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Kinetic Differences in the Phospholamban-Regulated Calcium Pump When Studied in Crude and Purified Cardiac Sarcoplasmic Reticulum Vesicles

A. Antipenko[1], **A.I. Spielman**[2], **M.A. Kirchberger**[1]

[1]Department of Physiology and Biophysics, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029-6574, USA

[2]Basic Science Division, New York University College of Dentistry, New York, NY 10010-4086, USA

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Abstract. Phospholamban (PLN) phosphorylation contributes largely to the inotropic and lusitropic effects of *beta*-adrenergic agonists on the heart. The mechanical effects of PLN phosphorylation on the heart are generally attributed solely to an increase in the apparent affinity of the Ca pump in the sarcoplasmic reticulum (SR) membranes for Ca^{2+} with little or no effect on $V_{max(Ca)}$. In the present report, we compare the kinetic properties of the cardiac SR Ca pump in commonly studied crude microsomes with those of our recently developed preparation of light SR vesicles. We demonstrate that in crude microsomes, the increase in the apparent affinity of the pump for Ca^{2+} is larger, while the increase in $V_{max(Ca)}$ is smaller, than in purified vesicles. The greater phosphorylation-induced increase in apparent Ca^{2+} affinity in crude microsomes may be further enhanced by an ATP-sensitive inhibitory effect of ruthenium red on the activity of the pump at subsaturating, but not saturating, Ca^{2+} concentrations as a result of a greater inhibition in unphosphorylated microsomes. Upon increasing the ATP concentration from 1 to 5 mM, an inhibition by 10 μM ruthenium red is eliminated in phosphorylated microsomes and reduced in control microsomes. Addition of the phosphoprotein phosphatase inhibitor okadaic acid produces a considerable increase in the phosphorylation-induced effects in both crude and purified microsomes. We conclude that the use of purified cardiac SR vesicles is critical for the demonstration of a major increase in $V_{max(Ca)}$ in addition to an increase in the pump's apparent affinity for Ca^{2+} in response to phosphorylation of PLN by protein kinase A.

Key words: Light sarcoplasmic reticulum — Heart — Phospholamban—Beta-adrenergic — Ruthenium red — Ca^{2+}-ATPase

Correspondence to: M.A. Kirchberger

Introduction

An abundant literature exists describing the properties and reaction mechanism of the calcium (Ca) pump of sarcoplasmic reticulum (SR) membranes isolated from rabbit fast skeletal muscle [16]. The study of the fast skeletal muscle SR Ca pump has been greatly facilitated by the availability of SR membrane preparations containing a high density of Ca pump sites yet free from the ryanodine- or ruthenium red (RR)-sensitive Ca^{2+} release channel present in the terminal cisternae. Ca^{2+} release channel-free microsomes, comprising the longitudinal component of the SR, are designated light SR vesicles while microsomes containing these channels are called heavy SR vesicles, based on their distribution on a sucrose density gradient after equilibrium centrifugation [19]. Analogous preparations of cardiac SR membranes have until recently been unavailable. Many in vitro studies on the myocardial SR Ca pump are carried out with preparations of whole homogenates of cardiac tissue [14] or a crude microsomal fraction obtained by differential centrifugation [4, 13, 18]. These SR-containing preparations are contaminated to varying extents with other subcellular constituents as well as with SR Ca^{2+} release channels. Chamberlain, Levitsky & Fleischer [5] further fractionated crude cardiac microsomes on a sucrose density gradient, however the resultant purified microsomes still exhibited low stoichiometric ratios of moles of Ca transported per mole of ATP hydrolyzed as a result of the presence of Ca^{2+} release channels. We have recently developed a simple preparation of purified cardiac SR vesicles that possess high Ca-uptake activity and are free

of the RR-sensitive Ca^{2+} release channels [2], thus representing light cardiac SR vesicles.

In the present report, we compare, side by side, the kinetic properties of the Ca pump measured in light cardiac SR vesicles and in crude microsomes under different experimental conditions in order to elucidate the basis for the conflicting conclusions by different laboratories (*see below*) regarding the kinetic mechanism of action of phospholamban (PLN), the pump's natural protein regulator [14]. Protein kinase A (PKA)-catalyzed phosphorylation of PLN in response to *beta*-adrenergic stimulation of the heart relieves an inhibition of the Ca^{2+}-ATPase protein exerted by unphosphorylated PLN. A significant finding in the history of PLN has been the demonstration of the release of PLN's inhibition of the Ca pump as a result of the binding of an anti-PLN monoclonal antibody [20]. Anti-PLN monoclonal antibody and PKA were originally reported to increase the apparent affinity of the pump for Ca^{2+} without affecting the $V_{max(Ca)}$ of microsomal Ca-uptake or Ca^{2+}-ATPase activity. Numerous other investigators, employing various preparations of unpurified cardiac SR membranes [e.g., 4, 22, 26, 27] as well as in vitro expression and reconstitution systems [23–25], reached similar conclusions. A few reports have appeared, however, providing evidence for a generally small [13] or poorly reproducible larger increase in $V_{max(Ca)}$ following partial digestion of crude microsomes with trypsin under mild conditions (*cf.* refs. 13 and 15). Evidence from a study with liposomes containing purified Ca pump protein and synthetic PLN also supported an effect of PLN on $V_{max(Ca)}$ of the SR Ca pump [9]. More recently using light cardiac SR vesicles, we have demonstrated a significant, highly reproducible increase in $V_{max(Ca)}$ as well as an increase in the apparent affinity of the pump for Ca^{2+} upon removal of PLN's inhibitory influence [1, 2].

