Functional Co-expression of the Canine Cardiac Ca\(^{2+}\) Pump and Phospholamban in *Spodoptera frugiperda* (Sf21) Cells Reveals New Insights on ATPase Regulation*

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The utility of the baculovirus cell expression system for investigating Ca\(^{2+}\)-ATPase and phospholamban regulatory interactions was examined. cDNA encoding the canine cardiac sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump (SERCA2a) was cloned for the first time and expressed in the presence and absence of phospholamban in *Spodoptera frugiperda* (Sf21) insect cells. The recombinant Ca\(^{2+}\) pump was produced in high yield, contributing 20% of the total membrane protein in Sf21 microsomes. At least 70% of the expressed pumps were active, Co-expression of wild-type, pentameric phospholamban with the Ca\(^{2+}\)-ATPase decreased the apparent affinity of the ATPase for Ca\(^{2+}\), but had no effect on the maximum velocity of the enzyme, similar to phospholamban’s action in cardiac sarcoplasmic reticulum vesicles. To investigate the importance of the oligomeric structure of phospholamban in ATPase regulation, SERCA2a was co-expressed with a monomeric mutant of phospholamban, in which leucine residue 37 was changed to alanine. Surprisingly, monomeric phospholamban suppressed SERCA2a Ca\(^{2+}\) affinity more strongly than did wild-type phospholamban, demonstrating that the pentamer is not essential for Ca\(^{2+}\) pump inhibition and that the monomer is the more active species. To test if phospholamban functions as a Ca\(^{2+}\) channel, Sf21 microsomes expressing either SERCA2a or SERCA2a plus phospholamban were actively loaded with Ca\(^{2+}\) and then assayed for unidirectional \(^{45}\)Ca\(^{2+}\) efflux. No evidence for a Ca\(^{2+}\) channel activity of phospholamban was obtained. We conclude that the phospholamban monomer is an important regulatory component inhibiting SERCA2a in cardiac sarcoplasmic reticulum membranes, and that the channel activity of phospholamban previously observed in planar bilayers is not involved in the mechanism of ATPase regulation.

Phospholamban is a pentameric transmembrane phosphoprotein regulator of the Ca\(^{2+}\)-transport ATPase of cardiac sarcoplasmic reticulum (1, 2). In the dephosphorylated state, phospholamban inhibits the Ca\(^{2+}\) pump by decreasing the apparent affinity of the ATPase for Ca\(^{2+}\) (3, 4). Inhibition of the Ca\(^{2+}\) pump is relieved by phosphorylation of phospholamban at serine 16 or threonine 17 or by the binding of a phospholamban monoclonal antibody to this cytoplasmic phosphorylation domain, resulting in a substantial increase in Ca\(^{2+}\) transport into cardiac sarcoplasmic reticulum vesicles at low ionized Ca\(^{2+}\) concentration (5–7). Purified phospholamban also forms Ca\(^{2+}\) channels in lipid bilayers (8), but the functional role of this channel activity is ill defined (9). The physiological importance of phospholamban is demonstrated by recent work with cardiomyocytes (7) and phospholamban knockout mice (10), where it was shown that ablation of phospholamban regulatory function greatly augments the intracellular Ca\(^{2+}\) transient and myocardial contractility, and at the same time attenuates the cardiac response to β-adrenergic agents such as isoproterenol.

To understand the molecular mechanism of phospholamban regulation, several mammalian cell expression systems have recently been developed in which phospholamban and the Ca\(^{2+}\)-ATPase are co-expressed after transient transfection of cells with plasmid expression vectors (11–13). These studies have provided useful insights into the mechanism of phospholamban inhibition, including identification of some of the amino acid residues of phospholamban required for Ca\(^{2+}\) pump regulation (14). However, the cell expression systems used to date have several drawbacks, including low transfection efficiencies, low expression levels, and low membrane yields, making detailed biochemical and kinetic characterizations with use of these systems difficult (11–13).

In the work described here, we have examined the utility of the baculovirus cell expression system for investigating phospholamban and Ca\(^{2+}\)-ATPase regulatory interactions. An important strength of this system is that virtually all of the insect cells are infected using viral expression vectors, ensuring that very high levels of foreign protein expression are achieved (15). We recently reported on the use of this system for the expression and mass purification of canine cardiac phospholamban and several of its protein mutants from Sf21 cells (16, 17). The purified, recombinant protein was successfully reconstituted into proteoliposomes to study its secondary structure (18) and oligomeric organization in the lipid bilayer (19). Successful co-reconstitution with Ca\(^{2+}\) pumps purified from rabbit skeletal muscle (16) and canine myocardium (20) was also achieved. Here, we report on the further development of this system for

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U94345.

