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Examination of the Role of Phosphorylation and Phospholamban in the Regulation of the Cardiac Sarcoplasmic Reticulum Cl^- Channel

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Abstract. Sarcoplasmic reticulum (SR) vesicles were prepared from either canine or sheep heart and fused into lipid bilayers to study their ionic channels. A 92 ± 5 pS anion-selective channel was recorded in asymmetric 50 mM trans/250 mM cis CsCl buffer system. Reversal potentials and theoretical equilibrium potentials for Cl^- ions obtained under various experimental conditions allowed us to confirm the Cl^- selectivity of this SR channel. The majority (69%) of channel recordings (n = 45) displayed steady-state kinetics and a slight voltage dependency of the open probability. However, 31% of the channels inactivated after their incorporation. We now report that the channel might be reactivated by depolarizing voltage steps. Furthermore, the use of either PKA or PKG in association with adequate phosphorylating buffers lengthens the deactivation process at the end of the voltage pulses, but does not prevent the inactivation. It was assumed that the change in gating mode was due to a voltage-sensitive association/dissociation mechanism with a phosphorylated protein of the SR membrane such as phospholamban (PL). We demonstrated that a specific monoclonal antibody raised against canine PL inhibited the activity of the channel and prevented its reactivation by depolarizing steps. 400 to 800 ng/ml of Anti-PL Ab consistently and sequentially turned off the channel activities. In contrast, heat inactivated Anti-PL Ab had no effect. We propose that phospholamban may be a primer of the SR Cl^- channel whereby Cl^- anions would play the role of counter-charge carrier during rapid Ca^{2+} release and Ca^{2+} uptake by the SR.

Key words: Sarcoplasmic reticulum — Phospholamban — Heart — Cardiomyocyte

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Introduction

The existence of a large chloride conductance in membrane vesicles derived either from skeletal or cardiac sarcoplasmic reticulum (SR) has been reported by several independent laboratories [11, 26, 30]. Although such anion conductance initially seemed to be a specific feature of isolated SR vesicles, this no longer appears to be the case since Cl^- channels have also been recorded, *in situ,* on SR membrane [29] and sarcoball preparations [9] from various striated muscles and in different species. The basic properties of the SR Cl^- channel from either cardiac or skeletal muscle have been previously described by this [26] and other laboratories [11, 30]. This channel is believed to be derived from the SR membrane since it has often, if not always, been associated with hte large conducting Ca^{2+} release channel recognized as the ryanodine receptor [24–25]. However the physiological role and regulation of the SR Cl^- channel remains obscure and needs to be clarified. It is speculated that the net Cl^- current across the SR membrane might represent one of the counter charge-transport during rapid Ca^{2+} release and uptake that characterizes the contractile cycle in cardiac cells. Up to now, the anion selective pathway of the SR membrane system which is quite different from the sarcolemmal Cl^- selective channels (2, 21) has not been under extensive study.

The planar lipid bilayer (PLB) technique is a convenient approach to probe ion channels from intracellular membranes which are not readily accessible by other electrophysiological methods [18]. The fusion of native SR vesicles are of interest since other specific membrane components are also transferred into the experimental bilayers along with channel proteins. The careful choice of experimental conditions allow us to record the activity of various types of ion-selective pathways [27].

Using this methodological approach, Kawano et al. [11] have reported that the activity of hte SR Cl^- chan-

nels from swine heart undergoes spontaneous deactivation and that a PKA-dependent phosphorylation was able to induce the reactivation of this channel under steady state conditions. Recently, the same group reported that the SR Cl^- channel was inhibited by the Ca^{2+}-calmodulin complex [12]. However, we had previously reported that the gating and conducting behaviour of the Cl^- channel was insensitive to a large range of free $[Ca^{2+}]$: 0.1 μM to 4 mM [26].

In the present report, we specify the SR localization of this channel and its biophysical properties under various experimental conditions. We confirm its susceptibility to spontaneous inactivation, and describe for the first time a voltage-dependent reactivation and deactivation process. Since we were not able to prove that the direct phosphorylation of the channel was responsible for its upregulation, we hypothesized an interaction of the Cl^- channel with a phosphorylated component of the SR membrane. Taking into account that phospholamban (PL) molecules in their dephosphorylated form down regulate the activity of Ca^{2+}-Mg^{2+} ATPase [28] and that their phosphorylated form do not bind to the Ca^{2+} pump [3, 6], it was of interest to investigate whether the PL molecules might also interact with other ion transporters of the SR membrane. Using a highly specific monoclonal antiphospholamban antibody we now report a consistent and positive interaction of phospholamban molecules with the SR Cl^- channel. Together, these observations support the concept that phospholamban molecules might represent a toggle switch between Ca^{2+} uptake supported by the Ca^{2+}-Mg^{2+}-ATPase and the necessary counter-charge mechanism that woud be sustained by the parallel anions flux.

A preliminary report of some of these findings has appeared elsewhere [4].

Material and Methods

Preparation of Sarcoplasmic Reticulum Vesicles

Microseomal fractions enriched in SR vesicles were prepared from canine heart using a method derived from the procedure initially proposed by Meissner and Henderson [17]. Canine hearts were obtained from mongrel dogs anesthetized with pentobarbital (40 mg/kg, i.v.) in accordance with Canadian and international standards for animal use. Sheep hearts, recovered from healthy animals, were obtained from the local slaughterhouse. Briefly, right and left ventricles were homogenized in a buffer containing (mM): 300 sucrose, 20 K-HEPES, 3 K-EGTA, 0.1 di-isopropylfluorophosphate, 2 dithiothreitol, 1 ascorbic acid, plus 1 μM pepstatin, 1 μM leupeptin and 2.5 UI/ml aprotinin, pH 7.4, and centrifuged at 7500 rpm for 20 min (7,500 × g) at 4°C in a Ti/6 rotor (Beckman). The supernatant was filtered through two layers of cheesecloth and centrifuged at 33,000 rpm for 1 hr 20 min (90,000 × g) in a Ti 35 rotor (Beckman). The pellet was resuspended in a buffer containing (mM): 300 sucrose, 400 KCl, 1 K-EGTA, 5 K-PIPES, 0.1 $MgCl_2$, pH 6.8. This crude fraction was either stored at −85°C or centrifuged overnight at 24,000 rpm (80,000 × g) through a discontinuous sucrose gradient 25, 30, 35, 40 and 45% w/w in a SW 28 rotor (Beckman). Six fractions were recovered from the sucrose gradient, diluted in a 1.5 volume of 0.4 M KCl, 5 mM K-PIPES, 0.1 mM EGTA, 0.1 mM $CaCl_2$, pH 7.0 and sedimented by centrifugation at 40,000 rpm for 1 hr 20 min (120,000 × g). The pellets wre resuspended in 300 mM sucrose and 20 mM Tris-maleate, pH 6.8, quickly frozen in liquid nitrogen and stored at −85°C, until used [15].

