Surface Charge Potentiates Conduction Through the Cardiac Ryanodine Receptor Channel

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ABSTRACT Single channel currents through cardiac sarcoplasmic reticulum (SR) Ca^{2+} release channels were measured in very low levels of current carrier (e.g., 1 mM Ba^{2+}). The hypothesis that surface charge contributes to these anomalously large single channel currents was tested by changing ionic strength and surface charge density. Channel identity and sidedness was pharmacologically determined. At low ionic strength (20 mM Cs^+), Cs^+ conduction in the lumen → myoplasm (L → M) direction was significantly greater than in the reverse direction (301.7 ± 92.5 vs 59.8 ± 38 pS, $P < 0.001$; mean ± SD, t test). The Cs^+ concentration at which conduction reached half saturation was asymmetric (32 vs 222 mM) and voltage independent. At high ionic strength (400 mM Cs^+), conduction in both direction saturated at 550 ± 32 pS. Further, neutralization of carboxyl groups on the lumenal side of the channel significantly reduced conduction (333.0 ± 22.5 vs 216.2 ± 24.4 pS, $P < 0.002$). These results indicate that negative surface charge exists near the lumenal mouth of the channel but outside the electric field of the membrane. In vivo, this surface charge may potentiate conduction by increasing the local Ca^{2+} concentration and thus act as a preselection filter for this poorly selective channel.

INTRODUCTION

In cardiac muscle, the ryanodine receptor (RyR) protein plays two roles. Structurally, the RyR is a primary component of the feet which physically span the gap between the transverse tubule and sarcoplasmic reticulum (SR) membranes (Wagenknecht, Grassucci, Frank, Saito, Inui, and Fleischer, 1989). Functionally, the RyR is the SR Ca^{2+} release channel (Fleischer, Ogunbunmi, Dixon, and Fleer, 1985; Lai, Erickson, Rousseau, Liu, and Meissner, 1988; Smith, Imagawa, Ma, Fill, Campbell, and Coronado, 1988). Little is known about the RyR's structure-function relationship.

The function of single cardiac RyR channels has been defined in planar lipid bilayer studies (Smith, Coronado, and Meissner, 1985; Fleischer et al., 1985; Smith et

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al., 1988; Lai et al., 1988). However, few specific structural features of the channel are known. In electron micrographs, the RyR appears as a large homotetrameric complex with most of its mass located on the myoplasmic side of the membrane (Wagenknecht et al., 1989; Inui, Saito, and Fleischer, 1987). The three-dimensional architecture of the RyR, deduced from sequential sections, shows that the relatively small portion of the protein which extends through the SR membrane has a surface area of roughly 196 nm^2 and contains the lumenal mouth of the channel (Wagenknecht et al., 1989). A site-directed mutagenesis approach to define more details of this channel's structure-function relationship is not yet possible.

In this study, a biophysical strategy was used to define a specific aspect of the structure-function relationship of the cardiac RyR channel. We show that surface charge exists near the lumenal mouth of the channel by demonstrating that (*a*) abnormally large and asymmetric conduction occurs at low charge carrier concentration; (*b*) screening of surface charge by high ionic strength attenuates conduction; and (*c*) removal of surface charge by esterification of carboxyl groups decreases conduction. The estimated surface charge density ($0.11e \cdot nm^{-2}$) could be produced by 22 ionized carboxyl groups distributed uniformly in an area of 196 nm^2. Alternatively, far fewer charges clustered in a much smaller area could produce the same effect. We propose that surface charge potentiates conduction by causing cations to accumulate near the lumenal mouth of the channel. Thus, the surface charge may act as a preselection filter for this poorly selective Ca^{2+} channel.

MATERIALS AND METHODS

SR Microsome Preparation

Heavy SR microsomes were isolated from dog cardiac muscle as previously described (Tate, Bick, Chu, Winkle, and Entman, 1985). Briefly, the left ventricle from dog hearts were cut into small pieces and homogenized. Microsomes were isolated by differential centrifugation and stored in 0.3 M sucrose, 0.9% NaCl, 10 mM Tris Maleate, PH 6.8 at −70°C until used.

SDS-Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed using 5–15% polyacrylamide gradient gels (Laemmli, 1970). SR microsomes were denatured before electrophoresis and overloaded to visualize the Ca^{2+} release channel (RyR) protein in the gel. Gels were stained with Coomassie blue or Stains-All (King and Morrison, 1976).

Channel Reconstitution and Solutions

Planar lipid bilayers were formed across a 150-μm diameter aperture in the wall of a Delrin (Small Parts Inc., Miami Lakes, FL) cup. The bilayer-forming solution contained a 8:2 (by volume) mixture (50 mg/ml in decane) of phosphatidylethanolamine and phosphatidycholine. Lipids were obtained in chloroform from Avanti Polar Lipids, Inc. (Pelham, AL).

