The Role of Sarcolipin and ATP in the Transport of Phosphate Ion into the Sarcoplasmic Reticulum

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ABSTRACT In a previous study, sarcolipin (SLN) was shown to form channels selective toward chloride ion when incorporated in a mercury-supported tethered bilayer lipid membrane (tBLM). Its incorporation had only a modest permeabilizing effect on phosphate ion. In this note the resistance of a tBLM membrane incorporating sarcolipin was investigated by electrochemical impedance spectroscopy in aqueous solutions of 0.05 M sodium phosphate of pH ranging from 5.3 to 8, in the presence of ATP, adenosine monophosphate, and phenylphosphonic acid. At pH 5.3, submicromolar additions of ATP increase the conductivity of the tBLM incorporating SLN up to a maximum limiting value. The dependence of the conductivity on the ATP concentration satisfies the Michaelis-Menten equation, with an association constant of 0.1 μM. Phenylphosphonium ion and adenosine monophosphate exert an inhibitory effect on membrane permeabilization to phosphate ions by ATP if they are added before ATP, but not if they are added after it. An explanation for this behavior is provided. In conclusion, SLN acts as an ATP-induced phosphate carrier exhibiting a behavior quite similar to that of the unidentified P_i transporter described previously. No ion-channel activity is exhibited by the T18A mutant of SLN.

INTRODUCTION

Sarcolipin (SLN) is a 31 amino-acid integral membrane protein that regulates the sarco(endo)plasmic reticulum Ca-ATPase (SERCA). It is highly expressed in fast-twitch skeletal muscle sarcoplasmic reticulum (SR), whereas it is expressed at ~10-fold lower concentrations in slow-twitch skeletal muscle SR, mimicking the expression pattern of the SERCA1 isoform (1). SLN is also localized in the cardiac SR membrane (2). By contrast, phospholamban (PLN), a major regulator of the kinetics of cardiac contractility, besides being highly expressed in cardiac SR, is expressed also in slow-twitch muscle SR, mimicking the expression pattern of SERCA2a (1). SLN is composed of a transmembrane helix consisting of 22 residues and of two short unstructured termini consisting of residues 1–6 in the N-terminus and residues 27–31 in the C-terminus (3,4). SLN spans lipid bilayers with its α-helical transmembrane domain, which is oriented perpendicularly to the membrane plane (3,4); the N-terminus faces the cytosol and the C-terminus protrudes into the lumen of the SR. SLN shares nearly 30% identity with the transmembrane domain of PLN.

Both SLN and PLN inhibit SERCA at low Ca^{2+} concentrations (1,5), due to an apparent decrease in the affinity of the calcium pump for Ca^{2+}. In contrast to PLN, SLN has been reported to stimulate maximal Ca^{2+} uptake rates (V_{max}) (1,6). However, Smith et al. (7) did not detect significant effects of SLN on the rate of ATP hydrolysis by SERCA or on the Ca^{2+}-dependence of SERCA activity. Although the inhibitory effect of PLN is relieved by its phosphorylation, no evidence of SLN phosphorylation was found originally (1). However, it was shown recently that mutation of the threonine residue (Thr^5) at the SLN amino terminus abolishes the inhibitory effect of SLN on SERCA function. These studies identified Thr^5 as a potential phosphorylation site and showed that serine-threonine protein kinase 16 (STK16) could phosphorylate SLN (8).

SLN forms aggregates in a lipid environment (6). We showed recently that SLN oligomers in the lipidic environment of a biomimetic membrane form anion-selective ion channels (9). The biomimetic membrane used is obtained by tethering a monolayer of a thiolipid, named DPTL, to the surface of mercury. DPTL, first used by Schiller et al. (10) and Naumann et al. (11), consists of a hydrophilic tetraethyleneoxy (TEO) chain terminated with a lipoic acid residue for anchoring to the mercury surface at one end and with two phytanoyl chains at the other end. This biomimetic membrane, often referred to as a tethered bilayer lipid membrane (tBLM), is completed by self-assembling a lipid monolayer on top of the thiolipid monolayer, so as to form a lipid bilayer interposed between the hydrophilic spacer and the aqueous solution that baths the tBLM. The hydrophilic spacer addresses the necessity of a submembrane space serving as an ionic reservoir and providing adequate space for incorporated membrane proteins. Thanks to its liquid nature, mercury provides a defect-free surface to the self-assembling film, and imparts a high fluidity to the tBLM by allowing the lateral movement of the thiolipid molecules anchored to its surface (12–14).