Biochemical Assays

Protein concentrations were determined by the Lowry method [14] using bovine serum albumin as a standard. Ryanodine binding assays were performed using the procedure previously described [15] with slight modifications. Briefly, SR vesicles (100 μg of protein) from each fraction were incubated in the presence of $[^3H]$ryanodine (with or without nonradioactive ryanodine) for 90 min at 37°C in the presence of 50 μM free Ca^{2+}. The mixture was then centrifuged twice and pellet radioactivity was determined by liquid scintillation. Specific $[^3H]$ryanodine binding was calculated by subtracting nonspecific binding (10 nM $[^3H]$ryanodine + 10 μM ryanodine) from total binding (10 nM $[^3H]$ryanodine alone). Free Ca^{2+} concentrations were calculated using apparent stability constants of $1.543\ 10^{-7}$ pH 7.4 for Ca-EGTA buffers along with computer programs published by Fabiato [7]. Mg^{2+} and ATP concentrations were also taken into account when these compounds were used. Western Blot analysis were performed after protein separation on 12% SDS-PAGE and transferred on nitrocellulose, typically 2 hr at 70 V and 4°C. The primary antibody was a mouse antiphospholamban (monoclonal, IgG_1) raised against canine cardiac phospholamban. The secondary antibody was an anti mouse IgG_1 coupled to horse radish peroxydase (HRP).

Chemical Reagents

Trizma base (Tris), HEPES (N-2-Hydroxyathylpiperazine-N′-2-ethanesulfonic acid), PIPES (Piperazine-N,N′-Bis(2-ethanesulfonic acid), protease inhibitors, protein kinase A and alkaline phosphatase were obtaned from Sigma (St. Louis, MO.). The isoform of bovine lung cyclic GMP-dependent kinase (PKG) used int this study was a gift from Dr. J. Huggings (Marion Merrell Dow, Strasbourg, France). Both PKA and PKG activities have been tested using *in vitro* phosphorylation assays prior planar lipid bilayer measurements. The monoclonal antiphospholamban antibody raised in mouse against the canine cardiac phospholamban isoform, (monoclonal IgG 1 — lot # 12255) was purchased from UBI, Lake Placid, NY. The radioligands used for binding assays were purchased from New England Nuclear (NEN-Dupont Canada). All other materials were of reagent grade. Deionized water from a Millipore Ro-Milli-Q-UF system (18 ± 0.2 M Ω/cm²) was used for preparing all buffer solutions.

Bilayer Formation and Vesicle Fusion

The planar lipid bilayers (PLB) were formed at room temperature from a lipid mixture containing phosphatidylethanolamine, phosphatidylserine and diphytanoylphosphatidyl choline in a ratio of 3:2:1 [24, 27]. The final lipid concentration was 25 mg/ml dissolved in decane. A 250 μm diameter hole, drilled in a DELRIN cup, was pretreated with the same lipid mixture dissolved in chloroform. Using a Teflon stick, a drop of the decane lipid mixture was gently spread across the aperture in order to obtain an artificial membrane. Membrane thinning was assayed by applying a triangular wave test pulse. Typical capacitance values were 150–300 pF. Aliquots of SR vesicles (10–60 μg of protein) were added to the cis chamber in the proximity of the bilayer.

The cis- and trans-chamber contained (in mM): 250/50 CsCl, respectively, plus 2 $CaCl_2$, 20 Tris-HEPES, pH 7.4. The fusions were either spontaneous, induced by stirring, or by applying negative holding potentials across the bilayer [26]. Followign a fusion event resulting in single or multiple channel incorporation, subsequent fusions were prevented either by lowering the free $[Ca^{2+}]$ cis to 10 μM (by addition of Tris-EGTA in the cis chamber) or by neutralizing the CsCl gradient.

Recording Instrumentation and Statistical Analysis

The currents were recorded using a low noise amplifier (Dagan 3900). The currents were then filtered (cut off frequency 5 kHz) and recorded on a digital audio tape recorder through a pulse code modulation device (75 ES-SONY, Unitrade). The currents were simultaneously displayed online on a chart recorder (DASH II MT, Astro Med.) and an oscilloscope (Kikusui, 5020A). Current recordings wre played back, filtered at 550 Hz and sampled at 2 kHz for storage on hard disk and for further analysis using a DTK-FEAT-5030 computer with programs kindly provided by Dr. M. Nelson, University of Vermont. The open probability values, (P_o) were determined from data stored in 40 to 90 sec-duration files unless specified otherwise, and the half-threshold discriminator method was used. Applied voltages were defined with respect to the cis-chamber, which was held at virtual ground. All bilayer experiments were performed at room temperature (20 ± 2°C).

The average values are given as means ± SEM and n represents the number of measurements. Regression curves were calculated by the least squares methods with the Windows version of the Sigma Plot program from Jandel Scientific.

Results

Steady-State Behavior and Electrophysiological Properties of the SR Cl⁻ Channel

Either single or multiple SR Cl⁻ channel activities were recorded in asymmetric CsCl concentrations (50 mM trans/250 mM cis pH 7.4) which represent our standard experimental conditions. Figure 1*A* shows unitary currents measured at various steady-state holding potentials. Although it is known that Cs^+ ion may permeate through the SR Ca^{2+} release channel (characterized as the ryanodine receptor) and generate outward currents at 0 mV in asymmetrical CsCl buffer 50 mM trans/250 mM cis [31], it is possible to record the activity of anion-selective channels either in choline chloride or CsCl buffer solutions [11, 26]. At a cut off frequency of 550 Hz, steady-state current traces showed a good resolution of the transition between the main open and closed levels. Under these experimental conditions, the current amplitude was minimal for potentials close to 30 mV. Below this voltage, the ionic currents shown as downward deflections flowed from the cis to the trans chamber. The current voltage relationships were obtained under various asymmetrical CsCl⁻ gradients (*see* Fig. 1*B*) or in symmetrical condition (250 mM trans/250 mM cis CsCl). The analysis of the unitary conductances revealed that they were dependent on Cl⁻ concentrations below 100 mM [Cl⁻] trans.

