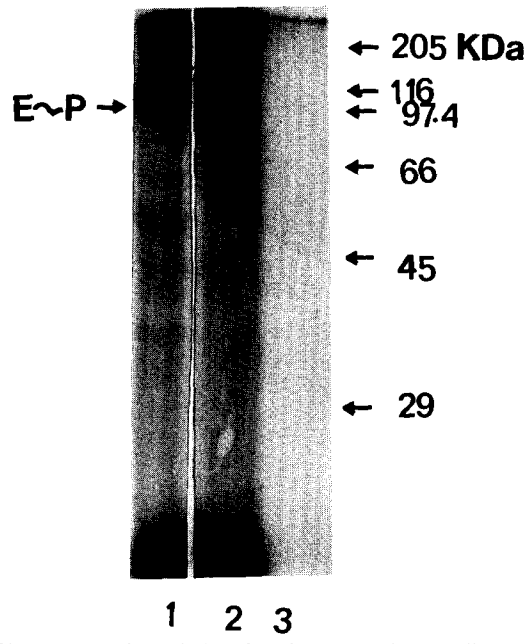

Fig. 5. Detection of the phosphoenzyme intermediate of the DTS Ca^{2+}-ATPase. Phosphorylation of the Ca^{2+}-ATPase was performed as described in the Materials and Methods. After analysis on 10% polyacrylamide gels, the destained gel was dried under vacuum at room temperature for 16 h and radiolabelled bands were identified by exposure to X-ray film for 6 days. Lanes: 1, 60 μM $CaCl_2$ and 1 mM $MgCl_2$; 2, as lane 1 plus 1 mM $MgCl_2$; 3, as lane 2 plus 1 mM EGTA (control). The arrow indicates the position of the phosphoenzyme intermediate of the Ca^{2+}-ATPase.

system and the reported value for cardiac sarcoplasmic reticulum is 1.0 [23], the same number as we observed in platelet membranes. Since cardiac

TABLE I

COMPARISON OF THE PHOSPHORYLATION LEVEL OF THE Ca^{2+}-ATPase WITH THE 22 kDa PROTEIN FROM PLATELET INTERNAL MEMBRANES

The 22 kDa protein was phosphorylated in the presence of 10 μg/ml C subunit for 12 min and the Ca^{2+}-ATPase was phosphorylated for 60 s under optimal conditions as described in the Materials and Methods. Values are averages of two determinations.

Experiment	Phosphate/protein ratio (pmol/mg protein)	Phosphoprotein molar ratio
Ca^{2+}-ATPase (E ~ P)	13	1.08
22 kDa protein	12	

phospholamban is a pentamer, the 1 : 1 molar ratio of phosphoproteins implies a 5 : 1 molar ratio of ATPase to phospholamban pentamer in cardiac sarcoplasmic reticulum, since each 5 kDa monomer contains one phosphate after cAMP-dependent phosphorylation. This stoichiometry is difficult to reconcile with the proposal that the phospholamban pentamer modifies ATPase activity by direct interaction. However, this problem does not arise with platelet membranes, since the platelet 22 kDa protein does not appear to dissociate.

Discussion

In this report, we have demonstrated that the DTS Ca^{2+} pump is stimulated by cAMP-dependent protein kinase in a manner dependent on the free Ca^{2+} concentration, like the cardiac Ca^{2+} pump. Our data suggests that the effect of cAMP on Ca^{2+}-ATPase is manifested mainly by an increase in the V_{max} of the enzymatic activity, rather than by modification of the affinity of the enzyme for Ca^{2+}. This is in contrast to observations made for the stimulation of the cardiac Ca^{2+}-ATPase by C subunit phosphorylation. In that system, the primary effect appears to be an increase in enzyme affinity for Ca^{2+}, with a negligible effect on the V_{max} of the enzyme, when measured either as Ca^{2+} transport or ATP hydrolysis at optimal Ca^{2+} concentration. Both these modes of regulation are different from the direct regulation of the plasma membrane Ca^{2+}-ATPase by calmodulin, which affects both the V_{max} and the K_m of the enzyme for Ca^{2+} [24].

We also showed that the 22 kDa protein from platelet internal membranes does not crossreact with antibodies against canine cardiac phospholamban. The polyclonal antibody was previously shown to crossreact with phospholamban in several different tissues and species (see Materials and Methods) [14]. Thus, failure to detect crossreaction in human platelets probably indicates immunochemical nonidentity and not failure of the antibody to recognize phospholamban in other species and tissues. The ability of the monoclonal antibody to recognize phospholamban from sources other than canine cardiac sarcoplasmic reticulum and internal membranes from platelets has not yet been determined.

The electrophoretic mobility of the phosphorylated 22 kDa protein was not altered by various treatments which are known to cause dissociation of cardiac phospholamban [11–14]. This provides additional evidence that the platelet 22 kDa phosphoprotein and cardiac phospholamban are physically distinct. It might be argued that the platelet 22 kDa protein is the light chain of myosin, which is a phosphorylatable protein in platelets with a similar molecular weight (20 kDa). This is highly unlikely, since myosin light chain phosphorylation is inhibited by cAMP in both smooth muscle [29] and platelets [30] in vitro and in vivo, whereas phosphorylation of the platelet protein was stimulated by cAMP [7].

We have shown that some aspects of regulation of cardiac Ca^{2+} pump are identical to regulation of the DTS Ca^{2+} pump in that the Ca^{2+} pump systems (1) are stimulated by calmodulin-dependent kinase alone, (2) are stimulated by cAMP-dependent kinase alone, (3) are stimulated additively by simultaneous phosphorylation by calmodulin- and cAMP-dependent protein kinases, and (4) exhibit coupling between stimulation of Ca^{2+} transport and phosphorylation of low-molecular-weight membrane-associated regulators. However, the primary effect of membrane phosphorylation in the platelet system is an increase in the V_{max} of the Ca^{2+}-ATPase at saturating concentrations of Ca^{2+} (Fig. 1), whereas the primary effect of membrane phosphorylation in the cardiac system is an increase in the apparent enzyme affinity for Ca^{2+} [10].

Of possible mechanistic importance, is our observation that the Ca^{2+} ionophore A23187, mimics the effect of C subunit phosphorylation in that it stimulates the Ca^{2+}-ATPase activity in a Ca^{2+}-dependent manner. The significance of this observation and its relevance to the mechanism of regulation of the Ca^{2+} pump by cAMP-dependent protein kinases is not known at present. It seems unlikely that phosphorylation of the 22 kDa protein could open a Ca^{2+} channel, since Ca^{2+} uptake is increased by phosphorylation of this protein [3,6,7].

The physical and immunochemical properties of the putative protein regulators of the Ca^{2+} pumps in the two membrane systems are different, suggesting that cardiac phospholamban and the

platelet 22 kDa protein are different proteins. Cardiac phospholamban has been purified, sequenced and well characterized [14,25]. A thorough study of the biochemical properties of the 22 kDa platelet membrane protein has not yet been performed, and this may be difficult, due to the low content of this protein in platelet membranes.

In conclusion, based on immunochemical and physical characteristics, cardiac phospholamban appears to differ significantly from the 22 kDa protein of internal platelet membranes which has been implicated in the regulation of the platelet DTS Ca^{2+} pump. The two proteins appear to be functionally related but structurally and immunochemically different. How two proteins with different chemical and physical properties perform similar functions in Ca^{2+} regulation remains to be tested. The possibility that these two proteins share homologous domains which modulate Ca^{2+} pump activity in response to phosphorylation is an attractive hypothesis for future testing.

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