We now provide data to document the effects of purification of crude cardiac microsomes on the kinetic properties of the SR Ca pump. We demonstrate, moreover, an inhibitory effect of RR on the cardiac Ca pump that may result in an overestimation of the increase in its apparent affinity for Ca^{2+} as a result of PLN phosphorylation.

Materials and Methods

The catalytic subunit of PKA, A23187, and ouabain were obtained from Sigma. All other reagents were obtained as described previously [1, 2].

Microsome Preparation

Crude microsomes were prepared from canine left ventricle and fractionated on a sucrose density gradient [2]. Briefly, crude microsomes were loaded on a gradient with discontinuous steps consisting of 25, 30, 33, and 40% sucrose in Buffer G (18 mM PIPES-KOH, pH 6.8, and 120 mM KCl) and centrifuged for 20 hr at 2°C in a Beckman SW28 rotor. Microsomes present at each interface between the steps in the gradient were collected and designated F1, F2, and F3, starting at the top of the tube. The F1 fraction, with a refractive index corresponding to 28.0 to 29.2% sucrose, is the source of the purified microsomes, also called light SR vesicles. Microsomes were quick-frozen in small aliquots and stored in liquid nitrogen. Since we have been unable to detect any consistent changes in the microsomes stored for variable lengths of time for a period of up to 3 years and subjected to up to 6 freezing/thawing cycles, unless otherwise specified, the data represent means of microsome preparations utilized under these conditions.

Ca-Uptake Measurement

Prior to the measurement of Ca-uptake, the microsomes were treated either with PKA catalytic subunit or boiled enzyme under conditions favorable for phosphorylation [1] or with trypsin or trypsin inhibitor-inactivated trypsin [2], as described previously. Ca-uptake was assayed under our standard assay conditions [2]. Briefly, microsomes (2.5 to 10 μg/ml) were incubated at pH 6.8 and 25 or 37°C in a reaction mixture containing: 40 mM histidine-HCl, pH 6.8, 120 mM KCl, 5 mM NaN_3, 4 mM phospho(enol)pyruvate, 0.2 mg/ml pyruvate kinase, 2 mM $MgCl_2$, 2.5 mM oxalate-Tris, 1 μM okadaic acid, 1 mM ATP, and the Ca^{2+} concentrations indicated in the text. The specified Ca^{2+} concentrations were maintained with a $CaCl_2$-EGTA buffer system and contained ^{45}Ca (1). When reactions were run at 37°C, the oxalate concentration was 5 mM and the pH of the reaction mixture was adjusted accordingly. After incubation of the microsomes in the temperature-equilibrated reaction mixture lacking Ca^{2+}, the Ca^{2+} was added to start the Ca-uptake reaction. In some experiments as indicated in the text, RR was present in the reaction mixture prior to the sequential additions of microsomes and Ca^{2+}. Samples were filtered 2 or 3 different times for up to 4 min to insure linearity of the reaction with time and protein concentration and were counted by liquid scintillation. Ca-uptake rates, measured as a function of Ca^{2+} concentration, were fit to the Hill equation, $V = V_{max}/[1 + (K_{m(Ca)}/[Ca^{2+}]^N]$, by a nonlinear least-squares procedure.

Ca^{2+}-ATPase Assay

Ca^{2+}-ATPase activity was assayed at 25 or 37°C by following the rate of decrease in NADH absorbency at 340 nm in an enzyme-linked ATPase assay [1]. The standard reaction mixture contained 40 mM histidine-HCl, pH 6.8, 120 mM KCl, 5 mM NaN_3, 2 mM $MgCl_2$, 1 mM ATP, 1 mM phosphoenolpyruvate, 10 U/ml pyruvate kinase, 28 U/ml lactic dehydrogenase, 0.2 mM NADH, either 9 μM Ca^{2+} or 2 mM EGTA, 0.3 μg/ml A23187, 2.4 μg/ml microsomes, and the additions indicated in the text. In some experiments, microsomes were pretreated with trypsin or trypsin inhibitor-inactivated trypsin [2] or 160 U/ml catalytic subunit of protein kinase A or boiled enzyme [1] (control), in which case 1 μM okadaic acid was included in the reaction mixture. Rates of ATPase activity were obtained using the kinetic program in a Shimadzu UV160U recording spectrophotometer. The reaction mixture lacking the microsomes was temperature equilibrated in the cuvette for 5 min, after which time the microsomes were added. After an additional min, Ca^{2+}, in the form of a $CaCl_2$-EGTA buffer system, or 2 mM EGTA was added and the time program begun. The ATPase reaction was followed for 5 min. During this time, ATP hydrolysis was linear with respect to time and microsomal protein concentration.

Table 1. Effect of ruthenium red on Ca-uptake activity assayed in crude cardiac microsomes and sucrose gradient-derived fractions

Microsomes	Ca-uptake (μmol/mg · min)			
	Ruthenium red (5 μM)		*N*	% Increase
	–	+		
Crude	0.20 ± 0.03*	0.25 ± 0.04*	7	25
F1 fraction	0.50 ± 0.07	0.50 ± 0.06	4	0
F2 fraction	0.30 ± 0.03†	0.35 ± 0.03†	5	17
F3 fraction	0.10 ± 0.01‡	0.14 ± 0.02‡	4	40

Fractions F1 to F3 were obtained upon centrifugation of the crude microsomes on a sucrose density gradient as described in Materials and Methods. Ca-uptake was determined under standard assay conditions at 25°C and 9 μM Ca^{2+}. The values are the means ± SEM. Differences in values identified by the same symbol are significant at $P < 0.05$ when tested by Student's *t* test for paired variates. *N*, number of microsome preparations.