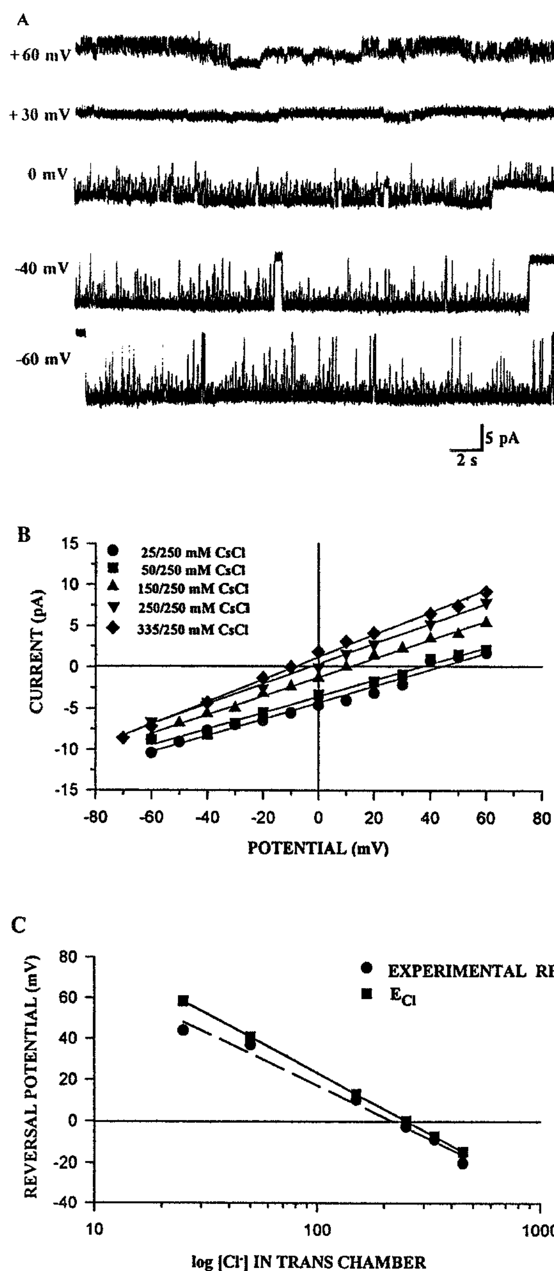

Fig. 1. Channel activity and conducting properties of the cardiac SR Cl⁻ channel. (*A*) Unitary current traces were recorded at various holding potentials (HP). The experimental conditions were (in mM): 50 CsCl⁻ trans/250 CsCl cis, 2 $CaCl_2$, 20 Tris-HEPES, pH 7.4. The zero current potential was just above +30 mV. Current fluctuations were shown as downward deflections for voltages below the reversal potential. (*B*) Mean *I/V* curves of the Cl⁻ channel determined in the presence of various $[Cl^-]_{trans}$- from 25 to 335 mM-, while that of the cis side was maintained at 250 mM. The respective conductances (in pS) were: ● 95.2 ± 5 (n = 3), ■ 91.7 ± 5.1 (n = 7), ▲ 114.3 ± 1.8 (n = 3), ▼ 121.3 ± 8.4 (n = 4), ♦ 135.8 ± 7.2 (n = 3). Error bars were omitted on this graph for clarity. (*C*) Semilogarithmic plot of the theoretical and experimental reversal potential (RP) as a function of $[Cl^-]_{trans}$. E_{Cl} was calculated according to the Nernst equation: E_{Cl} = 58.17 log ($[Cl^-]_{cis}/[Cl^-]_{trans}$). Experimental RP were obtained from the *I/V* curves (panel *B*). The slope values of the regression lines were respectively −58.18 (■) and −51.31 (●).

However the slope conductance value was similar across the range of high trans Cl^- concentrations (>100 mM; cis $[Cl^-]$ was maintained at 250 mM). Furthermore, the value and the shift of the experimental reversal potentials (zero current voltage) argue in favour of a Cl^- selectivity for this anion conducting channel. Figure 1*C* illustrates the variation of the reversal potential as a function of the various trans Cl^- concentrations used in different sets of experiments. The theoretical RP values were calculated according to the Nernst equation. The experimental reversal potential values were derived from the results in Fig. 1*B* and reported in a semilogarithmic plot; the slope coefficients were similar although not identical; −58.17 and −51.31 mV per decade, respectively. To facilitate the resolution of the unit current fluctuations, most of our experiments were performed in the presence of high Cl^- concentrations. However, a series of experiments was conducted at lower Cl^- concentrations and larger holding potentials (−100 to +100 mV) to determine the unitary conductance under physiological intracellular $[Cl^-]$: 5 to 25 mM in cardiac cells [1]. The results obtained in such experimental conditions (5 mM trans/25 mM cis) yielded a mean conductance value of 11 ± 1 pS (n = 3).

Voltage Dependence of the Open Probability

Our methodological approach allowed us to address the direct effects of the transmembrane voltage on activity of the SR channels. Data from steady state activated channels were collected from several single channel experiments performed under identical experimental conditions. As reported in Fig. 2*A*, the open probability (P_o)of the cardiac SR Cl^--selective channel was very high ($P_o \geqslant 0.90$) at negative voltage decreased for holding potentials toward 0 mV and remained above 0.45 for positive voltages. Despite some scattering, this voltage dependency summarized the steady-state behavior of the Sr Cl^- channel. Due to a small current amplitude, it was not possible to determine reliable P_o values for holding potentials close to the equilibrium potential for Cl^- ions, (+40 mV in 50 mM/250 mM CsCl). However, P_o values calculated at +60 mV attest that this channel might remain active over a large range of voltage.

We did observe variability in the gating behaviors of these Cl^- channels which also displayed a rapid flickering mode, instead of the slow one, characterized by long lasting open events as illustrated in Fig. 1*A*. Nevertheless, the voltage-dependent behavior of this fast-fluctuating Cl^- channel remained similar. These channels showed high P_o values for long periods of time, but we also recorded long-lasting inactivations of the cardiac SR Cl^- channel. These spontaneous inactivations of either single or multiple channel recordings at constant voltage were observed at various time intervals, ranging from 0.5 to 7 min (Fig. 2*B*). Kawano et al. [11] already reported such inactivations in swine SR preparations, which occurred after 20 min during long-lasting PLB recordings. In some experiments, careful scrutinizing of the recordings revealed that the complete inactivation was preceded by a transient behavior involving an increase in the number of long closed states. To get some insight concerning the inactivation process the gating behavior of the channel was challenged by various experimental protocols involving either voltage clamp steps or immunochemistry.

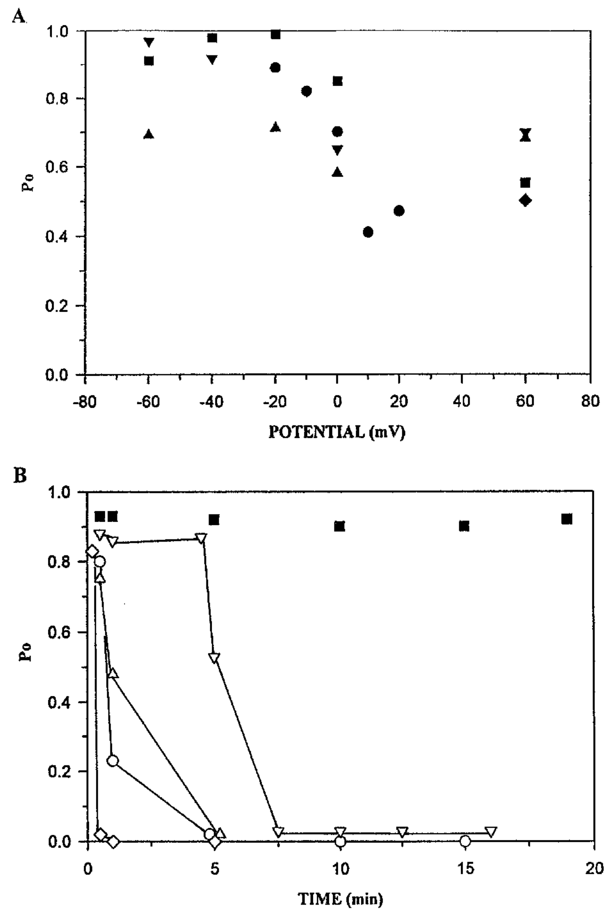

Fig. 2. Voltage and time dependence of the SR Cl^- channel open probability. (*A*) Data derived from four independent experiments under identical conditions, were combined on the same graph. Steady-state P_o values were calculated from 40- to 90-sec files for each potential. The open probability (P_o) of steady-state activated SR Cl^- channels was slightly voltage-dependent. P_o decreased as the holding potential increased, but remained above 0.45. (*B*) Time dependent variation of the open probability at −40 mV. Filled squares represent a channel that remained active for a long time, up to 19 min in this case. The other data points (open symbols) are measurements derived from 4 representative experiments where SR Cl^- channels inactivate at various time intervals. P_o values were determined from 30-sec digital files prior, during and after changes in gating behavior.