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1 The abbreviations used are: Sf21 cells, *Spodoptera frugiperda* insect cells; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid; SERCA, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase; SERCA2a, cardiac SERCA isoform; SERCA1a, adult fast twitch skeletal muscle isoform; SERCA3, ubiquitous SERCA isoform; L37A-PLB, canine phospholamban with leucine residue 37 in the transmembrane domain replaced by alanine; *K*\(_{\text{m}}^{\text{p}}\) value, ionized Ca\(^{2+}\) concentration giving half-maximal activation of the Ca\(^{2+}\) pump; WTV, wild-type virus.
functional co-expression of phospholamban with the canine cardiac Ca\textsuperscript{2+} pump (SERCA2a). Microsomes isolated from infected S21 cells exhibit high levels of ATP hydrolysis and active Ca\textsuperscript{2+} transport, and, furthermore, cardiac-like coupling between phospholamban and SERCA2a is retained. With the baculovirus system, we also demonstrate that a monomer-forming mutant of phospholamban (17, 19), unexpectedly, is a stronger inhibitor of SERCA2a activity than is the pentamer, suggesting that the monomer may be the key molecular species regulating the Ca\textsuperscript{2+} pump in sarcoplasmic reticulum membranes. No evidence for a Ca\textsuperscript{2+} channel activity of phospholamban was obtained.

EXPERIMENTAL PROCEDURES

Materials—(7-3\textsuperscript{P})ATP, \textsuperscript{45}CaCl\textsubscript{2}, and \textsuperscript{32}P-labeled protein A were purchased from DuPont NEN. Nucleic acid-synthesizing and -modifying enzymes were obtained from Promega. S21 cells were purchased from Invitrogen, and the BaculoGold\textsuperscript{TM} system was obtained from Pharmingen.

Cloning Canine SERCA2a cDNA—A canine cardiac c-kit10 cDNA library (21) was screened in duplicate with 5\textsuperscript{th} end-labeled oligonucleotide probes corresponding to base pairs 1–33 and 2935–2970 of rabbit SERCA2a cDNA (22). SERCA2a cDNA encoding the full-length canine cDNA was excised from 

\[ \text{XmaI} \]

and subcloned into the 

\[ \text{EcoRI} \]

polylinker site of pBluescript. The SERCA2a cDNA clone contained approximately 250 base pairs of 5\textsuperscript{th}-untranslated sequence, a 2991-base pair open reading frame, and an approximately 850 base pairs of 3\textsuperscript{rd}-untranslated sequence, a 2991-base pair open reading frame, and an approximately 850 base pairs of 3\textsuperscript{rd}-untranslated sequence. The entire 3\textsuperscript{rd}-untranslated region of the canine SERCA2a cDNA was sequenced in both directions by the dideoxy method (21).

Expression of SERCA2a and Phospholamban in S21 Cells—Wild-type phospholamban and L37A-PLB were expressed in S21 cells as recently described (16, 17). To express canine SERCA2a in S21 cells, the XmaI insert encoding the Ca\textsuperscript{2+} pump was excised from pBluescript and inserted into the 

\[ \text{XmaI} \]

site of the baculovirus transfer vector pVL1393 (15). This XmaI insert contained the entire protein coding region of the Ca\textsuperscript{2+} pump, 90 base pairs of 5\textsuperscript{th}-untranslated sequence, and the entire 3\textsuperscript{rd}-untranslated region including 11 base pairs excised from the EbcmR1 plasmid. Recombinant baculovirus containing the canine SERCA2a cDNA was obtained after co-transfection of S21 cells with pVL1393 and linearized baculovirus DNA using the BaculoGold\textsuperscript{TM} system (17). The Ca\textsuperscript{2+} pump and phospholamban were expressed in S21 insect cells grown in suspension (1.5 \times 10\textsuperscript{8} cells/ml) at 27 °C in Grace’s insect cell medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 0.1% Pluronic F-68 (Life Technologies, Inc.), 50 \mu g/ml gentamicin, and 2.5 \mu g/ml amphotericin B. Microsomes were isolated from insect cells harvested 48 h after infection with baculoviruses. For expression of the Ca\textsuperscript{2+} pump alone, a multiplicity of infection of 10 (viruses per cell) was used. For co-expression of the Ca\textsuperscript{2+} pump and phospholamban, a multiplicity of infection of 15 was used for SERCA2a, and of 5 for wild-type phospholamban and L37A-PLB.