In examining the channel-forming properties of SLN, we observed that SLN shares some features with a proposed phosphate transporter in SR, usually referred to as the P_i transporter (9). This transporter, whose nature is unknown, enhances the level of accumulation of calcium ions in SR vesicles, when SERCA is activated by addition of ATP in

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the presence of phosphate ions (15,16). This effect is ascribed to the transport of phosphate ion into the vesicles, with a resulting precipitation of calcium phosphate and a decrease in the concentration of free calcium ions. Such an effect was measured from the uptake of $[^{32}P]P_i$ or by following a slow phase of accumulation of calcium ions into the SR vesicles, which is only observed in the presence of phosphate. The P_i transporter is endogenous, because no effect of phosphate ions is observed in reconstituted vesicles containing purified Ca-ATPase. If ATP is added to the SR vesicles before the addition of phosphate, its enhancing effect on the level of accumulation of calcium ions decreases progressively with an increase in the time elapsed between the two additions (16). In practice, the enhancement vanishes after 10 min, during which Ca-ATPase continues pumping calcium ions in the absence of phosphate. The rate of the P_i transporter is higher at pH 5.3 than at pH 8, thus suggesting that the transport of $H_2PO_4^-$ is faster than that of HPO_4^{2-} (16). The P_i transporter is inhibited by phosphonocarboxylic acids and by phenylphosphonic acid (15). Modification of SR with phenylglyoxal also inhibits Ca^{2+} accumulation induced by the P_i transporter in the presence of phosphate, but the presence of 5 mM ATP reduces inhibition very markedly (15). Because phenylglyoxal modifies arginine residues (17), it was suggested that the P_i transporter has an ATP binding site including at least one arginine residue.

This study shows that SLN incorporated in a biomimetic membrane acts as a phosphate carrier, when in the presence of micromolar concentrations of ATP, and shares several features with the P_i transporter.

MATERIALS AND METHODS

The water used was obtained from an inverted osmosis unit, on distilling it once and then distilling the water so obtained from alkaline permanganate. Reagent grade Na_2HPO_4, NaOH, adenosine-5′-triphosphate disodium salt (ATP, ~97%), and adenosine-5′-monophosphate disodium salt (AMP >99%) were purchased from Fluka (Milwaukee, WI). Diphytanoylphosphatidylcholine (DPhPC) was purchased in chloroform solution from Avanti Polar Lipids (Birmingham, AL). Sphingomyelin (SM) was obtained from Lipid Products (Surrey, UK). Cholesterol (Chol) was purchased from Sigma (St. Louis, MO) and used without further purification. Phenylphosphonic acid was purchased from Aldrich (St. Louis, MO). The 2,3,di-O-phytanyl-*sn*-glycerol-1-tetraethylene-glycol-D,L-α lipoic acid ester lipid (DPTL) was provided by Prof. Adrian Schwan (Department of Chemistry, University of Guelph, Canada). SLN and its T18A mutant were prepared by following stepwise Fmoc solid-phase peptide synthesis and reverse-phase HPLC purification protocols described previously (3,6). DPhPC solutions were prepared by diluting proper amounts of stock solutions of this phospholipid with pentane. SM and Chol stock solutions were prepared by dissolving these lipids in chloroform. The lipid mixture used in all measurements had the composition DPhPC/SM/Chol (59:15:26) and was obtained by diluting proper amounts of each lipid stock solution with pentane. Solutions of 0.2 mg/mL DPTL in ethanol were prepared from a 2-mg/mL solution of DPTL in ethanol. Stock solutions of this thiolipid were stored at –18°C. Stock solutions of 1 mg/mL SLN in ethanol were stored at +4°C.