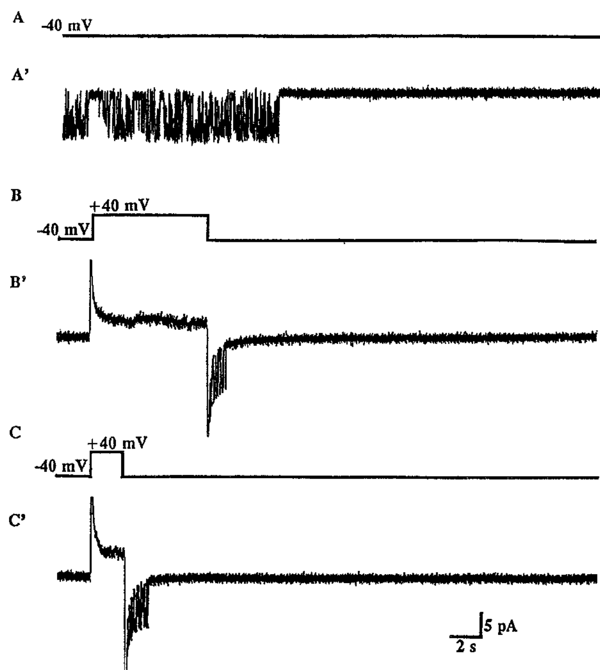

Fig. 3. Spontaneous inactivation, voltage dependent reactivation and deactivation of the SR Cl⁻ channel. In our standard experimental conditions (*see* Fig. 1*A*), the Cl⁻ channel could inactivate spontaneously at constant voltage: −40 mV (*A'*). It remained in this state until it was reactivated by long (*B'*) or short (*C'*) depolarizing voltage steps. For this recording, the reversal potential was estimated at +35 mV, thus the amplitude of the unitary current during the test pulses at +40mV was less than 1 pA. The channel deactivated rapidly when the potential was returned to the initial HP (*B',C'*). *A, B, C:* show the sequential voltage protocol. *A', B', C':* corresponding current traces. The large capacitive transients were partially wiped out, however the small leak current was not substrated.

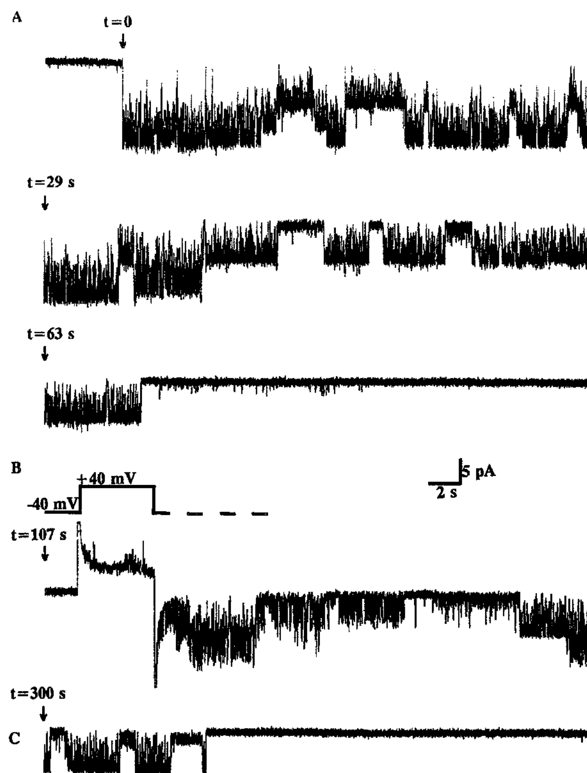

Fig. 4. Steady-state and voltage dependent behavior of a multiple channel recording under phosphorylating conditions. (*A*) Fusion of a vesicle (*t* = *0*) containing two active Cl⁻ channels (HP = −40 mV) in asymmetrical condition 50/250 mM CsCl trans/cis. However, the cis chamber contained 2 mM ATP-Mg and 10μg/ml Protein Kinase A. Even in the presence of a phosphorylation cocktail, the channels inactivate within 1 min, as illustrated in these sequential traces. (*B*) Voltage-dependent reactivation of the channels (*t* = 107 *sec*) followed by a long deactivation process of the Cl⁻ channel activities. (*C*) Complete channel deactivation. We conclude that, when compared to the channel behavior reported in Fig. 3, *B'* and *C'*, the deactivation process was delayed in the presence of the phosphorylation cocktail. Mean deactivation time was 20 sec instead of 1.5 sec in control conditions.

Inactivation, Voltage Dependent Reactivation and Deactivation

To test the status of the inactivated Cl⁻ channel, we performed a series of experiments on single- and multiple-inactivating channels. Figure 3 illustrates the inactivation of a SR Cl⁻ channel previously activated in standard experimental conditions and in the absence of any exogenous phosphorylation cocktail, G protein-activators, inhibitors or Ca^{2+} chelating agents. The presence of long-lasting closed states could be observed before complete inactivation occurred at −40 mV (upper trace). Upon 5 or 2 sec-depolarizing voltage-steps toward positive potentials (middle and lower traces, respectively), the channel reactivated within a few hundred milliseconds, then deactivated within a few sec after the end of the pulses and generally remained in this nonconducting state for long periods of time (exceeding 35 min). Despite the small amplitude of the unitary Cl⁻ current at positive holding potentials close to the zero current voltage value, consistent reactivations of the Cl⁻ channel were obtained with depolarizing voltage steps exceeding 0 mV.

Alternatively, we investigated channel behavior under phosphorylation conditions since it was previously reported that channel activity might be upregulated by the addition of a phosphorylation cocktail in the cis chamber corresponding to the cytoplasmic side of the conducting protein [11, 26]. However, we must emphasize that we have never been able to reactivate the inactivated SR Cl⁻ channel upon addition of the phosphorylation cocktail (2 mM ATP-Mg and 10 IU/ml of α catalytic subunit of PKA, ± 50 μM cAMP) under steady-state conditions (n = 11). In contrast, Fig. 4 shows that upon a single vesicle fusion (*t* = 0), two Cl⁻ channels fluctuate into the PLB; subsequently they inactivated (Fig. 4*A*,