Steady-State E_2P Formation and E_2P Decomposition

The microsomes were phosphorylated by PKA catalytic subunit or incubated under control conditions prior to measurement of steady-state E_2P (enzyme phosphate intermediate) formation from $^{32}P_i$ and E_2P decomposition, as described previously [2]. E_2P decomposition was followed in a Biologic QFM-5 rapid mixing apparatus [2].

Results

Cardiac microsomes that are conventionally prepared by differential centrifugation of tissue homogenates are sensitive to RR, an inhibitor of the SR Ca^{2+} release channel [6]. Thus when crude cardiac microsomes are assayed at 9 μM Ca^{2+} in the presence of 5 μM RR, a significant increase in Ca-uptake is observed as a result of the greater retention of transported Ca^{2+} (Table 1). Centrifugation of the suspension of crude microsomes on a simple 3-step sucrose gradient and collection of the microsomes found at the interfaces of the steps in the gradient (F1–F3, *see* Materials and Methods) results in 3 fractions with widely different sensitivity to RR in assays of Ca-uptake. The F3 fraction shows the largest increase in Ca-uptake in the presence of RR and therefore is highest in heavy SR content. The F1 fraction exhibits the highest Ca-uptake activity and is insensitive to RR, hence free of detectable Ca^{2+} release channels; these findings indicate the presence of light SR vesicles. The F2 fraction is intermediate with respect to these criteria.

To compare the kinetics of the Ca pump present in crude microsomes and light SR vesicles, crude microsomes were assayed for Ca-uptake and Ca^{2+}-ATPase activities after treatment with trypsin or PKA under conditions identical to those used previously for purified SR membranes [2]. Mild trypsin treatment of cardiac microsomes cleaves the cytoplasmic domain of PLN in the absence of deleterious effects on the Ca^{2+}-ATPase protein and thereby relieves the inhibitory effect of PLN on the pump [13]. In both trypsin-treated and phosphorylated microsomes and their respective controls, Ca-uptake in the light SR is more than 2-fold greater than that in crude microsomes when tested over a wide Ca^{2+} concentration range (Fig. 1). It is apparent, also, that in purified vesicles the increase in Ca-uptake at high Ca^{2+} concentrations attributable to trypsin treatment or PKA-catalyzed microsomal phosphorylation is greater than in crude microsomes, whereas the leftward shift in the Ca^{2+} concentration dependence curves is less pronounced (solid lines in Fig. 1). The significance of the broken line in the right panel of Fig. 1, which was derived as described in the figure legend, is considered in the Discussion. The foregoing differences in the Ca^{2+} dependence of Ca-uptake between crude and purified SR vesicles are statistically significant ($P < 0.05$), as shown in Table 2, which presents the means ± SEM of the optimized kinetic parameters resulting from separate fits of the data obtained in 3 independent experiments to the Hill equation (*see* Materials and Methods). The percent increase in $V_{max(Ca)}$ of Ca-uptake with trypsin treatment or phosphorylation in the purified microsomes is more than twice that observed with the crude microsomes and the decrease in $K_{m(Ca)}$ is considerably less in the latter microsomes as a result of these treatments. Consistent with previous reports [1, 2, 13], the Hill coefficient remains unchanged with trypsin treatment or phosphorylation of either crude or purified microsomes.

Ca^{2+}-ATPase activity was assayed in crude and purified vesicles as an additional criterion of the activity of the Ca pump protein. Basal ATPase levels measured at 25°C in the presence of 10 mM EGTA were 8 and 18% of the total activity measured in purified and crude vesicles, respectively, in the presence of 9 μM Ca^{2+} when 2.5 mM oxalate was present instead of the ionophore A23187 (*data not shown*). As no significant difference in Ca^{2+}-independent ATPase activity was detected when