Tethering a DPTL monolayer to a hanging mercury drop electrode (HMDE) and self-assembling a lipid monolayer on top of it were carried out as described elsewhere (9). The resulting tBLM was then subjected to several potential scans in aqueous 0.05 M NaH_2PO_4 over a potential range from −0.200 to −1.200 V while continuously monitoring the quadrature component, Y'' of the electrode admittance at 75 Hz until a stable curve of Y'' versus the applied potential E was attained. The minimum differential capacity of the resulting (DPTL|lipid)-coated mercury ranged from 0.55 to 0.65 μF cm^{-2}, and was therefore close to the capacity, ~0.8 μF cm^{-2}, of a solvent-free bilayer lipid membrane. The incorporation of SLN was carried out as described in Becucci et al. (9). Briefly, the applied potential was scanned from −0.300 to −0.950 V at 15 min intervals by alternating current voltametry in aqueous 0.05 M NaH_2PO_4 containing 0.7 μM SLN, while recording the in-phase component, Y', of the electrode admittance at 10 Hz. The Y' versus E curves exhibit a sharp peak that shifts progressively toward less negative potentials, until it is stabilized in the proximity of –0.85 V after ~1.5 h. This indicates that SLN incorporation into the tBLM practically attains equilibrium after this time. An identical procedure was adopted for the incorporation of the T18A mutant. In this case, the incorporation was denoted by an appreciable and stable increase in Y' at potentials negative of −0.8 V.

All measurements were carried out in aqueous solutions of 0.05 M sodium phosphate ranging from 5.3 to 8 pH. The pH was adjusted by small additions of aqueous NaOH. Use was made of a homemade HMDE described elsewhere (18). A homemade glass capillary with a finely tapered tip (~1 mm in outer diameter) was used. Capillary and mercury reservoir were thermostated at 25 ± 0.1°C in a water-jacketed box to avoid any changes in drop area due to a change in temperature. Electrochemical impedance spectroscopy measurements were carried out with an Autolab instrument PGSTAT12 (Echo Chemie, Utrecht, The Netherlands) supplied with FRA2 module for impedance measurements, SCAN-GEN scan generator and GPES 4.9005 Beta software. Potentials were measured versus a Ag|AgCl (0.1 M KCl) electrode and herein they are referred to this electrode. Impedance spectra of the tBLM in the absence of SLN and of its T18A mutant as well as on their incorporation, were recorded over the frequency range from 10^{-2} to 10^5 Hz at different potentials ranging from −0.3 to −0.9 V.

RESULTS

The tBLM used in this study was used in a previous study (9) to show that SLN incorporated in the lipid bilayer moiety of the tBLM forms channels that are impermeable to cations, such as K^+ or Na^+ ions, but are permeable to small inorganic anions, such as Cl^-. It was also shown that SLN channels are almost completely impermeable to phosphate ion (see Fig. 7 in Becucci et al. (9)). The tBLM consists of three substructures of different dielectric properties, namely the lipoic acid residue, the TEO hydrophilic moiety, and the lipid bilayer moiety. In Becucci et al. (9), the electrochemical impedance spectra of the tBLM incorporating SLN were fitted to an equivalent circuit consisting of four RC meshes in series (each mesh denoted as a circuit element consisting of a resistance and a capacity in parallel). The four meshes simulated the lipoic acid residue, the TEO hydrophilic moiety, the lipid bilayer moiety, and the aqueous solution bathing the tBLM. The potential dependence of the resistances and capacities of the TEO moiety and of the lipid bilayer moiety permitted a detailed examination of the properties of the tBLM incorporating SLN in the presence of 0.1 M KCl. In addition, the overall resistance (R_{tBLM}) of the tBLM was measured by fitting the impedance spectrum to a series of only two RC meshes, simulating the tBLM and the aqueous solution bathing it. This fitting was reported

on a Nyquist plot of Z'' versus Z', where Z' and Z'' are the in-phase and quadrature components of the electrochemical impedance. The latter approximate procedure avoids entering the details of the substructures composing the tBLM, when one is interested mainly in the resistance opposed by the membrane to ionic translocation. See the Supporting Material for the differences between the two approaches based on an equivalent circuit consisting of either four or two RC meshes in series. In this study, we will adopt the equivalent circuit consisting of a series of four RC meshes and we will consider the resistance, R_{LB}, exclusively ascribed to the lipid bilayer moiety of the tBLM. In practice, the R_{tBLM}/R_{LB} ratio turns out to be roughly constant in all series of measurements carried out on the same tBLM.