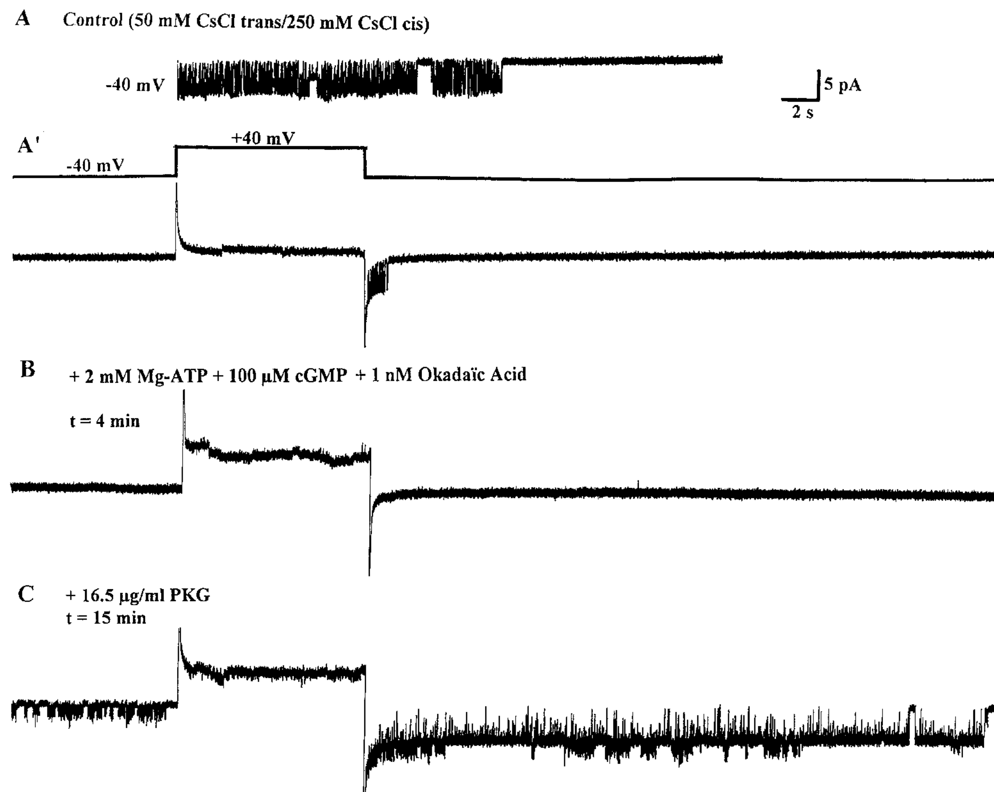

Fig. 5. Effect of the PKG-dependent phosphorylation on the inactivated channel in the presence of okadaic acid. (*A*) In our standard experimental condition, a single channel inactivated at negative steady-state voltage where it is known to maintain high P_o. The channel was subsequently reactivated during a positive square voltage pulse and deactivated at the end of the test pulse as already shown in Fig. 3. (*B*) The addition of 2 mM Mg-ATP + 100 µM cGMP in the presence of 1 mM okadaic acid had basically no effect. (*C*) The addition of PKG facilitated reactivation of the channel. It also increased the deactivation time and modified its gating behavior. The mean deactivation times were 2 ± 0.8 sec and 19 ± 2.4 sec. (n = 13 and 9) for control and PKG dependent-phosphorylating conditions, respectively. These results suggest that a phosphoprotein could be involved in the regulation of the SR Cl⁻ channel kinetics.

second and third traces) despite the presence of the phosphorylation mixture (*see* figure legend). Of interest was the fact that the Cl⁻ channels could be reactivated by a large depolarizing voltage step (as shown in Fig. 4*B*). Later on, the Cl⁻ channels deactivated slowly in the presence of the phosphorylating mixture. To reconcile current results with the conclusions proposed by Kawano et al. [11], we can argue that the presence of the phosphorylation cocktail might help to delay the deactivation process, at least in this specific experiment. At this point we should point out that we performed a series of experiments (n = 7) involving the use of a nonspecific protein phosphatase in order to induce the inactivation of the active channel that might have been phosphorylated. The addition of alkaline phosphatase in the cis chamber had no downregulating effect on the gating behavior of the cardiac SR Cl⁻ channel. Since maximum activity of the alkaline phosphatase is obtained at pH 9, we first had to raise the pH from 7.4 to 9. This step did not affect the P_o and gating behavior of the channel. Consequently, we were able to verify that the Cl⁻ channel activity was poorly pH-dependent, as previously reported [26].

To evaluate the putative involvement of a phosphorylation step mediated by cyclic GMP-dependent protein kinase (PKG) in the upregulation of the channel, a series of *in vitro* phosphorylation experiments were performed (n = 9) on inactivated channels. As illustrated in Fig. 5*A-C*, the addition of a phosphorylation cocktail in the presence of PKG and 1 nM okadaic acid, a potent inhibitor of protein phosphatase 1 and 2A, did not reactivate the SR Cl⁻ channel. However, the channels were consistently reactivated by long-lasting voltage steps. A few minutes after the addition of the phosphorylation cocktail, reactivation of the channel occurred earlier during the test pulse, while deactivation was slowed down (Fig. 5*C*) comparatively to the reactivation/deactivation phenomena observed under control conditions (Fig. 5*A′*). It appears that the gating behavior of the SR Cl⁻ channel was modified by the addition of PKG, cGMP and Mg-ATP. These observations do not prove that the channel protein is phosphorylated by PKG. Nevertheless, since the deactivation process was delayed by both PKG (and PKA) phosphorylation cocktails, resulting in 10-fold increase of the total deactivation time, it is thought

that a phosphoprotein might be involved in channel regulation.

Together, these results (Figs. 3, 4 and 5) strongly suggest that the gating behavior of the cardiac SR Cl⁻ channel is not directly controlled by a phosphorylation-dephosphorylation mechanism. However it is plausible that, during the cotractile cycles, the channel might interact with other protein of the SR membrane. Since we did observe that SR Cl⁻ channels could inactivate spontaneously and that phosphorylating conditions slow down the deactivation process at the end of voltage pulses, an association-dissociation mechanism with a phosphorylated SR protein was postulated. We hypothesized that phospholamban might be a likely candidate. This small molecular weight SR protein can be simultaneously transferred into PLB with other membrane proteins of the SR vesicles. Furthermore, this typical SR peptide is suitable for PKG and PKC dependent phosphorylation [3, 23].

Effect of Antiphospholamban Antibody

To test this working hypothesis, a highly specific monoclonal antibody raised against canine cardiac phospholamban was used. Phospholamban was found in our cardiac SR microsomal fractions mainly in the form of pentameres (*data not shown*), thus confirming the results from other laboratories [3, 19]. Figure 6 shows the results obtained from a typical experiment in which graded increases of antiphospholamban antibody (Anti-PL Ab) consistently turned off the SR channel activity. The Anti-PL Ab was tested at two- to four-fold the suggested concentrations normally used to run either radioimmunoassays or western blot experiments. A few minutes after the addition of Anti-PL Ab, the current traces resulted in long lasting nonconducting states, similar to those observed previously, when the channel inactivated. The Anti-PL Ab effect was observed in 8 our of 9 independent experiments. However, the channel displayed sporadic bursts of activity (Fig. 6*B* and 6*C*). These random reactivations were interpreted as transient associations of the SR Cl⁻ channel with other phospholamban molecules, freed of antibody interactions.

In control experiments, the SR Cl⁻ channel activity was challenged with the heat-denatured Anti-PL Ab. In all four experiments, the denatured antibodies had no effect on the steady-state channel activity (Fig. 6*A′* and *B′*) thus suggesting that the inhibition induced by Anti-PL Ab was specifically due to the neutralization of molecular interactions between phospholamban and Cl⁻ channel. Similar results were obtained in a time-dependent manner for a single concentration of Anti-PL Ab on a multiple channel recording as reported in Fig.7. A few minutes after the addition of the Anti-PL Ab, the SR Cl⁻ channels wre inactivated sequentially. This pattern of inhibition suggests that despite their functional clustering, the Cl⁻ channels are independently regulated by PL molecules. Although the channel displayed very short bursts of activity during the time course of the continuous recording of the silent bilayer, this unitary current activity is likely due to the sporadic activaiton of one of the Cl⁻ channels initially present in the artificial membrane and not to a new fusion. No effect of the Anti-PL Ab was delineated upon reconstitution of the SR Cl⁻ channel from the fast twitch rabbit skeletal muscle [25]. This protocol might be considered as a positive control experiment since SR membranes from rapid skeletal muscle are known to be virtually deprived of phospholamban molecules. As a matter of fact, western blot analyses show that the Anti-PL Ab initially raised against