Isolation of Microsomes from S21 Cells—Virus-infected S21 cells in 600 ml of suspension (9 \times 10\textsuperscript{8} cells) were sedimented, washed twice with phosphate-buffered saline, and resuspended in 50 ml of 

\[ 10 \text{mM} \text{NaHCO}_3, 0.2 \text{mM} \text{CaCl}_2, \text{plus the following protease inhibitors: aprotinin (10 \mu g/ml), leupeptin (2 \mu g/ml), pepstatin A (1 \mu g/ml), and Pefabloc (0.1 mm).} \]

which were included throughout the entire preparative procedure. Cells were disrupted by 

\[ \text{N} \_2 \text{cavitation method. } \]

Microsomes were isolated from infected cell cultures harvested at 48 h after infection with baculoviruses. For expression of the Ca\textsuperscript{2+} pump alone, a multiplicity of infection of 10 (viruses per cell) was used. For co-expression of the Ca\textsuperscript{2+} pump and phospholamban, a multiplicity of infection of 15 was used for SERCA2a, and of 5 for wild-type phospholamban and L37A-PLB.

\[ \text{Miscellaneous Methods—Procedure I canine cardiac microsomes enriched in sarcoplasmic reticulum were isolated as described previously (27). Purification of recombinant canine phospholamban from S21 cells was conducted as described elsewhere (16, 17). The Ca\textsuperscript{2+}-ATPase was purified from canine cardiac sarcoplasmic reticulum vesicles by 2A7-A1 monoclonal antibody affinity chromatography, using the methodology described by Reddy et al. (16). Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard.} \]

RESULTS

Cloning of Canine Cardiac SERCA2a cDNA—Most of the detailed biochemical work characterizing phospholamban and Ca\textsuperscript{2+}-ATPase regulatory interactions has been conducted with sarcoplasmic reticulum preparations isolated from dog heart (1, 3). These well-characterized preparations are enriched in the two proteins and are prepared in relatively high yield (27), which has greatly facilitated the biochemical work. Ironically, however, the canine cardiac Ca\textsuperscript{2+} pump has not been cloned or expressed to date. Therefore, to express canine SERCA2a in S21 cells, we first had to obtain the cDNA clone for this isoform. The SERCA2a cDNA clone was isolated and found to contain a 2991 base pair open reading frame encoding a polypeptide of 997 amino acid residues with a calculated molecular weight of 109,619. The deduced amino acid sequence...
of dog SERCA2a displays 98–99% identity with mammalian SERCA2a Ca\textsuperscript{2+}-ATPases cloned from human (29), rabbit (22), cat (30), rat (31), or pig (32) species, and 95% identity with avian SERCA2a Ca\textsuperscript{2+}-ATPase cloned from chicken (33) (Fig. 1).

**Expression and Functional Assay of SERCA2a in Sf21 Cells**—Microsomes were isolated from Sf21 insect cells infected with the SERCA2a-encoding baculovirus. Separation of the microsomal membrane proteins by SDS-PAGE followed by Coomassie Blue staining revealed the abundant expression of an 110-kDa protein, which migrated with identical mobility as the Ca\textsuperscript{2+} pump in canine cardiac sarcoplasmic reticulum vesicles (Fig. 2). The expressed protein was not visible in microsomes obtained from control (i.e. wild-type virus-infected) Sf21 cells. The identity of the expressed protein as the Ca\textsuperscript{2+} pump was

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**Fig. 1. Deduced amino acid sequence of canine cardiac Ca\textsuperscript{2+} pump.** The canine SERCA2a amino acid sequence deduced from the cDNA sequence is reported in single-letter amino acid code. SERCA2a sequences from human (29), rabbit (22), cat (30), rat (31), pig (32), and chicken (33) species are shown for comparison. Identical residues are indicated by hyphens and the asterisk denotes the stop codon. Residue numbers are in the right margin. The DNA sequence has been submitted to GenBank™ (accession no. U94345).
confirmed by immunoblotting with a monoclonal antibody recognizing SERCA2a (28) (Fig. 2B), demonstrating that the recombinant Ca\textsuperscript{2+} pump is expressed in Sf21 cell microsomes at levels approaching those of the native Ca\textsuperscript{2+}-ATPase in cardiac sarcoplasmic reticulum vesicles. Quantitative immunoblotting using the purified canine cardiac Ca\textsuperscript{2+}-ATPase as a standard revealed that the recombinant Ca\textsuperscript{2+} pump accounted for 20–25% of the total protein of Sf21 microsomes (data not shown), similar to Ca\textsuperscript{2+} pump content reported in cardiac sarcoplasmic reticulum vesicles (34). Thus Sf21 cells infected with the SERCA2a-encoding baculovirus readily express the full-length Ca\textsuperscript{2+} pump.