SR vesicles were added to one side of the bilayer (defined as *cis*). The other side was defined as *trans*, and was connected to the virtual ground input of the amplifier. Standard solutions were 350 mM $CsCH_3SO_3$ *cis* (20 or 50 mM *trans*), and 10 mM HEPES (pH 7.4), 20 μM free Ca^{2+} (unless specified). A simple gravity perfusion system was used to exchange bathing solutions. Unless stated, experiments were performed in solutions containing symmetrical $CsCH_3SO_3$. The free [Ca^{2+}] was confirmed by a Ca^{2+} electrode. After each experiment, the

[Cs^+] was confirmed by measuring solution conductivity. In the chemical modification experiments, 1 mM Carbodiimide (EDC) (Pierce Chemical Co., Rockford, IL) and 20 mM CH_3NH_2 were added. Cesium salts were used to isolate the conductance of the SR Ca^{2+} release channel from that of other ionic channels present in SR membranes (Fill, Coronado, Mickelson, Vilven, Ma, Jacobson, and Louis, 1990).

Single-Channel Recording and Analysis

A current/voltage conversion amplifier was designed to optimize single-channel recording from lipid bilayers (Hamilton, Alvarez, Fill, Hawkes, Brush, Schilling, and Stefani, 1989). Acquisition software (pClamp; Axon Instruments, Inc., Burlingame, CA), an IBM compatible 386 computer and a 12 bit A/D-D/A converter (Axon Instruments, Inc.) were used. Single-channel analysis was performed with commercially available software (pClamp, Axon Instruments, Inc.). Data were digitized at 5–10 KHz, filtered at 2 KHz and stored for future analysis. Current amplitudes were determined by individually measuring several long single channel openings and the accuracy of this method was confirmed by gaussian fits to the total amplitude histogram. Conductances were determined from the slope of current-voltage relationships. Data are plotted as means and standard deviation. When appropriate the difference between two means was statistically tested by a two-tailed *t* test (Instat program, Sigma Chemical Co., St. Louis, MO).

RESULTS

SDS-PAGE Characterization of the Cardiac SR Vesicles

The cardiac SR vesicles were characterized using SDS-PAGE analysis. The cardiac RyR, a 564-kD protein (Otsu, Willard, Khanna, Zorzato, Green, and Maclennan, 1990), represents a small fraction of total SR protein. To resolve a RyR band by coomassie blue staining (Fig. 1 *A*), gels were intentionally overloaded. Cardiac SR proteins were run in the lanes marked *C*. Skeletal SR proteins, a gel pattern reference, were run in the lanes marked *S*. The RyR, myosin, SR Ca^{2+} pump (*SRP*), and skeletal isoform of calsequestrin (*) are marked in the margin. Large myosin contamination in the cardiac lanes is consistent with the relatively crude nature of the cardiac SR vesicles used. It was our intent to perform experiments with a crude preparation in order to retain closely associated proteins which may influence RyR channel structure and/or function.

One protein which is thought to be closely associated with the RyR channel protein is calsequestrin (CSQ). CSQ is a low affinity, high capacity Ca^{2+} binding protein. At the Ca^{2+} levels (20 μM) present in our experiments, CSQ would be largely Ca^{2+}-free and negatively charged. Because the focus of our experiments is surface charge, the presence of CSQ could influence the interpretation of our data (see Discussion). To visualize the cardiac CSQ band, a parallel SDS-PAGE calorimetric assay (Stains-All) was used. Stains-All stains Ca^{2+} binding proteins dark blue (marked *) and other proteins light pink. The Stains-All gels (Fig. 1 *B*) show that the skeletal muscle SR vesicles contained large amounts of calsequestrin. However, the cardiac SR vesicles had little (if any) blue staining in the region where the cardiac isoform of CSQ should appear (55 kD). In the experiment shown, 50 μg (total protein) of skeletal and 200 μg of cardiac SR protein were loaded. This suggests that the cardiac SR vesicles used in this study contained very little calsequestrin.

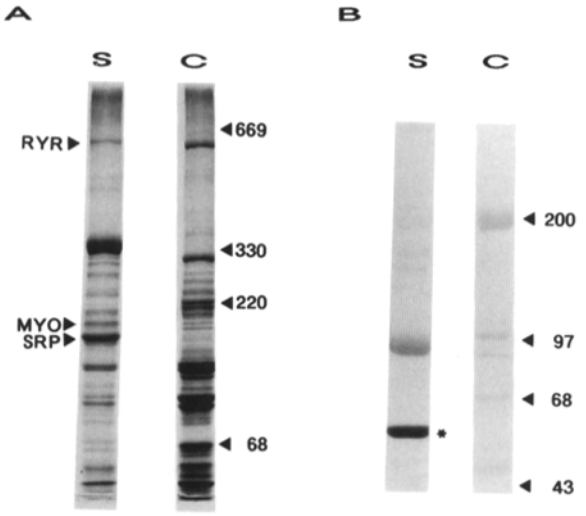

FIGURE 1. SDS-PAGE characterization of SR vesicle preparation. Skeletal and cardiac muscle SR vesicles were loaded in the lanes as marked at the top, *S* and *C*, respectively. Gels were stained by coomasie blue (*left*) and stains-all (*right*). In the stains-all gel, the marked (*) protein stained blue, all others stained light pink.

Pharmacological Characterization of the Channel

Single RyR channel activity was measured using Cs^+ or Ba^{2+} as charge carrier. To identify the channel, characteristic pharmacological properties were verified (Fig. 2). Single-channel activity was Ca^{2+}-dependent (Fig. 2, *A* and *B*). At 1 μM free Ca^{2+}, the open probability (P_o) of the channel was low ($P_o < 0.02$). At 50 μM free Ca^{2+}, channel activity was high ($P_o \approx 0.8$). Additionally, the channels were sensitive to the plant alkaloid ryanodine (Fig. 2 *C*). Ryanodine induced slow gating to a subconductance level, the characteristic action of ryanodine on single RyR channels (Smith et al., 1988). The Ca^{2+} dependence and ryanodine sensitivity confirm the identity of the channel.