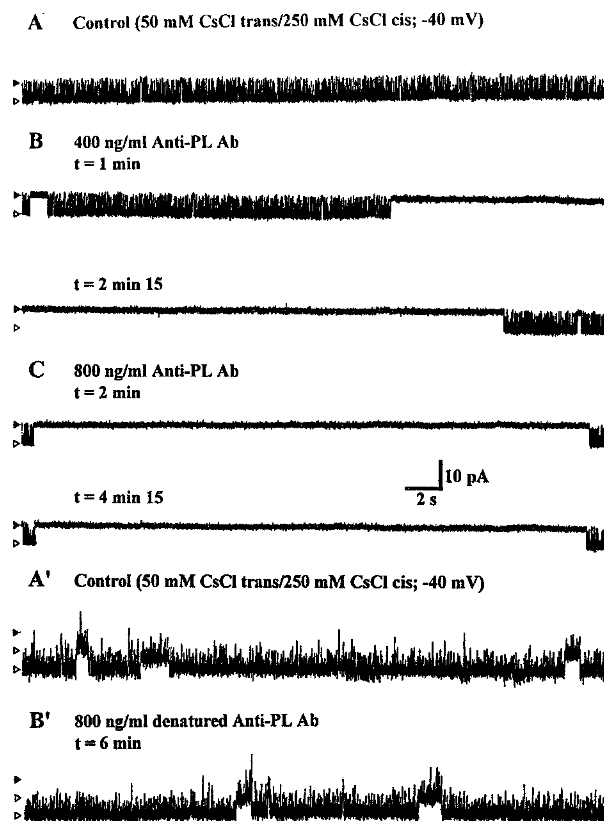

Fig. 6. Effect of anti-phospholamban antibody on the SR Cl⁻ channel activity. We hypothesized that a component of the SR membrane (which may not be the Cl⁻ channel itself) might be involved in the regulation of channel activity. (*A*) The activity of a single SR Cl⁻channel was recorded for up to 11 min in control conditions: 50 mM CsCl trans/250 mM CsCl cis (HP = –40mV). *B* and *C:* Sequential traces were recorded in the presence of graded increases of anti-Phospholamban antibody (Ant-PL Ab) 400 ng/ml and 800 ng/ml, respectively. Addition of Anti-PL Ab induced long-lasting, nonconducting closed states. In *A′* and *B′*, control experiments showed that the heat-denatured Anti-PL Ab (10 min at 95°C) had no effect on multiple channel activity. ▶ zero current levels; ▷open state levels. *t:* indicates the time after the sequential addition of the antibody.

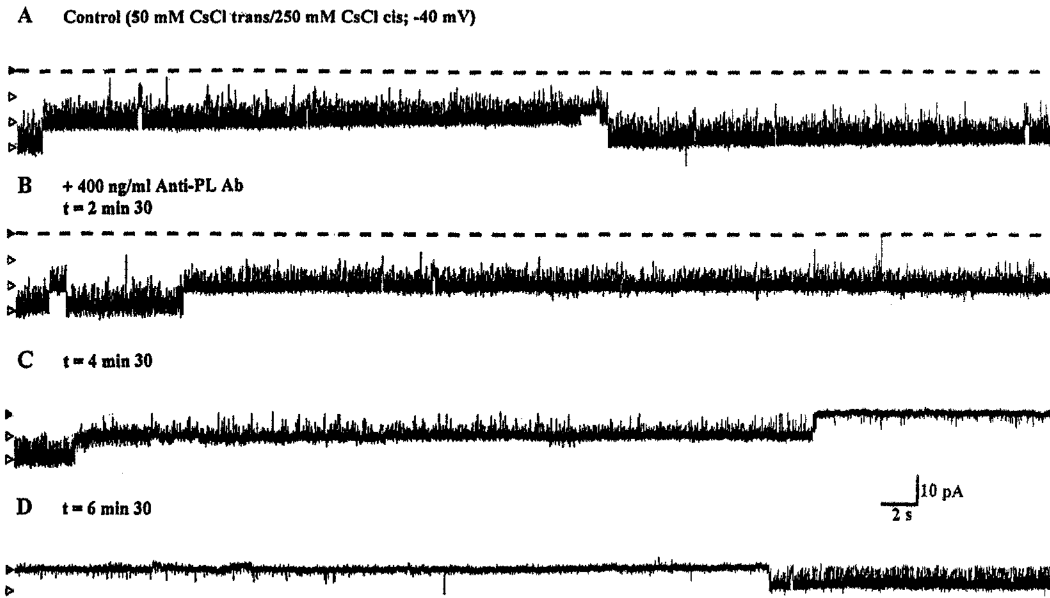

Fig. 7. Time-dependent effect of antiphospholamban antibody on multichannel activity. (*A*) Multichannel recording in control conditions (50 mM CsCl trans/250 mM CsCl cis, HP = −40 mV). Three channels were functional into this PLB for up to 12 min. (*B*) 2 min 30 sec after addition of 400 ng/ml of Anti-PL Ab, one channel became silent. (*C*) The two remaining channels closed successively. (*D*) After 2 min without channel activity, one channel transiently reactivates. ▶ zero current levels; ▷open state levels. *t:* indicates the time after the sequential addition of the antibody.

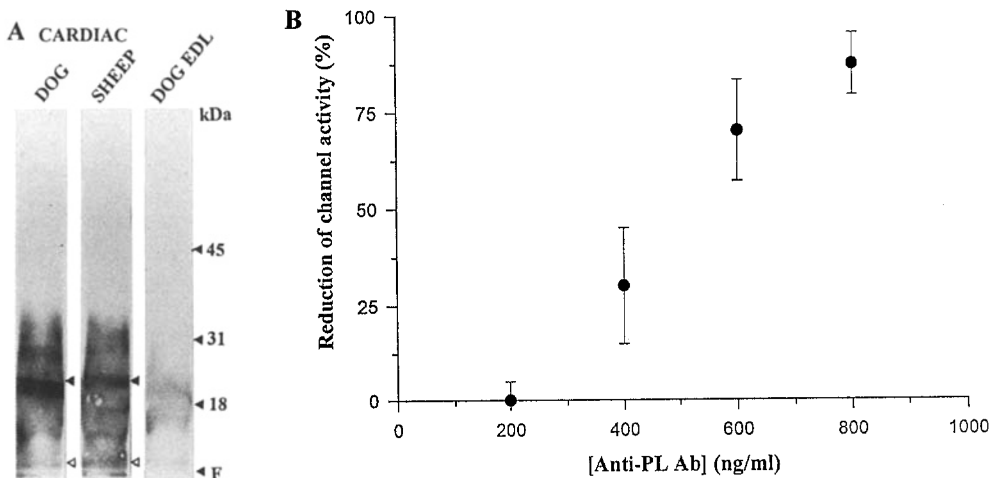

Fig. 8. Western blot analysis and dose-dependent effect of the Anti-Phospholamban antibody on the SR Ca^{2+} channel activity. (*A*) Immunoblot of various SR fractions using a monoclonal Antiphospholamban antibody. Lanes 1 and 2 show the immunoreactivity with a major protein band (≃ 28 kDa dark arrows) for dog and sheep cardiac SR, respectively. Open arrows: monomeric phospholamban. Lane 3 contained SR proteins derived from canine Extensor Digitarium Longus (EDL), which is essentially a fast twitch muscle. This fraction is deprived of immunoreactivity. Left column: molecular weight standards. (*B*) SR vesicles derived from sheep ventricular cells were prepared as described in the Materials and Methods section and fused into PLB. Cl⁻ channel activities were recorded in the absence (control) and the presence of various amounts of Anti PL antibody. Average reduction in channel activity as a function of the Anti-PL Ab concentration values were determined from three independent files and expressed as percentage of decrease ± SEM of total channel activities under control condition.

dog PL-isoform recognize various highly conserved PL isoforms among several species including sheep (Fig. 8*A*). However, Anti-PL does not recognize any protein in the SR fraction derived from fast twitch muscles (Fig. 8*A*, 3rd lane, canine EDL).