In the absence of ionic channels incorporated in the tBLM, R_{LB} is due to nonspecific penetration of ions across the lipid bilayer moiety. On applying an alternating current voltage, these ions move back and forth from the solution bathing the tBLM to the hydrophilic spacer that accommodates them and vice versa, imparting to the tBLM a resistance that is high, but not infinitely high. If incorporation of a protein in the tBLM bathed by an electrolytic solution does not cause a detectable decrease in R_{LB}, this implies that the protein does not act as a channel toward the ions present in the electrolytic solution. If the subsequent addition of a different ionic species to the bathing solution causes an appreciable decrease in R_{LB}, this provides clear evidence that the incorporated protein acts as a channel toward the added ionic species. Same conclusions hold if the decrease in R_{LB} is produced by the addition of an activating substance such as ATP, which is known not to permeate biological membranes; in this case, one must conclude that the added substance stimulates the protein to act as a channel toward some ionic species already present in the solution. In this study, Nyquist plots were used to show that the resistance R_{LB} of the lipid bilayer moiety of the tBLM incorporating SLN in contact with an aqueous solution of 0.05 M sodium phosphate is appreciably decreased by submicromolar additions of ATP. Because a tBLM incorporating SLN is almost impermeable to an aqueous solution of 0.04 M Na_2H_2ATP (see Fig. 4 in Becucci et al. (9)), such a decrease in R_{LB} must be necessarily ascribed to an ATP-induced permeation of the tBLM incorporating SLN by phosphate ions.

Fig. 1 shows Z'' versus Z' plots obtained at −0.500 V in a pH 5.3 solution of 0.05 M NaH_2PO_4, in the absence and in the presence of incorporated SLN, and on addition of different concentrations of ATP. The solid curves are fits of the curves to a series of four RC meshes. Proceeding in the positive direction of the abscissas, the first roughly semicircular arc is due mainly to the contribution from the lipid bilayer moiety. The subsequent hump, which is evident in curves *c* to *e*, is mainly due to the contribution from the lipoic acid residue (see the Supporting Material). The contributions from the hydrophilic TEO moiety and from the aqueous solution are not visible, because in this Nyquist plot they

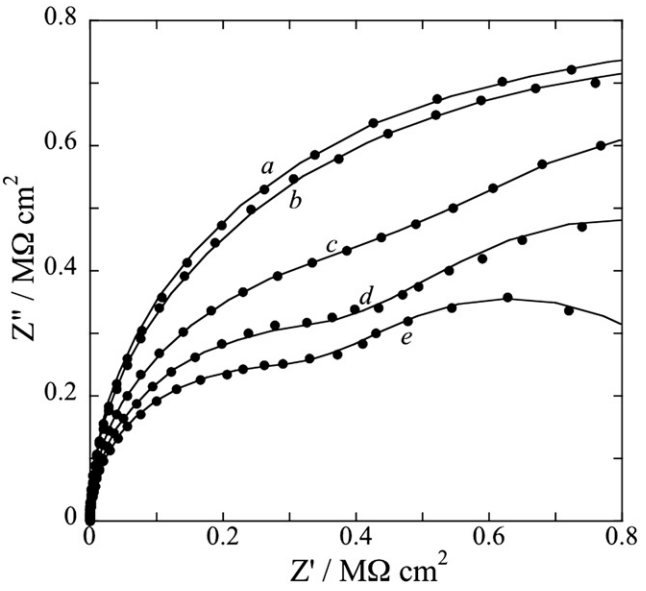

FIGURE 1 Solid circles are Z'' versus Z' plots at a tBLM in a pH 5.3 aqueous solution of 0.05 M NaH_2PO_4 at −0.500 V (*a*) in the absence of SLN, (*b*) after incorporation of SLN from its 0.7 μM solution, and after subsequent additions of (*c*) 0.1, (*d*) 0.3, and (*e*) 3 μM ATP. The solid curves are fits to an equivalent circuit consisting of four RC meshes in series. The R_{LB} values of the RC mesh are (*a*) 0.90, (*b*) 0.70, (*c*) 0.53, (*d*) 0.42, and (*e*) 0.35 MΩ cm^2. The corresponding C_{LB} values are all close to 1 μF cm^{-2}.

are very small and located very close to the origin of the coordinate system. The fitting is satisfactory and provides accurate values of the R_{LB} under the different experimental conditions. In the absence of ATP, the incorporation of SLN in the membrane had only a slight effect on phosphate permeation. Small progressive additions of ATP caused a decrease in the membrane resistance, which rapidly attained a minimum limiting value. The maximum percentage decrease depended to some extent on the initial resistance of the tBLM, and ranged from 60% to 70%. In fact, the resistance of the tBLM varied somewhat from one preparation to another. Consequently, all percentage changes in the tBLM resistance after incorporation of SLN or ionic additions to the bathing solution were measured on the same tBLM.