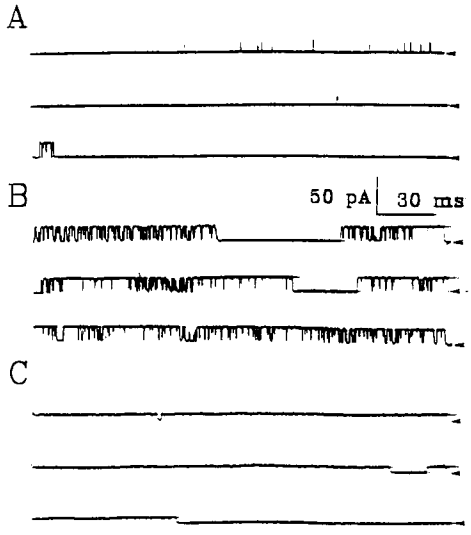

FIGURE 2. Pharmacological identification of the RyR channel. Cs^+ current were measured at 1 μM [Ca^{2+}] (*A*), 50 μM [Ca^{2+}] (*B*), and after addition of ryanodine (10 μM; *C*). *A*, *B*, and *C* have the same scale, the arrows indicate baseline (zero current level).

Because reconstituted RyR channels could be facing either direction in the bilayer, ATP sensitivity was used to determine channel sidedness. ATP is unlikely to cross the bilayer because it is a relatively large and charged molecule. The RyR protein has its ATP binding site on the myoplasmic side of the channel (Palade, Dettbarn, Brunder, Stein, and Hals, 1989; Takeshima, Nishimura, Matsumoto, Ishida, Kangawa, Minamino, Matsuo, Uedo, Hanaoka, Hirose, and Numa, 1989). In these experiments, ATP was added to one then the other side of the bilayer (Fig. 3). When ATP was added to the *trans* side (Fig. 3 *B*), channel activity did not change (0.14 ± 0.11 [n = 8] vs 0.13 ± 0.09 [n = 8]). When ATP was added to the *cis* side (Fig. 3 *A*), single channel P_o significantly increased (control 0.13 ± 0.12 [n = 8]; + ATP 0.48 ± 0.21 [n = 8]; t test $P < 0.002$). Reconstituted channels were sensitive to ATP only when

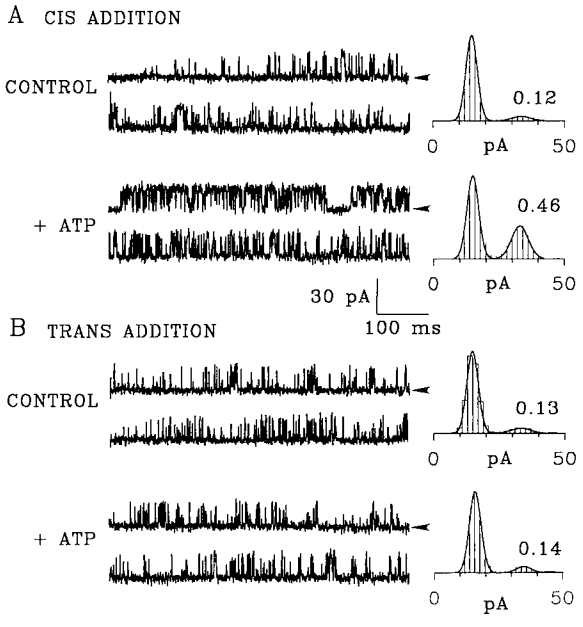

FIGURE 3. Determination of RyR channel sidedness by ATP action. Arrows indicate baseline (zero current level). Representative single channel records are shown before (*control*) and after (+ATP) addition of 2.0 mM ATP. Amplitude histograms generated from 5 min of recording under each condition. Open probability calculated from gaussian fits is indicated for each histogram. (*A*) Application of ATP to the *cis* side of the channel. Open probability significantly increased, control 0.13 ± 0.12 (n = 8) vs +ATP 0.48 ± 0.21 (n = 8; t test $P < 0.002$). (*B*) Application of ATP to the *trans* side of the channel. Open probability did not change, 0.14 ± 0.11 (n = 8) vs 0.13 ± 0.09 (n = 8).

added to the *cis* side of the bilayer indicating the channels were consistently oriented with the channel's myoplasmic face to the *cis* side.

Single Channel Current at Very Low Concentration of Charge Carrier

Single-channel currents were measured in 1 mM symmetrical Ba^{2+} (Fig. 4). Currents at 1 mM Ba^{2+} could only be resolved at large negative membrane potentials. There was no detectable currents at large positive potentials. The presence of a channel in the bilayer was verified in high Cs^+ (200 mM) solutions before and after each measurement. The absence of single-channel activity at positive potentials could be due to decreased channel gating (P_o) under these specific ionic conditions. Alterna-

tively, unusually large currents at only negative potentials could be explained if surface charge exists near one of the channel's mouths.