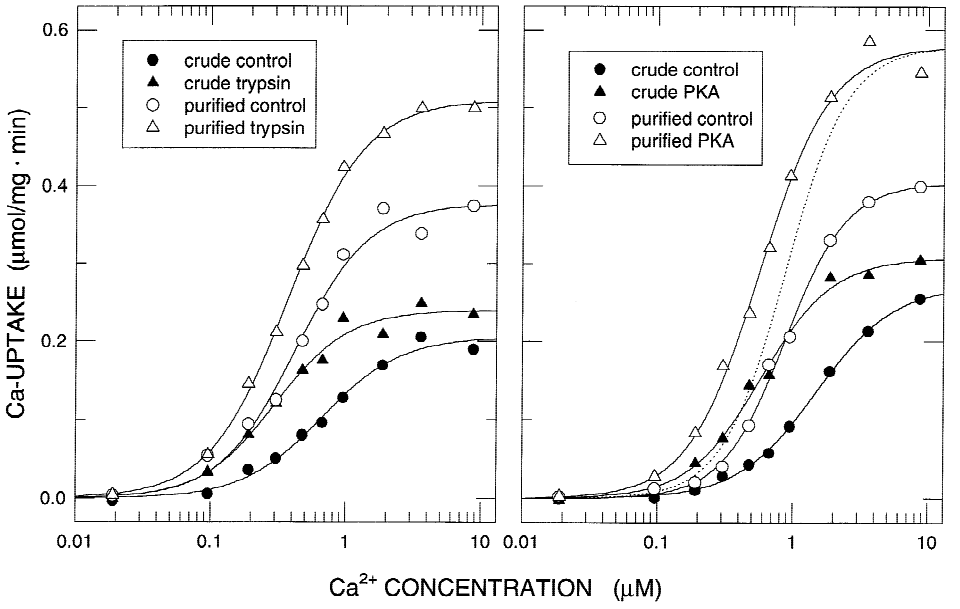

Fig. 1. Comparison of the Ca^{2+} concentration dependence of Ca-uptake by crude and purified cardiac microsomes. Ca-uptake was measured at the indicated Ca^{2+} concentrations under standard assay conditions in trypsin-treated (*left panel*) and PKA-phosphorylated (*right panel*) microsomes and their respective controls. Microsomes treated in this manner are represented by *triangles* and their respective controls by *circles. Closed symbols* represent crude microsomes, *open symbols* light SR vesicles. Prior to initiating the Ca-uptake reaction by the addition of Ca^{2+}, the microsomes were treated with trypsin or PKA catalytic subunit under conditions favorable for phosphorylation or under control conditions (1). Symbols represent Ca-uptake at each Ca^{2+} concentration in a representative microsome preparation. *Solid lines* represent fits of each of the four data sets (2 paired experiments) in each panel to the Hill equation. The optimized kinetic parameters, $V_{max(Ca)}$ (μmol/mg · min), $K_{m(Ca)}$ (μM), and Hill coefficient, are: *left panel* (●) 0.21, 0.66, 1.4; (▲) 0.24, 0.27, 1.5; (○) 0.38, 0.42, 1.6; (△) 0.51, 0.37, 1.5, respectively; *right panel* (●) 0.27, 1.49, 1.5; (▲) 0.31, 0.58, 1.6; (○) 0.40, 0.88, 1.9; (△) 0.58, 0.57, 1.7. The *broken line* in the right panel represents the result of a fit to the Hill equation using the $K_{m(Ca)}$ and Hill coefficient obtained for control microsomes and the $V_{max(Ca)}$ obtained for phosphorylated microsomes in order to illustrate the hypothetical effect of a PKA-induced increase in $V_{max(Ca)}$ alone. Microsomes were purified from either the crude microsomes used to obtain the data shown (*left panel*) or crude microsomes not represented by the data shown (*right panel*). Microsomes had been subjected to one prior freezing/thawing cycle. Data for light SR vesicles, shown for the purpose of comparison with crude microsomes, have previously been included in the means of multiple experiments reported in references 1 and 2 with permission from the American Chemical Society and The American Society for Biochemistry and Molecular Biology, respectively.

the EGTA concentration was varied from 2 to 10 mM, subsequent assays were carried out at 2 mM EGTA. The $(Na^+ + K^+)$-ATPase inhibitor ouabain decreased the apparent Ca^{2+}-ATPase activity in crude microsomes by about 15% but had no effect in purified microsomes, which suggests the absence of a significant plasma membrane contaminant in the latter preparation.

As in the Ca-uptake assay (Table 2), when Ca^{2+}-ATPase activity was assayed both at 25 and 37°C at saturating Ca^{2+} in PKA-phosphorylated and control microsomes (Table 3), a significantly smaller increase with phosphorylation was again seen in the crude microsomes compared to the percent increase obtained in purified microsomes. This indicates that while the apparent absence of Ca^{2+} release channels in the purified microsomes may contribute to a greater increase in $V_{max(Ca)}$ of Ca-uptake with phosphorylation compared to crude microsomes, it is not the only contributing factor. The omission of okadaic acid from the Ca^{2+}-ATPase assay mixture almost halved the increase attributable to PKA in its presence in purified vesicles (Table 3). This finding is consistent with a reduction in the PKA-induced stimulation of Ca-uptake in crude microsomes from 20% in the presence of okadaic acid to 11% in its absence when assays were carried out at 9 μM Ca^{2+} and 25°C: 0.20 ± 0.03 and 0.24 ± 0.03 μmol/mg · min in control and phosphorylated microsomes, respectively, in the presence of 1 μM okadaic acid *vs.* 0.19 ± 0.02 and 0.21 ± 0.02 μmol/mg · min in the presence of 1% dimethylsufoxide, the vehicle, alone. Hence the omission of okadaic acid from the incubation medium significantly decreases the stimulation of Ca-uptake and Ca^{2+}-ATPase activity by PKA, which indicates the presence of active phosphatases in the microsomal preparations.