Fig. 2 shows a plot of the average value of the lipid bilayer conductance (namely the reciprocal of its resistance, R_{LB}), as a function of the ATP concentration, estimated over four different series of measurements. Each series of measurements was carried out on the same tBLM. The conductance was normalized to the difference between the maximum and minimum value of each series, taken as unity. The error bars denote standard deviations, which necessarily vanish at the two extreme conductance values, due to the particular normalization procedure adopted. This experimental behavior satisfies the Michaelis-Menten equation. The association constant K, expressing the ATP concentration corresponding to half-saturation of a hypothetical SLN-ATP

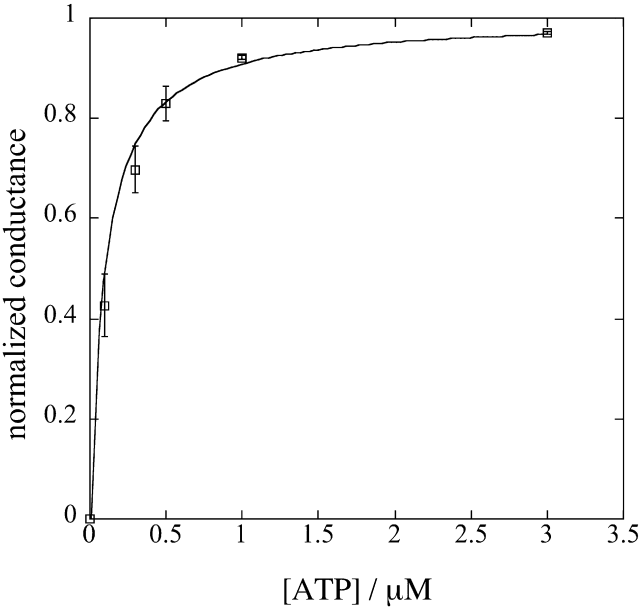

FIGURE 2 Normalized conductance of a tBLM incorporating SLN and immersed in a pH 5.3 aqueous solution of 0.05 M NaH_2PO_4 at −0.500 V, as a function of the ATP concentration. Error bars = SD. The solid curve is a fit of the experimental points to the Michaelis-Menten equation.

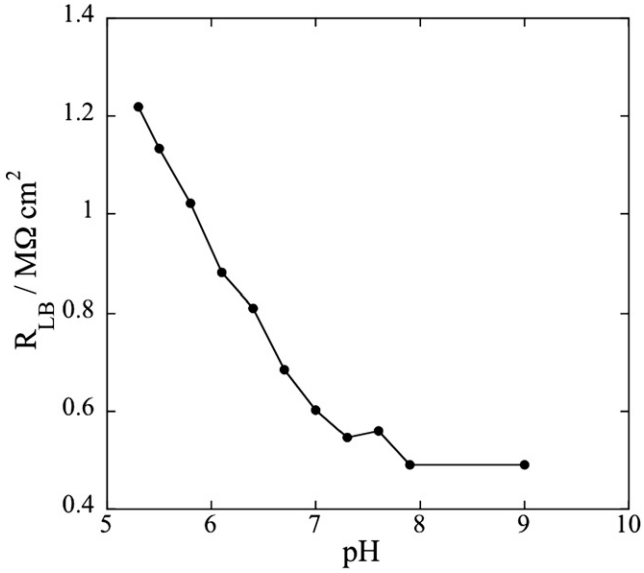

FIGURE 3 Resistance of the lipid bilayer moiety of a tBLM incorporating SLN and immersed in an aqueous solution of 0.05 M sodium phosphate + 1 μM ATP as a function of pH, at −0.500 V. The plot was obtained starting from pH 5.3 and increasing the pH by progressive additions of 0.5 M NaOH.