To confirm the functional integrity of recombinant SERCA2a, Ca\textsuperscript{2+} transport assays were conducted with microsomal membranes isolated after N\textsubscript{2} cavitation of Sf21 cells. When assayed at the saturating Ca\textsuperscript{2+} concentration of 1 \textmu M, the Ca\textsuperscript{2+} transport rate was increased 10-fold in Sf21 microsomes expressing SERCA2a compared with control microsomes, confirming that the recombinant Ca\textsuperscript{2+} pump was indeed functional (Fig. 3A). The Ca\textsuperscript{2+} transport rate and the maximal level of Ca\textsuperscript{2+} accumulation achieved was similar to that previously observed with use of canine cardiac microsomes (46). Ca\textsuperscript{2+} activation of Ca\textsuperscript{2+} transport was found to be half-maximal at approximately 0.1 \textmu M ionized Ca\textsuperscript{2+} (Fig. 3B), demonstrating that SERCA2a displays a high Ca\textsuperscript{2+} affinity when expressed in the absence of phospholamban (Fig. 3B; see Table II). This apparent Ca\textsuperscript{2+} affinity of SERCA2a is similar to that exhibited by the rabbit skeletal muscle Ca\textsuperscript{2+} pump when expressed in Sf9 cells and assayed under similar conditions (35). As expected, a phospholamban monoclonal antibody (4, 36) had no effect on Ca\textsuperscript{2+} transport when SERCA2a was expressed by itself (Fig. 3, A and B). Note that at the lower Ca\textsuperscript{2+} concentrations tested (\textlesssim 0.1 \textmu M), microsomes containing SERCA2a accumulated at least 20 times more Ca\textsuperscript{2+} than did control microsomes, due to the negligible activity of the endogenous Ca\textsuperscript{2+} pump in Sf21 cells at low Ca\textsuperscript{2+} concentration (Fig. 3B). Failure of the endogenous Ca\textsuperscript{2+} pump in insect cells to react with SERCA1 (35)- or SERCA2-specific antibodies coupled with its low apparent affinity for Ca\textsuperscript{2+} suggests that it may be the product of the SERCA3 gene (37), but this remains to be tested. In control experiments we observed that the low background level of Ca\textsuperscript{2+} uptake, as well as the Ca\textsuperscript{2+} uptake attributable to SERCA2a expression, was completely inhibited by thapsigargin (data not shown).

Functional Coupling between Recombinant SERCA2a and Phospholamban Assayed by Ca\textsuperscript{2+} Uptake—Previously we demonstrated that baculovirus-infected Sf21 cells express recombinant canine cardiac phospholamban with high efficiency (16, 17). To test for functional coupling between canine cardiac SERCA2a and phospholamban we therefore co-infected Sf21 cells with viruses carrying the cDNAs encoding both proteins and isolated membranes for Ca\textsuperscript{2+} transport assays. Fig. 2 shows that co-expression of phospholamban with SERCA2a in Sf21 cells produced similar levels of the two proteins in insect cell microsomes compared with the protein levels detected in canine cardiac sarcoplasmic reticulum vesicles. Phospholamban was visible in insect cell microsomes either by Coomassie

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**Table I.** ATPase Activity of Recombinant SERCA2a and Phospholamban in Sf21 Microsomes and Canine Cardiac Sarcoplasmic Reticulum Vesicles

| Protein/Condition | SERCA2a | PLB | ATPase Activity (nmol/mg/min)
|-------------------|---------|-----|-----------------
| WTV               | -       | -   | 50              |
| SERCA             | +       | -   | 150             |
| SERCA/PLB         | +       | +   | 600             |
| CSR               | -       | -   | 60              |

**Note:** ATPase activity was assayed at 1 \textmu M Ca\textsuperscript{2+} using microsomal membranes isolated after N\textsubscript{2} cavitation of Sf21 cells infected with recombinant viruses. Values are means of triplicate determinations.

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**Fig. 2.** SDS-PAGE and immunoblotting of canine SERCA2a and phospholamban expressed in Sf21 microsomes. Sf21 microsomes from wild-type (WTV), SERCA2a (SERCA), and SERCA2a plus phospholamban (SERCA/PLB) baculovirus-infected cells, as well as canine cardiac sarcoplasmic reticulum vesicles (CSR), were subjected to SDS-PAGE and immunoblotting. Panel A shows a Coomassie Blue-stained gel (50 \mu g of protein/lane), and panel B shows a corresponding immunoblot (5 \mu g of protein/lane) developed with SERCA2a (upper) and phospholamban (lower) monoclonal antibodies. Boil indicates whether microsomes were boiled in SDS prior to PAGE.
Blue staining (Fig. 2A) or by immunoblot analysis (Fig. 2B) and exhibited the characteristic conversion from the pentameric to monomeric forms (2) by boiling in SDS. Control Sf21 microsomes (WTV) contained no detectable phospholamban.