To ascertain the involvement of PL molecules in the functional modulation of the steady-state activated cardiac SR Cl⁻ channels from various species, the dose-dependent effect of the Anti-PL Ab was quantified from multiple channel recordings upon reconstitution of sheep SR proteins into PLB. Figure 8*B* illustrates the concentration-dependent inhibition of the SR Cl⁻ channel by

monoclonal Anti-PL Ab upon sequential addition. Cumulative concentrations of Anti-PL Ab in the cis chamber (cytoplasmic side) induced dose-dependent increases in teh duration of the long nonconducting states, causing significant decreases in P_o and subsequently in the total current. The inhibitory effect was quantified from digital recordings in the presence of various concentrations of the monoclonal antibody. It was determined that 500 ng/ml of antibody produces approximately a 50% inhibition, indicating that several of PL molecules might interact with SR Cl^- channels.

Upon reconstitution into PLB, cardiac SR Cl^- channels might dissociate from PL molecules leading to channel inactivation. Since the channel could be systematically reactivated by voltage pulses, a voltage-dependent associaton between the two SR membrane proteins was postulated. To prove that a voltage-dependent association mechanism between the SR Cl^- channel and the PL might be altered, experiments with both the addition of Anti-PL Ab in the cis chambers and voltage pulses were performed. As shown in Fig. 9, in which we first verified that voltage steps did not modify the gating behavior of the steady-state activated channel (Fig. 9*B*), channel activity was decreased by cumulative addition of Anti-PL Ab (Fig. 9*C, D* and *E*). Complete channel inhibition (inactivation) followed changes in bursting pattern as illustrated in Fig. 9*E*. Under these conditions, voltage steps failed to reactivate the channel despite the presence of very brief events attesting that the channel was still present in the PLB. Consequently, it was suggested that the binding of Anti-PL Ab on its epitope induces the dissociation of PL from the Cl^- channel and prevents the steady-state association as well as voltage-dependent reassociation with the channel protein. We have also verified that the Anti-PL Ab does not affect the behavior of another cardiac SR channel, the large conducting K^+-selective channel [18, 31]. In three out of three experiments, the Anti-PL Ab did not modify the gating nor the conducting behavior of either single or multiple channel recordings. Moreover, SR Cl^- channels are not affected by the addition of polyclonal antibodies initially raised against the cardiac ryanodine receptor-Ca^{2+} release channel (*data not shown*).

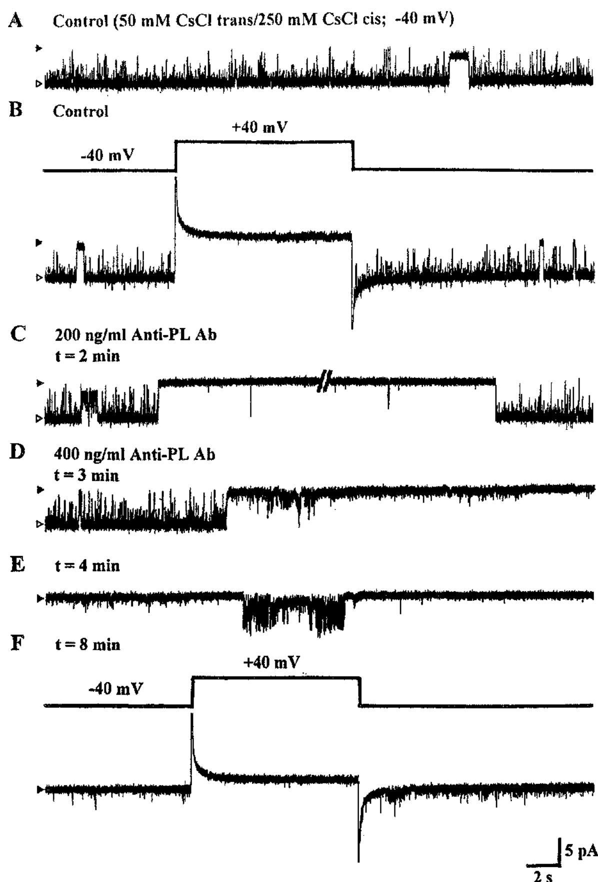

Fig. 9. Addition of antiphospholamban antibody prevents the reactivation of the Cl^- channel during voltage pulses. (*A*) A single canine Cl^- channel was incorporated in our standard condition as in Fig. 1. While the channel displayed a high P_o at −40mV its voltage-dependent behavior tested as illustrated in *B, C, D* and *E:* Upon subsequent addition of Anti-PL Ab, the channel activity displayed long lasting-inactivated-closed state as described earlier (Fig. 6, 7 and 8). (*F*) the application of square voltage pulses 8 min after the addition of 400 ng of Anti-PL Ab failed to reactivate the silent Cl^- channel thus suggesting that the Anti-PL Ab prevented the positive interaction of PL molecules with the SR Cl^- channel. This experimental data is representative of the results obtained from seven similar experiments.

Discussion

This study defines the conducting properties as well as the voltage- and time-dependent behavior of the cardiac SR Cl^- channel under various experimental conditions. The most important contribution of the present work was to uncover, for the first time, that the SR Cl^- channel might be regulated by cardiac phospholamban molecules. Using a monoclonal phospholamban-antibody we have obtained solid evidences that phospholamban molecules, simultaneously transferred with the SR Cl^- channels into the artificial membrane, modulate the gating of the anion selective channel.

Basic Properties of the Cl^- Channel

Despite several lines of indirect evidence obtained in varous laboratories [11, 25-26, 30], we have confirmed that the anion-selective channel under investigation was an SR channel. Although our experimental approach might not represent the ultimate proof for the specific SR localization of the Cl^- channel, most of the recordings obtained in KCl buffer do result in a dual K^+ and Cl^- channel activity upon single vesicle fusion. The SR K^+ channel from cardiac [10] and skeletal muscles [29]

preparations are well characterized. The use of CsCl gradients was justified in neutralizing most of the contaminating currents through cation selective channels. Furthermore, in our reconstitution system, all biochemical and immunological experiments were performed at negative voltage (–40 mV), close to the Cs^+ equilibrium potential, to minimize the contribution of cationic current either through SR K^+ [18] or Ca^{2+}-release channels [27].

Our measurements have shown a close similarity between the theoretical equilibrium potentials calculated for the different Cl^--gradients and the corresponding experimental zero-current potential values (FIg. 1*B* and *C*). This would argue in favor of the Cl^--selectivity over Cs^+ for this ionic pore. Since the slope coefficients of the linear regression (Fig. 1*C*) wre not identical, one might postulate that other anions ($HEPES^-$) could compete for the selective site and eventually permeate the channel, although a residual monovalent cation permeability cannot be ruled out. When Cl^- concentrations in the physiological range for cardiac cells are used –5 to 25 mM—according to Caillé [1], the SR Cl^- channel displays a mean conductance value of 11 ± 1 pS.

Voltage Dependency of the SR Cl^- Channel

The voltage-dependent behavior of the cardiac SR Cl^- channel ws carefully analyzed. The P_o, determined for channels activated under steady-state conditions, was sensitive to the potential applied across the artificial membrane (Fig. 2). However, channel activity was characterized by high P_o values. This observation is in agreement with the low-voltage sensitivity reported previously for the SR Cl^- channel isolated from swine heart [11]. However, it is at variance with the bell-shape relationship reported for the skeletal muscle isoform studied either in PLB or in sarcoball preparations [9, 30]. Our previous studies involving the reconstitution and analysis of the SR K^+ channels, allow us to point out that the voltage dependency of its open probability [27] is basically the mirror image of the relationship determined for the SR Cl^- channel (*see* Fig. 2*A*). The crossing-over between the two P_o/voltage curves occurs at +5 mV which, in the absence of direct electrophysiological measurements in cardiac SR, correspond to the best estimates for SR membrane potential (E_{SR} = 0 to +7 mV) in skeletal muscle [22, 29]. In fact, isotopic flux measurements have demonstrated the large permeability of the SR vesicles for monovalent cations and anions [16]. These observations support the concept that, *in situ,* the SR membrane is not highly polarized, although one cannot rule out that the large Ca^{2+} fluxes might generate variations of membrane potential. However, the extent of the membrane potential variations across the SR membrane is currently unknown. The large conductance to monovalent ions would maintain the SR potential away from the Ca^{2+} equilibrium potential, thus facilitating the rapid movements of Ca^{2+}.