Since SR Ca-uptake measurements are frequently made in the presence of RR in various cellular or subcellular preparations (e.g., refs. 14, 17), we tested the effect of different concentrations of RR on Ca-uptake by purified microsomes at both saturating (9 μM) and subsaturating (0.3 μM) Ca^{2+} concentrations. At 9 μM Ca^{2+}, 10 μM RR and other concentrations between 1 and 40 μM had no significant effect when tested under standard

Table 2. Comparison of the kinetic parameters for calcium uptake by trypsin-treated, phosphorylated, and control crude and purified cardiac microsomes

Treatment	$V_{max(Ca)}$		$K_{m(Ca)}$		Hill coefficient	
	μmol/mg · min	% change	μM	% change		% change
			CRUDE MICROSOMES			
Control	0.21 ± 0.00*		0.74 ± 0.09*		1.5 ± 0.0	
Trypsin	0.24 ± 0.00*	+14	0.36 ± 0.04*	−51	1.6 ± 0.0	+7
Control	0.23 ± 0.03¶		1.21 ± 0.17¶		1.5 ± 0.1	
Phosphorylation	0.26 ± 0.03¶	+13	0.53 ± 0.06¶	−56	1.5 ± 0.1	—
			PURIFIED MICROSOMES			
Control	0.43 ± 0.06†		0.42 ± 0.02†		1.7 ± 0.1	
Trypsin	0.58 ± 0.09†	+35	0.38 ± 0.01†	−10	1.7 ± 0.1	—
Control	0.51 ± 0.05‡		0.59 ± 0.14‡		1.7 ± 0.1	
Phosphorylation	0.68 ± 0.06‡	+33	0.46 ± 0.06‡	−22	1.6 ± 0.1	−6

Values are the means ± SEM (3 independent experiments) of the optimized parameters obtained in separate unweighted fits to the Hill equation of the four data sets. Differences between values identified by the same symbol in each column are significant at $P < 0.05$ when tested by Student's *t* test for paired variates. The microsomes were treated with trypsin or trypsin inhibitor-inactivated trypsin (control) or with either the catalytic subunit of PKA under conditions favorable for phosphorylation or with boiled enzyme (controls). Previously published data for purified microsomes, seen shaded in this and some subsequent tables, are shown for the purpose of comparison with the data for crude microsomes. Data for purified trypsin-treated and control microsomes were reproduced from Table I in reference 2 with the permission of The American Society for Biochemistry and Molecular Biology; data for purified phosphorylated and control microsomes were taken from Table 1 in reference 1 with the permission of The American Chemical Society.

Table 3. Comparison of the stimulatory effect of protein kinase A on Ca^{2+}-ATPase activity assayed at saturating Ca^{2+} in crude and purified cardiac microsomes: effects of temperature and okadaic acid

Microsomes	°C	Okadaic acid	Ca^{2+}-ATPase (μmol/mg · min)		% Increase
			Control	PKA	
Crude	25	+	0.18 ± 0.03	0.24 ± 0.03	33
Purified	25	+	0.48 ± 0.03	0.73 ± 0.07	52
Crude	37	+	0.51 ± 0.03	0.67 ± 0.05	31
Purified	37	+	1.72 ± 0.18	2.49 ± 0.30	45
Purified	37	−	1.61 ± 0.04	2.00 ± 0.05	24

Ca^{2+}-ATPase activity was assayed at 9 μM Ca^{2+} in the presence or absence of 1 μM okadaic acid at the specified temperature (°C) under the standard assay conditions given in Materials and Methods. Prior to initiation of the ATPase reaction, the microsomes were treated with 160 U/ml PKA catalytic subunit or boiled enzyme (control). Values are the means ± SEM of 3 experiments with different microsome preparations. All differences between PKA-treated and control microsomes and between purified microsomes assayed in the presence and absence of okadaic acid are significant at $P < 0.05$ when tested by Student's *t* test for paired and unpaired variates. Data in the shaded rows were taken from Table 2 in reference 1 with the permission of the American Chemical Society.

assay conditions in PKA-phosphorylated and trypsin-treated microsomes and their respective controls (Fig. 2). At 0.3 μM Ca^{2+}, however, RR produced a marked concentration-dependent inhibition that was reduced when the microsomes were either phosphorylated or trypsin treated. Similar results were obtained when testing the effect of RR on Ca^{2+}-ATPase activity (Table 4). Thus at saturating Ca^{2+}, no effect of RR on Ca^{2+}-ATPase activity was observed in trypsin-treated or control microsomes. However at 0.3 μM Ca^{2+}, Ca^{2+}-ATPase activity was only 58% of the rate observed in the absence of RR, whereas in trypsin-treated microsomes the inhibition was less with 77% of the activity remaining in the presence of RR. When Ca-uptake was assayed at 5 mM ATP instead of 1 mM, the inhibition seen previously at 10 μM RR in control microsomes was completely abolished in both the phosphorylated and trypsin-treated microsomes and was much reduced in the respective control microsomes (Fig. 3). These findings demonstrate a direct, PLN-sensitive action of RR on the Ca pump protein at subsaturating Ca^{2+}.

Finally, in order to estimate the relative density of

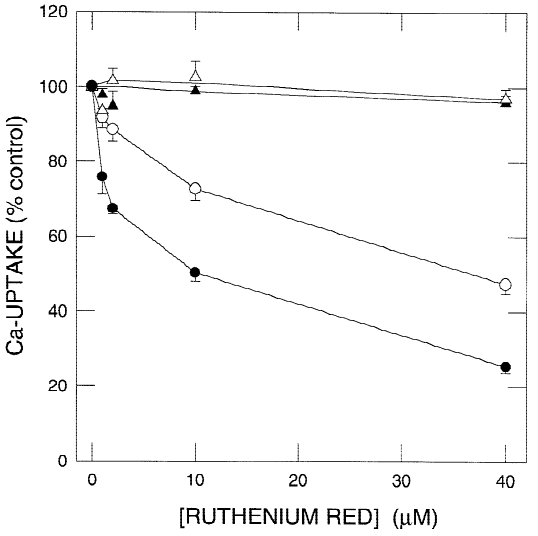

Fig. 2. RR sensitivity of Ca-uptake by light cardiac SR vesicles assayed at saturating and subsaturating Ca^{2+}. Ca-uptake was assayed under standard conditions at 37°C in trypsin-treated (△, ○) and control (▲, ●) microsomes at the indicated concentrations of RR. The Ca^{2+} concentration was either 9 μM (*triangles*) or 0.3 μM (*circles*). Values are expressed as percent control where 100% control represents Ca-uptake in the absence of RR. The symbols are the means ± SEM of the values obtained in 3 experiments with different microsome preparations.