To test for functional coupling between SERCA2a and phospholamban in Sf21 microsomes, Ca\(^{2+}\) transport assays were conducted at low (30 nM) and high (1 mM) Ca\(^{2+}\) concentrations in the presence and absence of anti-phospholamban monoclonal antibody 2D12, which blocks the inhibitory interaction between phospholamban and the cardiac Ca\(^{2+}\)-ATPase (4, 7, 23, 36). At low Ca\(^{2+}\) concentration, Ca\(^{2+}\) transport by Sf21 microsomes containing both proteins was stimulated 8-fold by addition of the phospholamban monoclonal antibody (Fig. 4A). However, at high Ca\(^{2+}\) concentration, Ca\(^{2+}\) transport by the same microsomes was unaffected by the antibody (Fig. 4B). Since the same monoclonal antibody increased Ca\(^{2+}\) uptake by canine cardiac sarcoplasmic reticulum vesicles approximately 10-fold at a low Ca\(^{2+}\) concentration, but had no effect at saturating Ca\(^{2+}\) concentration (4, 23), we conclude that the recombinant SERCA2a Ca\(^{2+}\) pump is tightly coupled to phospholamban in Sf21 microsomes in a similar fashion to that in cardiac sarcoplasmic reticulum vesicles.

Ca\(^{2+}\) Affinities Monitored by ATP Hydrolysis—It has proven problematical to measure ATP hydrolysis by recombinantly expressed Ca\(^{2+}\) pumps in previous studies, due to the low protein expression levels obtained and the interference by endogenous ATPase activities present in the microsomal preparations (38, 39). The abundant expression of canine SERCA2a in Sf21 cells suggested that it would be possible to measure ATP hydrolysis by this recombinant Ca\(^{2+}\) pump, which is a more direct method than assay of Ca\(^{2+}\) transport for estimating apparent Ca\(^{2+}\) affinities. Table I demonstrates that Ca\(^{2+}\)-independent (basal) ATPase activity was very low in Sf21 microsomes (WTV) compared with that exhibited by SERCA2a. The maximal Ca\(^{2+}\)-ATPase activity

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**Fig. 3.** Ca\(^{2+}\) uptake by Sf21 microsomes expressing canine SERCA2a. Panel A shows a time course of Ca\(^{2+}\) uptake measured at 1 µM ionized Ca\(^{2+}\), and panel B shows the ionized Ca\(^{2+}\) dependence of Ca\(^{2+}\) transport, determined at 8 min of Ca\(^{2+}\) uptake. Sf21 microsomes were from cells infected with SERCA2a-encoding baculovirus (SERCA) or wild-type baculovirus (WTV). **Fig. 4.** Ca\(^{2+}\) uptake by Sf21 microsomes co-expressing canine SERCA2a and phospholamban. Panel A shows results of Ca\(^{2+}\) uptake measured at 30 nM ionized Ca\(^{2+}\) concentration, and panel B shows results of Ca\(^{2+}\) uptake measured at 1 µM ionized Ca\(^{2+}\) concentration. Sf21 microsomes were obtained from cells co-infected with SERCA2a and phospholamban-encoding baculoviruses (SERCA/PLB) or wild-type baculovirus (WTV). Filled symbols denotes microsomes pretreated with phospholamban monoclonal antibody (Ab).
TABLE I

| [Ca$^{2+}$] | ATPase activity |
|-------------|-----------------|
| nM          | SERCA2a         | WTV             |
| 0           | 207             | 204             |
| 30          | 1025            | 222             |
| 47          | 1455            | 257             |
| 67          | 1992            | 300             |
| 89          | 2422            | 335             |
| 115         | 2760            | 361             |
| 179         | 3636            | 430             |
| 328         | 4471            | 544             |
| 625         | 5008            | 661             |
| 2412        | 4851            | 727             |

Ca$^{2+}$-ATPase activity of SERCA2a expressed in Sf21 microsomes

Ca$^{2+}$-ATPase activity was measured in microsomes isolated from Sf21 cells infected with wild-type baculovirus or baculovirus encoding canine SERCA2a. Pi release was monitored colorimetrically as described under "Experimental Procedures" and was found to be linear with time for at least 20 min. ATP hydrolysis at 8 min is reported.