Modulaton of the Gating Behavior

Aside from their conductance and selectivity, canine and sheep (this study) as well as swine SR Cl^- channels [11] share similarities in their gating properties. All three channel isoforms can inactivate spontaneously after several minutes of activity into the PLB (*see* Fig. 3, 4 and 5). We initially assumed, that the vanishing Cl^- channel activity might result from a change in the micro-environment in the vicinity of the activated channel protein. However 69% of the channels do not inactivate while some of them (15%) display variations in their gating behavior, including longer closed states and shorter open times resulting in a bursting pattern. These kinetic changes have not been quantified in the present report. Nevertheless such variations are readily illustrated in 4 out of the 9 figures presented herein. Of importance is that the depolarizing voltage-steps were systematically able to reactivate the silent-inactivated-channel, as demonstrated in Figs. 3, 4 and 5 as well as in the Table (left column). Using either canine or sheep SR preparations, we were not able however to reproduce the rapid Mg-ATP dependent reactivation of the inactivated Cl^- channel either in the absence or presence of exogenous α catalytic subunit of PKA under steady state conditions as reported previously [11]. It is difficult to give a straight forward explanation to account for the differences of responsiveness to the phosphorylation condition between teh swine [11] and the canine and sheep (this study). Apart from interspecies variations, one might also evoke experimental conditions such as the PLB composition: Kawano et al.[11] used highly negatively charged PLB (PE/PS^-: 1/1) while we currently use a lipid mixture ($PE/PS^-/PC$: 3/2/1) more similar to the lipid composition of the intracellular membrane. Nevertheless, we have observed that the addition of a phosphorylation cocktail on the cytoplasmic face of the Cl^- channel could slow down the deactivation process after the end of the voltage pulses (*see* Figs. 4 and 5). It can also be pointed out that when a voltage-step protocol (similar to the one used in Fig. 3*B* and *C*) was applied to the active SR Cl^- channel (Fig. 9*B*), the P_o was decreased durng the positive voltage pulses but no deactivation was recorded at the pulse offset, suggesting that the SR Cl^- channel might display two distinctive functional gating behaviors. Consequently, our results are consistent with the idea that a phosphorylated SR compound might be involved in the instantaneous regulation of the channel gating. However they are both slightly at variance and complementary to those reported by Kawano et al. [11] who proposed a direct phosphorylation-dephosphorylation of the Cl^- channel protein. We now report the voltage-dependent reactivation and deactivation of the inactivated SR Cl^- channel which may correspond to the association and dissociation of a regulatory subunit. The overall behavior of the cardiac SR Cl^- channel is now sumarized in the Table.

Table 1. Time and voltage dependent behavior of the cardiac SR Cl^- channel

Type of channel activity	Number of experiments	Mean duration of channel activity (min)	% of recording analyzed	Voltage dependent reactivation
Channels displaying a steady state P_o	31/45	17 ± 4 min (n = 31)[a]	69%	Not applicable
Spontaneously inactivating channel	14/45	3 ± 2 min 30 sec (n = 14)[b]	31%	7/7
Inactivated channel + MgATP + PKA	11/11	No reactivation	100%	10/11
PL-induced inactivation	8/9	4 min 30 sec (n = 7)[c]	72%	0/4

65 experiments, performed under identical basic control condition, are classified in this table. Several other experiments were discarded from the analysis since experimental conditions were changed.

[a] This value represents the average duration of steady state recordings, which generally ended by the rupture of the P.L.B.

[b] Average time for the first inactivation following channel fusion, then the channel was reactivated by voltage pulses.

[c] Average value determined for 400 ng of Anti-Pl.AB/ml.

The Phospholamban Connection

Taken together, our data prompted us to look for another mechanism to explain the important changes in gating behavior observed under steady state conditions as well as during voltages pulses.

The specific localization of phospholamban molecules in the SR membrane and the fact that this peptide displays multiple phosphorylation sites, as well as an ability to modulate the activity of Ca^{2+}-Mg^{2+}-ATPase [28] another well characterized ion transporter, lead us to test the effect of a monoclonal antibody raised against canine cardiac PL. The phospholamban-dependent activation of the cardiac Ca^{2+}-pump is explained by a removal of inhibition [3, 28] when phospholamban molecules are phosphorylated by various kinases [20, 23]. It is of interest to note that the phospholamban displays up to three consensus amino acid sequences that might be independently phosphorylated by PKG and/or PKC [6, 23] and thus represent relevant biochemical targets for these kinases under physiological condiitons. Variations in their accessibility might explain why it was impossible to reactivate the SR Cl^- channel under steady state condition in the presence of an adequate phosphorylation cocktail and either PKG or PKA. This was achieved only when the polarity of the holding potential was switched toward positive potentials.

In this context, it was also possible to demonstrate that single or multiple Cl^- channel activities were down-regulated by the addition of sensitive amounts of anti-PL antibody in a dose dependent manner, to the cytoplasmic face (cis side). According to these results, the PL molecules would be able to upregulate the Cl^- channels and Cl^- movement across the SR membrane. The physiological relevance of this interpretation is related to the suspected role of SR Cl^- permeability in the modulation of rapid Ca^{2+} fluxes during the E-C coupling mechanism in cardiac cells. Monovalent anions (mainly Cl^-) and cations (K^+) fluxes might be used as concomitant counter-charge systems during rapid Ca^{2+} uptake and, Ca^{2+} release supported by the ryanodine sensitive-Ca^{2+} release channel. The imperative presence of permeable ions such as K^+ and Cl^- in the experimental buffers used to measure the Ca^{2+} uptake [2] and release [16, 22] is an indirect evidence of this assumption. However, the main physiological role for the monovalent conductances would be to maintain the SR membrane potential away from the Ca^{2+} equilibrium potential, facilitating the rapid and alternative movement of Ca^{2+} across the SR membrane during the beat to beat regulation of the SR function.

Up to now, it was not possible to establish whether cardiac SR Cl^- channels interact with either monomers or pentamers of PL molecules. Native SR membranes essentially contain pentameric PL complexes, but vesicle fusion into PLB might alter both protein-complex stability as well as the degree of interaction with the channel protein. Alternatively, Anti-Pl antibody might bind to the phospholamban-Cl^- channel complex and produce its effects that way; rather than by dissociating the phospholamban from the channel. Finally, the antibody itself might be responsible for influencing the channel activity, although this hypothesis is unlikley according to the Western Blot experiments.

In conclusion, despite the fact that we cannot rule out that other intracellular second messengers might be implicated in the modulation of the SR Cl^- channel [11–12], the present study using the simultaneous reconstitution of multiple native proteins of the SR membrane and a highly specific monoclonal antibody, reveals an unexpected and thus challenging functional interaction between two peptide moieties of the SR membrane and provides further evidences attesting to the biochemical control of the gating mechanism of the SR Cl^- channel in mammalian heart.

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