Directional Asymmetry of Channel Conduction

Cs^+ conduction (gCs) in both the myoplasm → lumen (M → L) and lumen → myoplasm (L → M) directions were determined at several [Cs^+]'s (Fig. 5). The [Cs^+] was always symmetric. After each experiment, the [Cs^+] was confirmed by measuring solution conductivity. Single-channel currents were monitored at large negative (−50 → −120 mV) and large positive (50 → 120 mV) potentials. The net current at these potentials (> 50 mV from reversal potential) was essentially a unidirection flux. Current amplitude at each potential was determined from several long single-channel openings and the accuracy confirmed by gaussian fits to total amplitude histograms. Conductance (gCs) was determined from the slope of the current-voltage relationship. At each [Cs^+], data were collected from four to six channels and plotted as

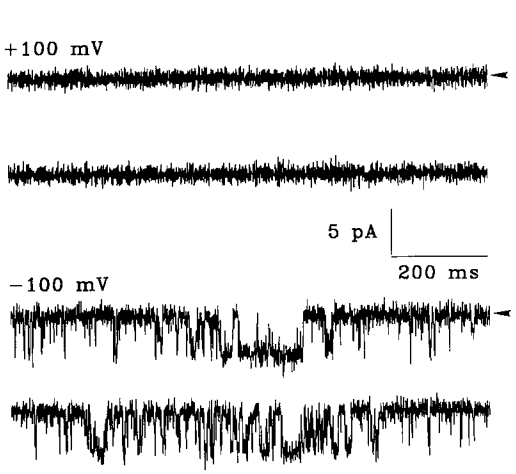

FIGURE 4. Large single channel currents at low ion concentration. Solutions contained 1 mM symmetrical $Ba(CH_3SO_3)_2$, 10 mM Hepes-tris (pH 7.4), and 20 μM free Ca^{2+}. Arrows indicate baseline (zero current level). No single channel events were observed at large (+100 mV) positive potentials. Relatively large well resolved single channel currents were observed at large (−100 mV) negative potentials.

mean ± standard deviation (Fig. 5 *A*). The difference between the means at each [Cs^+] was statistically tested by a two-tailed *t* test. At low [Cs^+]'s, gCs in the L → M direction (recorded at negative potentials; *filled symbols*) was significantly ($P < 0.0001$) larger than gCs in the M → L direction (recorded at positive potentials; *open symbols*). However, this difference became progressively smaller as the [Cs^+] was increased. Conduction in both directions saturated at ≈ 550 pS at high [Cs^+]'s.

The relatively large changes in current carrier concentration often involved significant changes in osmolarity. We were concerned that the results may be due to osmotic changes which may alter the hydration of the channel protein. To rule out this possibility, glycine was added as a noncharged osmotic effector. Certain osmotic effectors like sucrose or mannitol were not used because viscosity changes would affect diffusion limited steps (if any) associated with ion conduction. Further, some sugars may actually pass through the channel (Kasai and Kawasaki, 1993). To make the 20 mM $CsCH_3SO_3$ solution roughly isoomotic to the 500 mM $CsCH_3SO_3$

solution, 0.8 M glycine was added. At high osmolarity (0.8 M glycine; *squares*), the conduction asymmetry remained. The data were fit using the Gouy-Chapman-Stern formulation (see Discussion).

To determine if the conduction asymmetry arises within the membrane electric field, the relationship between current amplitude and [Cs^+] at three different membrane potentials (60, 75, and 90 mV) were plotted (Fig. 5 *B*). The intercept on the abscissa indicates the half saturation concentration value (K_d). The [Cs^+] at which gCs (L → M) reaches half saturation ($K_d \approx 30.0$ mM; *filled symbols*) was smaller than if gCs was in the M → L direction ($K_d \approx 222.0$ mM; *open symbols*). The difference in half

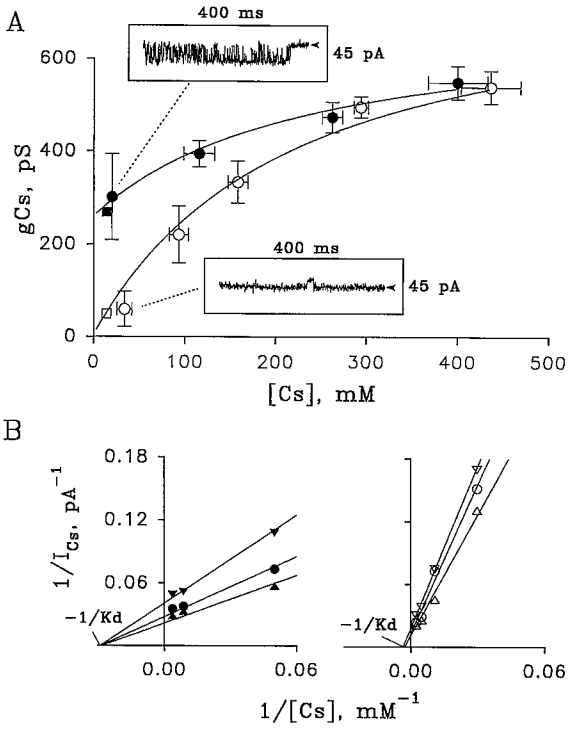

FIGURE 5. Directional asymmetry of ion conduction of cardiac RyR channel. (*A*) Cs^+ conduction in *cis* → *trans* (M → L; *open circles*) and *trans* → *cis* (L → M; *filled circles*) directions were measured at several concentrations. Single channel currents recorded in 20 mM symmetrical Cs-CH_3SO_3 were shown in the boxes. Data points were fit using Gouy-Chapman-Stern equations and plotted. Open and filled squares were gCs in the presence of 0.8 M glycine. All data were recorded at least 50 mV from the reversal potential. (*B*) Unit Cs^+ current vs [Cs^+] relationships were plotted as Lineweaver-Burk double reciprocal plot at different voltages (top to bottom, 90, 75, and 60 mV). K_d in L → M direction (*filled symbols*) was significantly smaller than K_d in M → L direction (*open symbols*). The arrows indicate baseline (zero current level).

saturation concentrations was not dependent on membrane potential. This suggests that the conduction asymmetry arises outside the membrane electrical field.