Ca pump sites in the membranes, we compared crude microsomes and light SR vesicles with respect to steady-state formation of E_2P from P_i. Mean steady-state E_2P formation was 33% higher in control light SR vesicles than in control crude microsomes (Table 5). Trypsin treatment of crude microsomes decreased the steady-state E_2P formation by the same percentage as was reported for light SR vesicles (Table 5) as a result of an increase in E_2P decomposition rate [2]. PKA-treated crude microsomes exhibited a virtually identical E_2P decomposition rate compared to the light SR vesicles, also studied previously [2] (Fig. 4). Thus, as trypsin and PKA produce similar effects on the kinetics of the SR Ca pump, the trypsin-induced decrease in steady-state E_2P formation is likely to be attributable to an increase in the rate of E_2P decomposition.

Discussion

Light SR vesicles exhibit a more than twofold greater increase in $V_{max(Ca)}$ of Ca-uptake than crude microsomes when both membrane preparations are treated with either PKA catalytic subunit in the presence of okadaic acid or trypsin in order to remove the inhibitory influence of unphosphorylated PLN (Table 2). The same treatments produce a considerably smaller decrease in $K_{m(Ca)}$ in purified microsomes than in crude vesicles. A simple explanation for the inverse relationship in the magnitude of the changes in $K_{m(Ca)}$ and $V_{max(Ca)}$ of Ca-uptake with phosphorylation or trypsin treatment of crude and purified microsomes is the known activation of Ca^{2+} release channels at Ca^{2+} concentrations greater than approximately 1 μM [29]. Release of transported Ca^{2+} at extravesicular Ca^{2+} concentrations >1 μM would decrease the apparent Ca-uptake rate and diminish the apparent effects of treatment with PKA, trypsin, or anti-PLN monoclonal antibody on $V_{max(Ca)}$ because the rate of Ca^{2+} leakage through the release channel may increase as a result of the greater downhill driving force attributable to the higher rate of Ca^{2+} transport into the vesicle. The latter assumption requires that transported Ca has greater accessibility to the Ca release channel than to intravesicular oxalate, which would fix the intravesicular Ca concentration at a constant value depending on the product of the activities of Ca^{2+} and oxalate. However as an increase in $V_{max(Ca)}$ is equivalent to an increase in the number of functional Ca pump units, such an increase would already be evident as increased Ca pump activity at the lower end of the Ca^{2+} concentration range (Fig. 1, broken line in right panel). Thus at Ca^{2+} concentrations <1 μM, the effect of PLN phosphorylation or related treatments on both $V_{max(Ca)}$ and $K_{m(Ca)}$ would be fully apparent.

Contamination of membranes by Ca^{2+} release channels is not a confounding factor when Ca^{2+}-ATPase activity is assayed in the presence of the ionophore A23187. Therefore it is not surprising to find that, following phosphorylation or trypsin treatment, the increase in Ca^{2+}-ATPase activity at saturating Ca^{2+} in crude microsomes is considerably larger than the increase in Ca-uptake (Tables 2 and 3). However, the increase in Ca^{2+}-ATPase activity with phosphorylation is still smaller in crude compared to purified microsomes despite elimination of the problem associated with Ca^{2+} release channels. In fact, even in the purified microsomes the increase with PKA was greater in the Ca^{2+}-ATPase assay than in the Ca-uptake measurement. The major difference in the assay conditions used in these two measurements is the presence of A23187 in the Ca^{2+}-ATPase assay *vs.* oxalate in the Ca-uptake assay. The maintenance of low intravesicular Ca^{2+} concentration (i.e., the same as is present extravesicularly) by inclusion of an ionophore appears permissive for high rates of Ca pump activity in the phosphorylated microsomes. A limitation of oxalate entry into the vesicle at high rates of Ca-uptake could be one possible explanation for this finding. The present data show, moreover, that the absence of okadaic acid, a phosphoprotein phosphatase inhibitor, from the assay medium results in a significantly smaller increase in Ca pump activity in response to PKA treatment in both crude and purified vesicles (Table 3).

Although one cannot eliminate the possibility of an

Table 4. Effect of ruthenium red on Ca^{2+}-ATPase activity in trypsin-treated and control light cardiac SR vesicles

Treatment	$[Ca^{2+}]$	10 μM Ruthenium red		
		–	+	+/–
	μM	μmol/mg · min		
Control	0.3	0.19 ± 0.01	0.11 ± 0.01*	0.58
Trypsin	0.3	0.31 ± 0.03	0.24 ± 0.03*	0.77
Control	9	1.41 ± 0.07	1.42 ± 0.08	1.01
Trypsin	9	1.89 ± 0.14	1.88 ± 0.14	1.00

Ca^{2+}-ATPase activity was assayed at 37°C at the specified Ca^{2+} concentrations under the standard assay conditions given in Materials and Methods. Prior to measurement of the ATPase activity, the microsomes were treated with either trypsin or trypsin inhibitor-inactivated trypsin (control). Values are the means ± SEM of 3 experiments with different microsome preparations. Values marked with an asterisk differ significantly from the control values when tested by Student's *t* test for paired variates at $P < 0.05$. The column headed +/– represents the fraction of activity remaining in the presence of RR (+) in the ATPase reaction mixture compared to that in its absence (−).