...) reported in Table I for SERCA2a expressed in Sf21 microsomes (33 µmol of P/mg of protein/h) is 55 times greater than the ATPase activity recently reported for SERCA2a expressed in HEK-293 membranes (0.6 µmol of P/mg of protein/h) (40).

Plots depicting Ca$^{2+}$ activation of ATP hydrolysis by SERCA2a, expressed in the presence and absence of phospholamban, are shown in Fig. 5. SERCA2a expressed alone had a high apparent Ca$^{2+}$ affinity ($K_{Ca}$ value = 105 ± 10 nM), which was unaffected by the phospholamban antibody (Fig. 5A and Table II). Co-expression of phospholamban with SERCA2a decreased the Ca$^{2+}$ affinity by a factor of two, but this decrease in Ca$^{2+}$ affinity was removed by the phospholamban monoclonal antibody, shifting the Ca$^{2+}$ activation curve to the left (Fig. 5B and Table II). At the saturating Ca$^{2+}$ concentration of 2.4 µM, the antibody had no effect on ATP hydrolysis (Fig. 5B). Thus phospholamban primarily decreases the Ca$^{2+}$ affinity of the pump, whether measured by assay of Ca$^{2+}$ transport or by ATP hydrolysis, but has no effect on the $V_{max}$ of the enzyme. It should be pointed out that the Ca$^{2+}$-ATPase activities determined in these studies were about 2–3 times greater than the Ca$^{2+}$ transport rates measured, giving apparent coupling coefficients (Ca$^{2+}$ ions transported per ATP molecule hydrolyzed) of approximately 0.3–0.5. Similar low coupling coefficients are obtained with the use of canine cardiac sarcoplasmic reticulum vesicles, and are believed to be due to a significant proportion of leaky vesicles that hydrolyze ATP but are unable to retain accumulated Ca$^{2+}$ (4, 23). We detected no differences in coupling coefficients between microsomes expressing SERCA2a alone, or microsomes expressing SERCA2a plus phospholamban.

Co-expression of a Monomeric Mutant of Phospholamban

Purified phospholamban incorporated into planar phospholipid membranes is the same, in that the apparent $K_{Ca}$ value is $3.7\pm0.1$ nM, as for SERCA2a expressed in Sf21 microsomes from SERCA2a (SERCA)-infected cells (panel A) and SERCA2a plus phospholamban (SERCA/PLB) co-infected cells (panel B). Microsomes from wild-type virus (WTV) infected cells were also assayed. Microsomes pretreated with the phospholamban monoclonal antibody (+Ab) are denoted by the filled symbols. ATPase activities were determined at 8 min of incubation, and basal (Ca$^{2+}$-independent) ATPase activities have been subtracted from the values reported.

$^{45}$Ca$^{2+}$ Efflux from Sf21 Microsomes Expressing Phospholamban—Purified phospholamban incorporated into planar
lipid bilayers forms Ca\textsuperscript{2+} channels (8), and it has been proposed that Ca\textsuperscript{2+} efflux through phospholamban is involved in its mechanism of ATPase regulation (9). This hypothesis has been difficult to test with cardiac sarcoplasmic reticulum vesicles, due to the presence of other channels in these membranes and the lack of adequate control membranes that contain SERCA2a but no phospholamban. Here we tested for a Ca\textsuperscript{2+} efflux role for phospholamban by using Sf21 microsomes expressing SERCA2a alone, or microsomes expressing SERCA2a plus wild-type phospholamban. Microsomes were actively preloaded with \textsuperscript{45}Ca\textsuperscript{2+} in the presence of 25 mM phosphate, a Ca\textsuperscript{2+}-precipitating agent that readily exchanges Ca\textsuperscript{2+} (24). \textsuperscript{45}Ca\textsuperscript{2+} efflux was then initiated under active transport conditions by diluting extravesicular \textsuperscript{40}Ca\textsuperscript{2+} with excess unlabeled Ca\textsuperscript{2+}-EGTA buffer, or under conditions in which the Ca\textsuperscript{2+} pump was inactivated by chelating extravesicular Ca\textsuperscript{2+} completely with EGTA. Figure 8A shows a time course of Ca\textsuperscript{2+} uptake measured at 30 nM ionized Ca\textsuperscript{2+} concentration, and panel B shows the Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+}-ATPase activity determined at 8 min of incubation. Phospholamban antibody was used as described in the legend to Fig. 3.