High Ionic Strength Attenuates L → M Current

Ba^{2+} currents were measured at 0 mV in the presence of different symmetrical [Cs^+]'s. Barium was added only to the *trans* chamber so that current was unidirectional. Under different experimental conditions, single channel currents were measured as a function of [Ba^{2+}] (Fig. 6). At very low ionic strength (*inverted triangles*), Ba^{2+} current increased hyperbolically and saturated. In the presence of symmetrical

Cs^+ (50 mM), Ba^{2+} current amplitude was reduced (*filled squares*). In the presence of 400 mM Cs^{2+} (*symmetric*), current was further attenuated (*filled circles*). Because the RyR channel conducts Cs^+, at least part of the attenuation could be due to competition between charge carriers (PBa/PCs ≈ 5). However, Ba^{2+} current was attenuated to the same degree by 400 mM symmetrical $Tris^+$ (PBa/P Tris ≈ 15; *open circles;* Smith et al., 1988).

It is important to note that $Tris^+$ is also permeant and may compete as charge carrier as well. However, well resolved unit Ba^{2+} currents (L → M direction) at 0 mV have been routinely measured in the presence of a large opposing $Tris^+$ gradients (e.g., 225 to 25 mM; Smith et al., 1985). This implies that relatively large [$Tris^+$]'s do

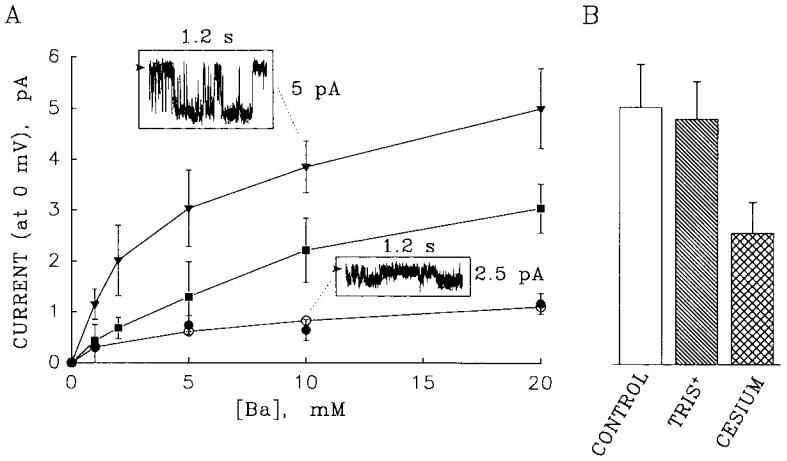

FIGURE 6. Attenuation of unidirectional (*trans → cis;* L → M direction) Ba^{2+} current by high ionic strength. Ba^{2+} (1–20 mM) was added only to the *trans* chamber. Solutions also contained 10 mM HEPES-Tris, PH 7.4, 20 μM free Ca^{2+}. Ba^{2+} current were measured at 0 mV. (*A*) In the presence of no other cations (very low ionic strength; *inverted triangles*), current increased hyperbolically and saturated. In the presence of symmetrical Cs^+ (50 mM; *filled squares*), Ba^{2+} current amplitude was reduced. In the presence of 400 mM Cs^{2+} or $Tris^+$ (*filled and open circles*), current was further attenuated. (*B*) Unit Ba^{2+} current (L → M direction; 20 mM Ba^{2+} *trans;* 0 mV) were measured; in the absence Cs^+ and $Tris^+$, with 200 mM Cs^+ added to *cis* chamber or, with 200 mM $Tris^+$ added to the *cis* chamber. The ionic strength in the *trans* chamber to remained constant.

not compete effectively with Ba^{2+} as charge carrier. To demonstrate the degree to which Cs^+ and $Tris^+$ compete with Ba^{2+}, unit Ba^{2+} currents (L → M direction; 20 mM Ba^{2+} *trans;* 0 mV) were measured under three conditions (Fig. 6 *B*); in the absence Cs^+ and $Tris^+$, with 200 mM Cs^+ added to *cis* chamber or, with 200 mM $Tris^+$ added to the *cis* chamber. This experimental protocol allows the ionic strength in the *trans* chamber (lumenal face of channel) to remain constant. The degree to which Cs^+ and $Tris^+$ compete as charge carrier is proportional to the extent of Ba^{2+} current attenuation. *Cis* Cs^+ attenuated Ba $^{2+}$ current amplitude indicating significant interion competition. However, *cis* $Tris^+$ did not significantly attenuate Ba^{2+} current amplitude indicating little interion competition. Thus, the attenuation Ba^{2+}

current by symmetrical Cs^+ and $Tris^+$ is not solely due to interion competition (Fig. 6 *A, circles*). Instead, the attenuation of Ba^{2+} current by Cs^+ and $Tris^+$ is probably due to the high ionic strengths of the solutions. The high ionic strengths apparently attenuate Ba^{2+} current by screening surface charge.