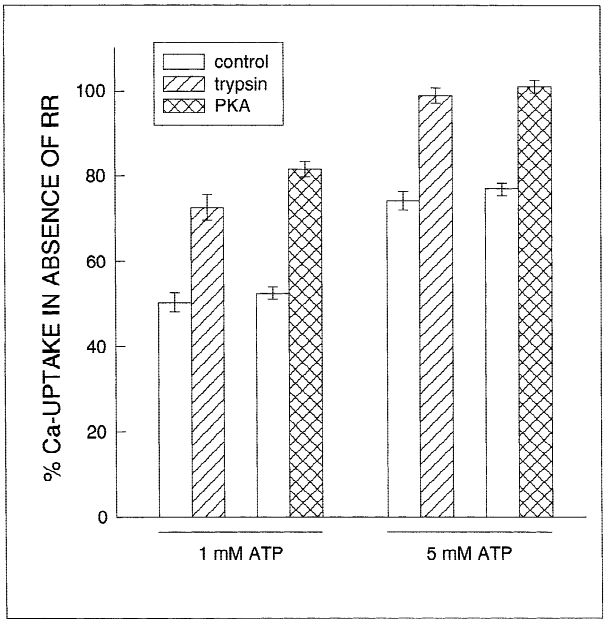

Fig. 3. Effect of ATP concentration on the inhibition of Ca-uptake by 10 μM RR in trypsin-treated or PKA phosphorylated microsomes and their respective controls. 100% Ca-uptake represents Ca-uptake assayed in the absence of RR. Ca-uptake was assayed under standard conditions in light SR subjected to trypsin treatment or PKA-catalyzed phosphorylation, as described under Materials and Methods. The data represented by the left-hand pair of bars were taken from Fig. 2 and are included to facilitate comparison with other data shown in the figure. Bars represent means ± SEM of 3 experiments with different microsome preparations. All differences between treated samples and their respective controls were significant at the $P < 0.05$ level when tested by Student's *t* test for paired variates.

enrichment, upon microsomal purification, of an unknown activator of Ca^{2+}-ATPase activity at saturating Ca^{2+} that is dependent on some change in either PLN or the Ca^{2+}-ATPase protein when the inhibitory influence of PLN is removed, there is no experimental evidence to support such an explanation. A more likely contributory factor in the greater increase in $V_{max(Ca)}$ of Ca^{2+}-ATPase activity (as estimated by activity at saturating Ca^{2+}) in purified microsomes is enrichment of light SR (Tables 1 and 5) and removal of contaminants present in crude microsomes that specifically decrease $V_{max(Ca)^{2+}}$ without increasing the apparent affinity of the pump for Ca^{2+}. An example of such selective inhibition is the decrease in $V_{max(Ca)}$ of Ca-uptake produced by incubation of trypsin-treated microsomes with certain amphiphiles, namely $C_{12}E_8$ and other nonionic detergents [15]. However, unlike such amphiphiles which increase E_2P decomposition rate [7], any putative inhibitory contaminant does not interfere with the phosphorylation-induced increase in E_2P decomposition rate, which was found to be identical in crude and purified microsomes (Fig. 4).

An additional complication in kinetic studies of the SR Ca pump protein may be introduced by the use of RR to inhibit Ca^{2+} release channels in crude SR membrane preparations or whole homogenates of cardiac tissue. Whereas fast skeletal muscle Ca^{2+}-ATPase is known not to be inhibited by up to at least 20 μM RR [21], the present results demonstrate an inhibition of the cardiac SR Ca pump by μM concentrations of RR at subsaturating Ca^{2+} that is reduced when the ATP concentration is increased from 1 to 5 mM (Table 4, Figs. 2 and 3). These results are consistent with a report that appeared while the present manuscript was under review [11]. Our results, moreover, show that the degree of inhibition differs in PKA or trypsin-treated microsomes. A greater inhibition of both Ca-uptake and Ca^{2+}-ATPase activity when unphosphorylated or nontrypsin treated microsomes are assayed at subsaturating Ca^{2+} will increase the change in apparent affinity of the pump for Ca^{2+} produced by phosphorylation or trypsin treatment. Moreover, this effect together with the relief of the inhibition by RR at satu-

Table 5. Steady-state E_2P formation from $^{32}P_i$ in crude cardiac microsomes and light SR vesicles

Treatment	Crude microsomes		Light SR vesicles	
	nmol E_2P/mg	% Decrease	nmol E_2P/mg	% Decrease
Control	1.01 ± 0.19		1.34 ± 0.07	
Trypsin	0.74 ± 0.11	27	0.98 ± 0.02	27

Microsomes were treated with trypsin inhibitor-inactivated trypsin (control) or trypsin, centrifuged, and resuspended in K^+-free buffer (2). Steady-state E_2P formation from $^{32}P_i$ was measured at 25°C in crude microsomes and previously at room temperature (23°C) in light SR vesicles. Values are the means ± SEM of 3 determinations each with different crude and light SR preparations. Differences between control and trypsin-treated microsomes are significant at $P < 0.05$ when tested by Student's *t* test for paired or unpaired variates. Data for purified microsomes were taken from Table IV in reference 2 with the permission of The American Society for Biochemistry and Molecular Biologists.