**FIG. 6.** Immunoblot showing co-expression of monomeric phospholamban and Ca\textsuperscript{2+} pump. 10 ng of microsomes from Sf21 cells expressing monomeric phospholamban and the Ca\textsuperscript{2+} pump (SERCA/L37A) were subjected to SDS-PAGE and immunoblotting, along with 10 ng of microsomes expressing wild-type phospholamban and the Ca\textsuperscript{2+} pump (SERCA/PLB). Blots were probed with antibodies as described in Fig. 2. Note that boiling in SDS (Boil) was required to dissociate the wild-type phospholamban pentamer into monomers, whereas L37A-PLB was entirely monomeric without boiling in SDS.

**TABLE II**

Phospholamban regulation of SERCA2a Ca\textsuperscript{2+} affinity in Sf21 microsomes

The ionized Ca\textsuperscript{2+} concentrations giving half-maximal stimulation of Ca\textsuperscript{2+}-ATPase and Ca\textsuperscript{2+} uptake activities (K\textsubscript{Ca} values) in Sf21 microsomes are reported. Microsomes expressed SERCA2a alone, SERCA2a plus wild-type phospholamban (WT-PLB), or SERCA2a plus L37A-PLB. Assays were conducted in the presence and absence of phospholamban monoclonal antibody 2D12 (Ab) as described under “Experimental Procedures.” Results were obtained from three separate microsomal preparations for each condition and are the means ± S.E.

| Protein expressed | Ca\textsuperscript{2+} -ATPase | Ca\textsuperscript{2+} uptake |
|-------------------|---------------------------|---------------------------|
| SERCA2a           | 105 ± 10                  | 106 ± 8                   |
| SERCA2a + WT-PLB  | 224 ± 44                  | 125 ± 19                  |
| SERCA2a + L37A-PLB| 537 ± 77                  | 185 ± 24                  |

**FIG. 7.** Phospholamban monomer effect on Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+}-ATPase activities. Microsomes were isolated from Sf21 cells infected with SERCA2a and L37A-PLB encoding baculoviruses (SERCA/L37A) or wild-type baculovirus (WTV). Panel A shows a time course of Ca\textsuperscript{2+} uptake measured at 30 nM ionized Ca\textsuperscript{2+} concentration, and panel B shows the Ca\textsuperscript{2+} dependence of Ca\textsuperscript{2+}-ATPase activity determined at 8 min of incubation. Phospholamban antibody was used as described in the legend to Fig. 3.

Phospholamban ATPase Regulatory Interactions
pumps resulted in levels of cellular protein expression much lower than those presently reported. For example, expression of SERCA1a in COS cells yielded 150 μg of microsomal protein containing 2 μg of Ca^{2+}-ATPase (38). SERCA2a and SERCA3 Ca^{2+} pumps have been expressed at comparable levels (37), where microsomal membranes contained approximately 50–100 pmol of Ca^{2+} pump per mg of total protein, or about 1–2% Ca^{2+}-ATPase. SERCA1a has also been expressed in yeast cells (yielding 0.6% of total membrane protein in secretory vesicles) (39) and in baculovirus-infected Sf9 cells (yielding 1–3% of total membrane protein in microsomes) (35). A drawback to some of these systems, however, is that Ca^{2+}-ATPase activities could not be measured, due to the low expression levels achieved and interference from basal ATPase activities present in the microsomal preparations (38, 39). Additionally, in some of these studies a significant fraction of the recombinant Ca^{2+} pumps were apparently inactive (12, 41). As reported here, SERCA2a accounted for 20% or more of the total microsomal protein from infected Sf21 cells. Moreover, we estimate that 70–80% of the expressed Ca^{2+} pumps were active, relative to activity in cardiac sarcoplasmic reticulum membranes, by comparison of the ATPase hydrolysis rate to the amount of protein expressed determined by immunoblotting. Thus we have achieved expression levels of a highly active SERCA Ca^{2+} pump 10–20 times greater than those previously reported. In the study of Skerjanc et al. (35) the same baculovirus system was used to express the rabbit skeletal muscle Ca^{2+} pump in Sf9 cells, but the level of the recombinant protein obtained was about 10-fold lower than that presently reported. The higher expression level observed presently could be due to the use of Sf21 cells instead of Sf9 cells or simply due to the fact that canine SERCA2a expresses more efficiently in insect cells than does rabbit SERCA1a; however, we have not investigated either of these possibilities. Even with the lower expression level in insect cells achieved by Skerjanc et al. (35), it was still possible to measure direct binding of {sup 45}Ca^{2+} to a recombinant Ca^{2+} pump for the first time. Thus, we anticipate that Sf21 microsomes expressing canine SERCA2a will be a very useful system for future studies investigating detailed biochemical aspects of enzyme function.