If Cs^+ not $Tris^+$ attenuate the Ba^{2+} current by inter-ion competition, then how can 400 mM symmetrical Cs^+ or $Tris^+$ attenuate Ba^{2+} current to exactly the same degree? A small additional Ba^{2+} current attenuation should be anticipated in the presence of symmetrical Cs^+. Several parameters may explain why additional Ba^{2+} current attenuation was not observed. First, $Tris^+$ ($C_4H_{11}NO_3$; mol wt 121) and Cs^+ (mol wt 132) may not be equally effective in screening the surface charge. Binding affinities and relative accessibilities of Cs^+ and $Tris^+$ to the surface charge moieties are unknowns. The dissimilar charge distributions of Cs^+ and $Tris^+$ (charged only at one end) are consistent with different screening effectiveness. Second, if additional

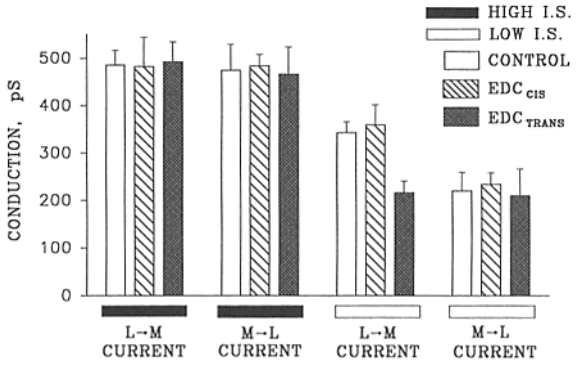

FIGURE 7. EDC treatment reduces Cs^+ conduction in the L → M direction at low ionic strength. At two different [Cs^+]'s (50 mM and 300 mM Cs^+), Cs^+ conduction in both the L → M and M → L directions were measured as described for Fig. 5. The slope conductance was determined under three experimental conditions; (*a*) control (*open bars*); (*b*) EDC treatment *cis* side (*hatched bars*); and (*c*) EDC treatment trans side (*dense cross-hatched bars*). EDC treatment consisted of a 10-min equbation in the presence of 1 mM EDC and 20 mM CH_3NH_2. Solutions also contained 10 mM HEPES, 20 μM free Ca^{2+}, PH 7.4. EDC treatment on either side of the channel did not alter conduction at high ionic strength. At low ionic strength, L → M conduction was significantly reduced (333.0 ± 22.5 (n = 5) to 216.2 ± 24.4; n = 3) by *trans* EDC treatment.

current attenuation were to occur, it would be difficult to resolve. The smallest Ba^{2+} currents resolved in our experiments were ≈0.33 pA (Fig. 6 *A*, low [Ba^{2+}]'s) with a standard deviation of ≈0.25 pA. Third, the estimates of inter-ion competition may not be precise. The degree of inter-ion competition had to be estimated under nonideal conditions, with the current carrier (20 mM Ba^{2+}; *trans*) and the competing ions ($Tris^+$ or Cs^+; *cis*) on opposite sides of the bilayer (Fig. 6 *B*).

Neutralizing Negative Charge Attenuated L → M Current

Carbodiimides are commonly used to modify carboxyl groups on proteins (DeTar and Silverstein, 1965; Dudley and Baumgarten, 1993). These agents promote the reaction of carboxyl groups and cationic donor compounds. The cationic donor used in our experiments was CH_3NH_2 (20 mM). One advantage of carbodiimide, over similar reagents like trimethyloxonium (TMO) or triethyloxonium (TEO), is that

carbodiimides can be used in low concentrations at physiological pH. The carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDC), was chosen since it is highly hydrophilic and thus will react only with carboxyl groups on the surface of the protein. In separate experiments, 1 mM EDC was added to either the *cis* or the *trans* side of the channel. The action of EDC was measured after a 10-min incubation. Conduction was measured in both the L → M and M → L directions (as described for Fig. 5) under three experimental conditions (*a*) control (no EDC treatment); (*b*) EDC treatment on the *trans* side; and (*c*) EDC treatment on the *cis* side. These experiments were done at both high and low ionic strength (300 or 50 mM symmetrical $CsCH_3SO_3$). The results are summarized in Fig. 7. At high ionic strength, conduction in both directions was equal and unaffected by EDC treatment on either side of the channel. At low ionic strength under control conditions (*open bars*), conduction was dependent on current direction (L → M or M → L; predicted by Fig. 5). EDC treatment on the *cis* side of the channel did not significantly change conduction in either direction. EDC treatment on the trans side, however, significantly reduced conduction in the L → M direction. This is consistent with the hypothesis that negative charges near the lumenal mouth of the channel potentiate L → M conduction.