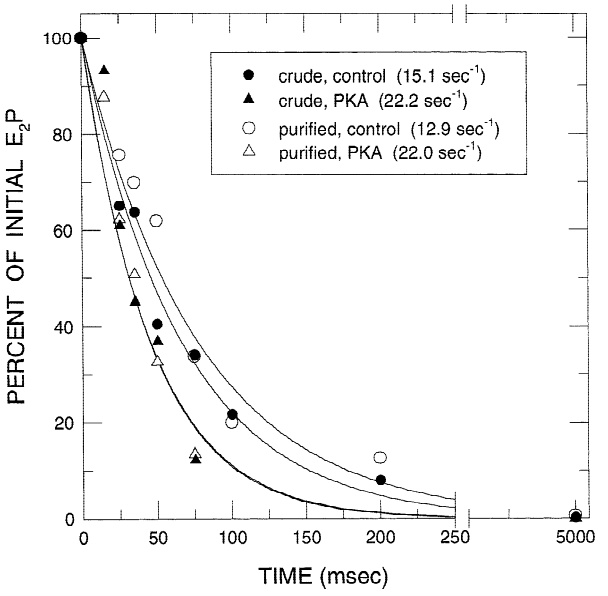

Fig. 4. Comparison of E_2P decomposition in crude microsomes and light SR vesicles under control conditions and after PKA treatment. Membranes were incubated in the presence (○, △) and absence (●, ▲) of PKA catalytic subunit as described previously (1). The crude microsomes (*circles*) and light SR vesicles (*triangles*) were then incubated in the presence of $^{32}P_i$ to form E_2P and mixed at 15°C in a QFM-5 rapid mixing system with 16 volumes of a chase solution. At the indicated times the reaction was quenched with an equal volume of 10.3% trichloroacetic acid. Each data point represents an average of two determinations. The lines represent single exponential fits with the rate constants shown in the *inset* to the figure. The results of similar measurements made previously with light SR vesicles are shown for the purpose of comparison and are taken from Fig. 4 in reference 2 with the permission of The American Society for Biochemistry and Molecular Biology. Initial E_2P concentrations correspond to 1.11 (●), 0.97 (○), 1.52 (▲), and 1.50 (△) nmol E_2P/mg microsomal protein.

rating Ca^{2+}, will produce an overestimated apparent cooperativity with respect to Ca^{2+} when measuring Ca pump activity. This Ca^{2+}-sensitive inhibitory effect of RR may be the result of a preferential affinity of RR for the pump protein in a conformational state that is stabilized by unphosphorylated or uncleaved PLN. The inhibition of the Ca^{2+}-ATPase by RR is different from that caused by certain polycationic compounds studied previously [28], which, unlike RR, decrease $V_{max(Ca)}$. Interestingly, inhibition of the cardiac SR Ca pump by thapsigargin (which is not polycationic in nature) is also reduced by PLN phosphorylation [12]. Obviously the phosphorylation status of PLN plays an important role in determining the accessibility of inhibitory or stimulatory molecules to the Ca^{2+}-ATPase protein.

Recently, evidence for an essentially equivalent effect of treatment with PKA or anti-PLN monoclonal antibody on both $V_{max(Ca)}$ and $K_{m(Ca)}$ of the Ca pump was obtained independently by another laboratory [10]. In this case, Ca-uptake by the sucrose gradient purified vesicles described by Chamberlain et al. [5] was monitored by use of the fluorescent Ca^{2+} indicator Fura 2 in the presence of 10 mM oxalate. This demonstrates that although this preparation of microsomes may still contain ryanodine-sensitive Ca^{2+} release channels, it yields essentially the same results that we have obtained with light SR vesicles using a filtration method [1]. Interestingly, Chiesi and Schwaller [8], using a microsome preparation similar to that of Chamberlain et al. [5], reported a 30 to 50% stimulation of $V_{max(Ca)}$ of Ca^{2+}-ATPase activity measured in the presence of A23187. These findings are in accord, moreover, with measurements of the declining phase of intracellular Ca^{2+} transients with the fluorescent indicator indo-1 in rat heart cells under conditions where the SR is the major means of removal of Ca^{2+} from the cytoplasm [3]. In these experiments, an increase in $V_{max(Ca)}$ was the major effect of isoproterenol on the kinetics of SR Ca-uptake; the $K_{m(Ca)}$ was, in fact, slightly increased. Taken together, these studies (1–3, 8, 9) and various previously cited reports constitute strong evidence for a significant regulation by PLN of both $V_{max(Ca)}$ and apparent $K_{m(Ca)}$ of the cardiac SR Ca pump.

In conclusion, the present comparative study of the properties of the Ca pump in crude and purified SR vesicles documents the contribution of several factors to

the modulation of the Ca pump kinetics and helps in understanding the origin of the discrepancies in the conclusions reached by various laboratories using different microsome preparations. These factors are the use of crude *vs.* purified microsome preparations, the absence of a phosphoprotein phosphatase inhibitor in studies involving PLN phosphorylation, and the presence of RR in the assay medium. Additional factors related to the use of in vitro reconstitution and expression systems were discussed in a previous publication [1]. The information presently reported will facilitate a reexamination of the extensive literature on crude cardiac microsomes and tissue homogenates and provide a basis for the interpretation of kinetic data obtained with in vitro reconstitution and expression systems.

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