complex, amounts to 0.1 μM. The solid curve in the same figure expresses the normalized conductance calculated from the Michaelis-Menten equation with this K value. Measurements carried out at pH 7.3 under otherwise identical conditions show an analogous trend with a progressive increase in ATP. However, the maximum percentage decrease of the tBLM resistance is appreciably smaller than at pH 5.3, amounting to ~20%. No detectable decrease was observed at pH 7.9. Surprisingly, if the pH of the 0.05 M sodium phosphate solution containing 1 μM ATP is progressively increased from pH 5.3 by gradual additions of NaOH, the resistance of the tBLM undergoes a further decrease with an increase in pH up to 7.5 and then levels off. This is shown in Fig. 3. The time spent at pH values >7.5 in the latter measurements amounts to ~10 min, and is, therefore, short enough to exclude appreciable ATP hydrolysis at these relatively high pH values (see Wach and Gräber (19)).

The following results were obtained in aqueous 0.05 M NaH_2PO_4 bathing tBLMs in which SLN had been incorporated previously from its 0.7 μM solution. The presence of AMP at pH 5.3 inhibited the effect of ATP on the permeabilization of SLN channels to phosphate ions, if added before ATP. However, it did not remove this effect if added after ATP. Thus, addition of 3 μM AMP to a pH 5.3 solution of 0.05 M NaH_2PO_4 caused a slight decrease of ~15% in R_{LB}; subsequent additions of ATP up to 6 μM caused no further decrease in resistance. Addition of 0.1 μM ATP to a pH 5.3 solution of 0.05 M NaH_2PO_4 caused a decrease in resistance of ~40%, in accordance with the plot in Fig. 2; a subsequent addition of 1 μM AMP caused a further slight decrease in resistance of ~5%; no further decrease in resistance was produced by ATP additions up to 3 μM. If ATP was added to a pH 5.3 solution of 0.05 M NaH_2PO_4 at a concentration of 3 μM, high enough to cause the maximum permeabilizing effect on phosphate ions, a further addition of 1 μM AMP did not affect R_{LB}, namely it did not remove such a permeabilization. The above behavior strongly suggests that ATP and AMP compete for the same binding site in the SLN molecules; however, the effect of ATP on the permeabilization of the SLN channel to phosphate ions is much greater than that of AMP. An analogous behavior was shown by phenylphosphonic acid. Thus, addition of 2.5 mM phenylphosphonic acid to a pH 5.3 solution of 0.05 M NaH_2PO_4 caused a decrease of ~30% in resistance; subsequent additions of ATP up to 3 μM caused no further decrease in resistance. On the other hand, addition of 2.5 mM phenylphosphonic acid to a pH 5.3 solution of 0.05 M NaH_2PO_4 that already contained 3 μM ATP left the lipid bilayer resistance unaltered, and hence did not remove the permeabilizing effect of ATP.

We may, therefore, conclude that both the inhibition and the pH dependence of the permeabilization of SLN channels to phosphate ions by ATP are notably influenced by the order with which the different additions are carried out.

DISCUSSION

The similarity between the amino acid sequences of the transmembrane domains of SLN and PLN suggests that these two proteins belong to the same family. However, we have shown recently that, although SLN monomers tend to aggregate in

a lipid environment forming anion-selective channels, the PLN pentamer is impermeable to ions (20). This difference in behavior can be explained by one particularly significant difference in the amino acid sequences of SLN and PLN. The transmembrane domain of SLN includes two hydrophilic threonines, Thr^{13} and Thr^{18}, whereas that of PLN includes two hydrophobic cysteines, Cys^{41} and Cys^{46}, at about the same position along the transmembrane helix. The two threonines are rotated by ~90° with respect to the helix axis of the SLN monomer and are almost equidistributed along the membrane thickness. The face of the SLN helix containing the two threonines is therefore moderately hydrophilic, whereas the opposite face is definitely hydrophobic. In a lipid environment, SLN is therefore expected to aggregate into a bundle of four or five monomers, with the hydrophilic faces directed inward, making the hydroxyl groups available for favorable interactions with permeant ions and/or water molecules. Conversely, the hydrophobic faces are directed outward, to establish favorable interactions with the hydrocarbon tails of the lipid bilayer. A similar model is assumed for channel-forming peptides, such as melittin (21) and alamethicin (22), and also for bulky proteins, such as potassium channels.