DISCUSSION

Evidence for a Surface Charge Effect

Historically, single Ca^{2+} channel measurements have been performed in near saturating levels of Ca^{2+} or Ba^{2+} in order to increase the signal/noise ratio (50–100 mM; Tsien, 1983; Smith et al., 1985; Hamilton et al., 1989). Assuming Michaelis-Menten behavior, one would predict that it should be near impossible to resolve single-channel currents at physiological Ca^{2+} levels ($\approx 2 \times 10^{-3}$ M, external; 1×10^{-7} M, internal). In this study, anomalously large single channel currents were observed in solutions containing only 1 mM Ba^{2+}. We tested the hypothesis that surface charge near one of the channel's mouths causes the local cation to be significantly higher than in the bulk solution. The channel examined in this study was the cardiac SR Ca^{2+} release channel, also called the ryanodine receptor channel.

In the absence of electrostatic effects, the conduction-concentration relation of a singly occupied channel, like the RyR channel (Tinker, Lindsay, and Williams, 1992), will follow the Michaelis-Menten relationship (Hille, 1984). If surface charges exist near one channel entrance, conduction at low permeant ion concentration would be larger than expected in one direction due to electrostatic attraction. But at high ion concentrations, the surface charge will have less effect on conduction due to screening of the charge. Our results demonstrate that at low [Cs^+]'s conduction in one direction (L → M) was significantly larger than in the opposite direction (M → L; see Fig. 5 *A*). Conduction in both directions saturated at the same value at high [Cs^+]. Similar conduction asymmetry has been seen on L-type Ca^{2+} channels (Prod'hom, Pietrobon, and Hess, 1989) and Ca^{2+}-activated K^+ channels (MacKinnon, Latorre, and Miller, 1989), where existence of fixed surface charges near one channel mouth has been suggested.

If surface charge potentiates RyR conduction, then high ionic strength should attenuate conduction. To test this premise, the amplitude of unidirectional Ba^{2+} current at 0 mV in symmetrical Cs^+ and $Tris^+$ was measured (Fig. 6). As ionic strength increased, the Ba^{2+} current amplitude decreased. Because $Tris^+$ does not compete effectively with Ba^{2+} as charge carrier (Fig. 6 *B*), attenuation of Ba^{2+} current by the high $[Cs^+]$ and $[Tris^+]$ can not be solely due to interion competition. Instead, most of the attenuation can reasonably be attributed to the high ionic strength of the solutions screening the charge.

If surface charge potentiates conduction, then reducing the surface charge density will attenuate conduction. To test this premise, negative charge on the channel protein was neutralized using a carbodiimide (EDC). Conduction was unaffected at high ionic strength or when EDC was applied to the myoplasmic side of the channel (Fig. 7). However, EDC applied to the lumenal side of the channel reduced gCs significantly only at low ionic strengths.

Three lines of evidence support the hypothesis that conduction through the SR Ca^{2+} channel was potentiated by negative surface charge near its lumenal mouth. First, gCs was directionally asymmetric at low ionic strength. Second, unidirectional ($L \rightarrow M$) Ba^{2+} currents were attenuated at high ionic strengths. Third, reduction of negative surface charge on the lumenal side of the channel attenuated conduction.

Location of the Surface Charge

At low ionic strength, gBa and gCs were asymmetric (Figs. 4 and 5). One explanation is that this phenomenon arises from asymmetrical energy barriers in the conduction pathway. However, we show that the ion affinity of the channel was asymmetric and independent of membrane potential (Fig. 5). This indicates that the factors which create the asymmetric conduction we observe are outside the electrical field of the membrane and, thus, not due to asymmetrical energy barriers in the conduction pathway.

At low ionic strength, conduction in the $L \rightarrow M$ (lumen $\rightarrow$ myoplasm) direction was several fold larger than in the reverse direction. Accepting the premise that surface charge is involved, there are two possibilities: (*a*) positive charge near the myoplasmic mouth attenuates $M \rightarrow L$ conduction; or (*b*) negative charges near the lumenal mouth potentiates $L \rightarrow M$ conduction. To discern these possibilities, the net negative charge on the lumenal surface of the channel was reduced. Carbodiimide (EDC) was used to neutralize carboxyl groups on the RyR protein. These compounds have been used in the same fashion on sodium channels to abolish sensitivity to tetrodotoxin (Shrager and Profero, 1973). Alternatively, other carboxyl-specific reagents like trimethyloxonium (TMO) have been used to reduce negative surface charge on sodium and Ca^{2+}-activated K^+ channels (Sigworth and Spalding, 1980; MacKinnon et al., 1989). We show here that neutralizing carboxyl groups on the lumenal surface of the RyR channel reduced gCs in an ionic strength dependent manner. Control experiments with EDC added to the myoplasmic side of the channel revealed that EDC did not alter gCs. These results suggest that the negative surface charge which potentiates $L \rightarrow M$ conduction are located near the lumenal mouth of the channel.

Surface Charge On Other Proteins

Single cardiac RyR channels were reconstituted using native heavy SR vesicles. If the cardiac RyR channel is closely associated with other unknown proteins, then these proteins are likely to be present in the bilayer as well. Our results show that surface charge exists near the lumenal pore enterance outside the electrical field of the membrane. The data does not conclusively demonstrate that the charge is part of the RyR channel itself. It is possible that the surface charge resides on a closely associated protein. Such a protein would have to be very near the lumenal pore enterance. Two Ca^{2+} binding proteins, calsequestrin and annexin VI, are known to be closely associated with the lumenal surface of the RyR channel (Volpe, 1989; Diaz-Munoz, Kaetzel, Dedman, and Hamilton, 1990).