Previously we demonstrated that Sf21 cells express canine cardiac phospholamban very effectively, allowing purification of milligram quantities of the protein (16, 17), and here we have shown that, when canine SERCA2a is co-expressed with phospholamban, the two proteins are functionally coupled. Both Ca^{2+}-ATPase and Ca^{2+}-uptake activities of Sf21 microsomes were stimulated manifold at low ionized calcium concentration by the phospholamban monoclonal antibody, which produces the same effect as phosphorylation of phospholamban by protein kinases (7), but no effect of the antibody was noted at saturating Ca^{2+} concentration. Thus, use of the baculovirus cell expression system provides additional strong evidence that the main regulatory effect of phospholamban is on the apparent Ca^{2+} affinity of the Ca^{2+} pump, but not on the $V_{\text{max}}$ of the enzyme. A similar regulatory effect of phospholamban on the Ca^{2+} pump has been reported with use of canine cardiac sarcoplasmic reticulum vesicles (4, 6, 23, 42), with use of the purified and reconstituted proteins (16, 20), with use of the recombinant proteins expressed in HEK-293 membranes (43), and with use of phospholamban knockout mice (10). Although Kirchberger and co-workers (44) have recently challenged the idea that the main regulatory effect of phospholamban is on the $K_c$ value for Ca^{2+} transport, most evidence concurs that de-phosphorylated phospholamban strongly suppresses the Ca^{2+}-affinity of the enzyme, with an insignificant effect on $V_{\text{max}}$. The argument by Kirchberger and co-workers (44) that the {sup 45}Ca^{2+}
uptake assay used by others (4, 6, 16, 43) artificially reports a Ca\(^{2+}\) affinity change is negated by our results with ATPase activity measurements, where a similar \(K_m\) shift by phospholamban is observed by a completely independent and more direct method.

Use of the SP21 system allowed us to test if the phospholamban pentamer is essential for SERCA2a regulation. To examine this issue, SERCA2a was co-expressed with L37A-PLB, which is known to exist on SDS-PAGE (17) and, more importantly, also when reconstituted in lipid membranes (19). Surprisingly, we observed that L37A-PLB is a more effective suppressor of SERCA2a Ca\(^{2+}\) affinity than wild-type phospholamban. Using an electron paramagnetic resonance technique, Cornea et al. (19) recently demonstrated that wild-type phospholamban exists in two physical states in the lipid bilayer; one state is composed of pentamers (80% of total phospholamban) and the other monomers (20% of total phospholamban), giving about an equimolar ratio of monomers to pentamers. These two states are in dynamic equilibrium, and phosphorylation of phospholamban by cAMP-dependent protein kinase shifts the equilibrium completely toward pentamers (19). Since we show here that the phospholamban monomer is a more effective inhibitor of ATPase activity than is the pentamer, the results suggest that phosphorylation of phospholamban in the sarcoplasmic reticulum membrane could relieve inhibition of the Ca\(^{2+}\) pump at least to two mechanisms: one, by disrupting the physical interaction between phospholamban and the Ca\(^{2+}\) pump (45) and, two, by promoting the more complete association of phospholamban into pentamers (19), which are relatively ineffective inhibitors compared with monomers. More sophisticated techniques will be required to test if the phospholamban pentamer by itself is capable of inhibiting the Ca\(^{2+}\) pump, but the results presented presently in combination with those of Cornea et al. (19) clearly indicate that at least one monomeric mutant of phospholamban is a much stronger inhibitor of SERCA2a activity than is wild-type phospholamban. Our results are consistent with a recent preliminary report of Kimura et al. (20).

Although we reported earlier that purified phospholamban forms Ca\(^{2+}\) channels in lipid bilayers (8), we found no evidence here for phospholamban acting as a Ca\(^{2+}\) efflux channel when co-expressed with SERCA2a under conditions in which the two proteins are tightly coupled. A similar inability of phospholamban to form Ca\(^{2+}\) channels was noted by Reddy et al. (16) in a study in which phospholamban was successfully co-reconstituted with the skeletal muscle Ca\(^{2+}\) pump in phospholipid vesicles. Thus we believe that a Ca\(^{2+}\) efflux role for phospholamban in its mechanism of regulation of SERCA2a is very unlikely and that recent models proposing such a role (9) should be viewed with some skepticism.

In conclusion, we have demonstrated that the baculovirus cell expression system is ideally suited for investigating phospholamban and SERCA2a regulatory interactions. High levels of protein expression are achieved, with preservation of functional coupling. With use of this system, it is now possible to carry out detailed biochemical and kinetic analyses while investigating both normal and mutated proteins.

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