To confirm the role played by the threonine residues in the formation of a hydrophilic channel, impedance spectra of the tBLM, both in the absence of SLN and its T18A mutant and on their incorporation, were recorded over the frequency range from 10^{-2} to 10^{5} Hz at different potentials ranging from −0.3 to −0.9 V in 0.1 M KCl. Taking the in-phase admittance Y' as an approximate measure of the tBLM conductance, the maximum increase in Y' for SLN over the potential range of physiological transmembrane potentials is observed at low frequencies. Incidentally, in the absence of ionic charges in the hydrophilic spacer moiety of the tBLM, the transmembrane potential ϕ_2 (namely, the potential difference across the lipid bilayer moiety of the tBLM) is approximately given by $\phi_2 = 0.72 \times (E$ versus Ag|AgCl|0.1 M KCl + 0.460 V) (14). Fig. 4 shows $\Delta Y'$ versus E plots at 0.1 Hz for both SLN and the T18A mutant, where $\Delta Y'$ is the increase in Y' at each applied potential after the incorporation of the given protein. The error bars measure standard deviations estimated from four different sets of independent measurements. It is apparent that, although SLN attains a maximum conductivity at −0.600 V, corresponding to a transmembrane potential $\phi_2 = 0.72 \times (-0.600 + 0.460)$ V = −0.100 V, its mutant causes no detectable increase in the conductance of the tBLM at physiological transmembrane potentials.

The behavior of the SLN channel is similar to that of the still unidentified P_i transporter, described in detail by Stefanova et al. (15,16). Thus, both species are induced to transport phosphate ions by ATP; moreover, with both species the permeability of the membrane to phosphate ions induced by ATP decreases in passing from a slightly acidic to a slightly alkaline medium and is inhibited by the presence of phenyl-

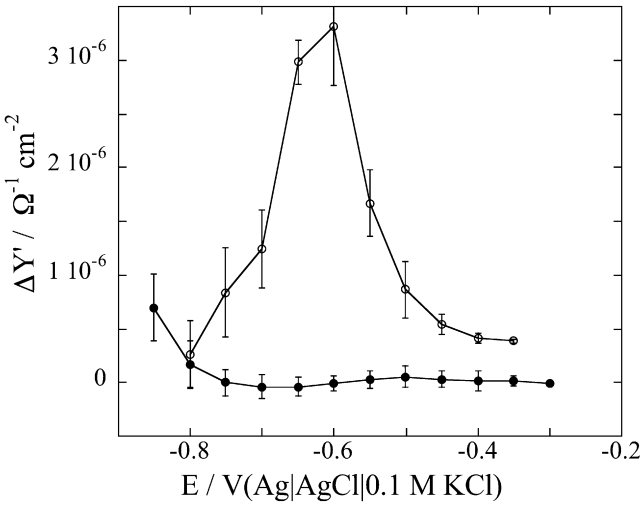

FIGURE 4 $\Delta Y'$ versus E plots at 0.1 Hz for a tBLM incorporating either SLN from its 0.7 μM solution in 0.1 M KCl (*open circles*) or its T18A mutant (*solid circles*). $\Delta Y'$ is the increase in Y' at each applied potential after the incorporation of the given protein.

phosphonic acid. It has been suggested that the binding site of the P_i transporter for ATP is an arginine residue (15), and SLN has indeed Arg^6 in its cytoplasmic domain, in close proximity to the outer leaflet of the SR membrane. Arg^6 is directly linked by peptide bonds to Glu^7 and Thr^5. Phosphorylation of SLN at the Thr^5 residue by ATP is to be excluded in the absence of a suitable kinase (8). Quite probably, the interaction of ATP with Arg^6 is mainly electrostatic, although a modest specific contribution cannot the excluded. The pK value for the protonation of ATP^{4-} to $HATP^{3-}$ in 0.1 M KCl is equal to ~6.5 (23). If the ATP molecules bind to the Arg^6 residues of some or all the monomeric units of a SLN channel, the electrostatic repulsion among the highly charged ATP molecules is expected to keep the SLN helices away from each other, widening the channel lumen and allowing the passage of phosphate ions.