Calsequestrin (CSQ) is a high capacity low affinity Ca^{2+} buffer ans may actually be bound to the lumenal surface of the channel (Vople, 1989). When purified annexin VI was directly applied to the channel, the annexin interacted with the lumenal surface of the channel and modified channel behavior (Diaz-Munoz et al., 1990). In the low [Ca^{2+}]'s used in our study, CSQ would be largely Ca^{2+} free and thus negatively charged. Stains-All gels (Fig. 1) indicate that little CSQ was present in the SR vesicles used. It is, however, possible that CSQ was associated with some of the channels studied. The observed surface charge effects were present in all channels studied ($n > 100$) from many different vesicle preparations. If CSQ washes out of cardiac vesicles easily (Volpe, 1989), it is not likely that the surface charge was on CSQ. However, the possibility that the charge resides on CSQ, annexin VI, or another closely associated protein can not be ruled out.

What is the Surface Charge Density?

To estimate surface charge density it is necessary to measure the electrostatic potential adjacent to the RyR protein. This is technically impossible. It is, however, possible to estimate the potential adjacent to a charged planar surface using the Gouy-Chapman-Stern (GCS) double-layer theory (Mclaughlin, 1977). Among its many assumptions, the GCS theory assumes that the surface charge is smeared uniformly over the surface. Although channels are not ideal charged surfaces, the GCS theory has been applied to interpret surface charge effects on many types of ion channels (MacKinnon et al., 1989; Dani, 1986; Jordan, 1987). Thus, the GCS theory was used here to estimate the surface charge density required to produce our results.

At equilibrium the local concentrations are given by the Boltzmann equation:

$$[C]_s = [C]_b \cdot \exp(-z \cdot FV_s/RT) \tag{1}$$

where $[C]_s$ and $[C]_b$ are the surface and bulk concentrations, respectively, V_s the surface potential, z the valence of the ion, R the gas constant, and T the temperature in Kelvin scale. The relation between the surface potential (V_s), the charge density (σ), and the aqueous electrolyte composition is given by:

$$\sigma = \{2\epsilon_r \epsilon_o RT \Sigma_i C_i [\exp(-z_i FV_s/RT) - 1]\}^{1/2} \tag{2}$$

where the ϵ_r is the relative dielectric constant, ϵ_o the permitivity of free space, F the Faraday constant. The data in Fig. 5 *A* were fit by applying Eqs. 1 and 2 to the

Michaelis-Menten equation:

$$g = g_{max}/(1 + K_M/[C]_s) \quad (3)$$

Cesium conduction in the L → M direction was best fit when $\delta = 0.11 e \cdot nm^{-2}$. Conduction in the M → L direction was best fit when $\delta = 1.2 \cdot 10^{-10} e \cdot nm^{-2}$ (essentially zero).

It has been reported that the lumenal face of the RyR channel has a surface area of 196 nm^2 (Wagenknecht et al., 1989). Thus, 22 uniformly distributed negatively charged amino acids would be required to produce the observed surface charge effect. Alternatively, far fewer charges clustered in a much smaller area could produce the same effect.

Physiological Significance

In vivo, the role of the SR Ca^{2+} release (RyR) channel is to mediate a large Ca^{2+} flux on a millisecond time scale. The large conductance and fast gating of the channel are consistent with this role (Fill and Coronado, 1988). However, it is not clear how this channel can have such a large conductance and be selective. Large conduction be achieved if the ion exit rate or entry rate were exceptionally fast. The exit rate could be optimized by a very short pore. The ion entry rate could be optimized by a large capture radias (i.e., large vestibule) or by fixed charge which concentrates ions near the pore enterance. Electron micrographic evidence (Wagenknecht et al., 1989) suggests the pore length may be quite long and that the capture radias on the lumenal side of the channel may be relatively small.

In this study, evidence is presented with indicates that negative surface charge exists near the lumenal mouth of the channel. This fixed charge would create a local electrostatic potential causing local cation concentration to be significantly higher than in the bulk solution. Accumulation of cations near the lumenal mouth of the channel may potentiate conduction in vivo.

The RyR channel is a poorly selective Ca^{2+} channel. The permeability ratio $P_{Ca}/P_K \approx 6$ is significantly less than that of surface membrane Ca^{2+} channels that have a $P_{Ca}/P_K > 25$ (Smith et al., 1988). In vivo, the lack of K^+ gradients across the SR (Somlyo, Shuman, and Somlyo, 1977) implies that the $[K^+]$ inside the SR is equal to the myoplasmic $[K^+]$ (≈ 100 mM). The Ca^{2+} binding affinity of CSQ suggests the free $[Ca^{2+}]$ inside the SR is near 1 mM. Thus, K^+ and Ca^{2+} must compete as charge carrier in vivo. How could the channel optimize Ca^{2+} conduction under these conditions? The existence of negative surface charge near the lumenal mouth of the channel may also resolve this issue. The surface charge may act as a pre-selection filter. Fixed negative charge would concentrate Ca^{2+} near the pore mouth. Thus, the local $[Ca^{2+}]$ would be quite different than that predicted in the bulk solution. It is possible that preferential (divalent over monovalent) accumulation of Ca^{2+} near the channel mouth allows Ca^{2+} to compete more effectively as a charge carrier.

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