When ATP is added progressively to a phosphate solution at constant pH, it causes a gradual increase in the conductance of a SLN-containing tBLM. This conductance increase diminishes as the pH passes from pH 5.3 to pH 7.3, and practically vanishes at pH 8. This behavior can be explained by an increasing repulsion of phosphate ions from the mouth of the SLN channel as they pass from the $H_2PO_4^-$ to the HPO_4^{2-} form. In fact, the pK value for the ionization of $H_2PO_4^-$ to HPO_4^{2-} amounts to 7.2. A similar explanation was provided for the analogous behavior exhibited by the P_i transporter (16). The electrostatic repulsion between phosphate ion and ATP may also be enhanced by the concomitant increase in the negative charge of ATP from −3 to −4.

In apparent contrast with the above behavior, the resistance of the lipid bilayer decreases if the pH of the phosphate solution that baths the SLN-containing tBLM is gradually increased from 5.3 to 7 by progressive additions of NaOH at constant ATP concentration, ultimately attaining a constant

minimum value (see Fig. 3). This behavior can be tentatively explained by the close proximity of Glu^7 to Arg^6 in the cytoplasmic domain of SLN. The pK value of the carboxyl group of the side chain of glutamate is ~4.3. However, it was reported recently that the close proximity of two glutamate residues in *Bacillus circulans* xylase causes one of the two residues to have the abnormally high pK value of 6.8 (24). In the bundle of SLN monomers forming a SLN channel, the Glu^7 residues are close to each other, practically in contact with the lipid leaflet exposed to the cytoplasmic side of the membrane. Even though they are also close to the positively charged Arg^6 residues, it is possible that their pK value may be >4.3, approaching a value of 6. If this is the case, then at pH 5.3 the Glu^7 residues are almost completely protonated and do not oppose the electrostatic attraction between the Arg^6 residue and ATP. When the solution pH in the presence of ATP is progressively increased from pH 5.3, the $HATP^{3-}$ molecules bound to Arg6 create a highly negative environment that prevents the Glu^7 residues from undergoing deprotonation, even at pH 8. Hence, the only effect of the increase in the solution pH is an increase in the negative charge of the bound ATP molecules from −3 to −4, with a resulting increase in the separation of the helices of the SLN channel. Moreover, the Glu^7 residue being neutral has no effect on phosphate ion, thus creating more favorable conditions for its translocation than those encountered in resistance measurements carried out at pH values >6, where Glu^7 is negatively charged before the addition of ATP.

Anions such as phenylphosphonium ion and AMP exert an inhibitory effect on membrane permeabilization to phosphate ions by ATP if they are added before ATP, but not if they are added after it. This strongly suggests that they compete with ATP for the same binding site, probably via their common $-PO(OH)_2$ group. Because they are all negatively charged, it is reasonable to assume that the binding site is the positively charged Arg^6 residue, and that the nature of their interaction with this residue is mainly electrostatic. The electrostatic repulsion among these polyvalent anions bound to the cytoplasmic domain of the monomeric units of the SLN channel widens the lumen of the channel the more, the higher their negative charge is. This may explain why the AMP and phenylphosphonium anions, whose charge varies from −1 to −2 straddling pH 6.1 (23) and 7.1 (25) respectively, have only a modest effect on the translocation of phosphate ions across the lipid bilayer, as compared to the effect exerted by the tri- or tetravalent ATP. Once the binding of a given anion to Arg^6 has been established, its replacement by a different anion, subsequently added to the solution, is apparently very slow, even in the presence of a strong mass action by the added anion. Stefanova et al. (16) reported that, when ATP is added to SR vesicles before the addition of phosphate, the enhancing effect of the P_i transporter on the level of accumulation of calcium ions decreases progressively with an increase in the time elapsed between the two additions. It is tempting to expect a similar behavior from SLN. As SERCA continues pumping calcium ions into the SR vesicles, the concentration of ADP increases progressively at the expense of ATP, whereas the local pH on the cytoplasmic side of the pump decreases due to the countertransport of protons. This causes the interaction of ADP with Arg^6 to prevail over that of ATP the more, the longer the time elapsed before the phosphate addition. This ADP interaction with Arg^6 may also be favored by the possible protonation of Glu^7, after the local decrease in pH. Due to its lower negative charge, ADP is expected to widen the SLN channel much less effectively than ATP.

In conclusion, SLN acts as an ATP-induced phosphate carrier exhibiting a behavior quite similar to that of the unidentified P_i transporter described by Stefanova et al. (15,16). The question of whether SLN is merely similar to the P_i transporter or may be identified with it is yet to be answered.

SUPPORTING MATERIAL

Two figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)01422-2.

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