Cardiac Sarcoplasmic Reticulum Chloride Channels Regulated by Protein Kinase A

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In heart cells, several plasma membrane ion channels are targets for phosphorylation. However, it is not known whether sarcoplasmic reticulum (SR) ion channels, which are also essential in the regulation of cardiac function, are regulated by second-messenger systems. Here, we show that a Cl\(^-\) channel in the cardiac SR membrane is activated by the catalytic subunit of protein kinase A (PKA). Purified cardiac heavy SR vesicles were incorporated into planar lipid bilayers. This channel spontaneously inactivated within a few minutes after the channel was incorporated into the bilayer. Mg-ATP (2–5 mM), but not the nonhydrolyzable ATP analogue S'-adenyllylimidodiphosphate, added to the cis solution prevented this spontaneous channel inactivation. After the inactivation process occurred, the catalytic subunit of PKA (with 0.05 mM Mg-ATP) reactivated this channel. These effects of Mg-ATP and PKA on the Cl\(^-\) channel were prevented by an inhibitor of PKA. Thus, these results suggest that this Cl\(^-\) channel is a novel target of PKA-dependent phosphorylation in cardiac muscle regulation. (Circulation Research 1992;71:585–589)

Key Words: chloride channel • sarcoplasmic reticulum • phosphorylation • protein kinase A • single-channel recording

In heart myocytes, several ion channels in the sarcoplas-lemma\(^1-^5\) and intracellular proteins\(^6-^8\) in the contractile apparatus are regulated by cAMP-dependent phosphorylation. As a result of these phosphorylation processes, cardiac contraction is increased, and relaxation is accelerated. During excitation–contraction coupling, Ca\(^{2+}\) is released from the sarcoplasmic reticulum (SR) through the ryanodine-receptor Ca\(^{2+}\) release channel and is also taken up via a Ca\(^{2+}\) pump in the SR.\(^9-^11\) Ca\(^{2+}\) mobilization from the SR may also be influenced by the movement of other ions such as K\(^+\) and Cl\(^-\) through the SR membrane. The flow of these ions may serve to maintain charge neutrality across the SR membrane.\(^12\) Several ion channels on the SR membrane have been studied in detail using the bilayer technique.\(^13-^16\) It is not known, however, whether ion channels in the SR can be modulated by second messengers.\(^17\) In this report, we investigated whether the chloride channel of the cardiac SR membrane is regulated by protein kinase A (PKA)–mediated phosphorylation.

Materials and Methods

Preparation and Current Measurement

Porcine cardiac heavy SR was isolated by discontinuous sucrose gradient centrifugation as described by Meissner and Henderson\(^18\) and stored at −80°C until use. \(^19\)Ryanodine binding to this preparation has been examined to characterize the purity of the SR membrane.\(^19\) Purified cardiac heavy SR vesicles were incorporated into planar lipid bilayers. The planar lipid bilayer was composed of brain phosphatidylethanolamine and brain phosphatidylserine (Avanti Polar Lipids, Alabaster, Ala.), at a ratio of 1:1, dissolved in decane (20 mg/ml). SR vesicles were added to the cis chamber and fused into the lipid bilayer formed in the hole (0.3 mm in diameter) in a Lexan polycarbonate partition. In the present experiments, the cis chamber was defined as the side to which SR vesicles were added, and the opposite side was referred to as the trans chamber. The cis chamber was equivalent to the cytoplasmic side of the incorporated channel, and the trans chamber was equivalent to the lumen of the SR as determined previously.\(^10,^13\) Applied voltages were defined with respect to the trans chamber held at ground. Channel activities were recorded at room temperature (22±1°C), amplified by a patch-clamp amplifier (Axopatch, Axon Instruments, Inc., Foster City, Calif.), and stored on a videocassette tape recorder through a PCM converter system (RP-880, NF Instruments, Yokohama, Japan) digitized at 40 kHz. Data were reproduced and low-pass-filtered at 500 Hz (−3 dB) by a bessel filter (48 dB per octave attenuation), sampled at 5 kHz, and analyzed off-line on a computer (PC 286, IBM). For single-channel analysis, the threshold used to judge the open state was set at half of the single-channel amplitude.\(^20\) Data were expressed as mean±SD. Statistical analysis using Student’s t test was performed whenever appropriate.

Solution and Drugs

The cis bath solution contained 500 mM CsCl, 1 μM free Ca\(^{2+}\), 1 mM EGTA, and 10 mM HEPES. The trans solution contained 50 mM CsCl, 1 μM free Ca\(^{2+}\), 1 mM EGTA, and 10 mM HEPES. The pH values of these
solutions were adjusted to 7.4 adding CsOH. Various concentrations of Mg2+ and Na2ATP were added to the cis solution. One micromolar of free Ca2+ in the solution was maintained by adding various amounts of CaCl2 calculated with the computer program described by Fabiato.21

Distilbene, 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) was purchased from Pfaltz and Bauer Inc., Waterbury, Conn. Concentrated stock solutions of DNDS were dissolved in dimethyl sulfoxide (100 mg/ml). The stock solution was kept at 5°C and diluted with the cis solution just before use. The final concentration of dimethyl sulfoxide was less than 0.1%. The catalytic subunit of cAMP-dependent PKA was purified from fresh porcine cardiac muscle by the method of Beavo and Krebs22 in our laboratory (Mie University). The catalytic subunit was eluted with a linear gradient of 30–300 mM potassium phosphate buffer containing 0.1 mM EGTA. Protein concentration was determined by protein assay (Bio-Rad Laboratories, Richmond, Calif.).23 ATP, 5'-adenylylimidodiphosphate (AMP-PNP, a nonhydrolyzable analogue of ATP), and a protein kinase inhibitor specific to PKA (Sigma P3294) were purchased from Sigma Chemical Co., St. Louis, Mo. Enzymes were dialyzed against the bath solution at 0°C for 1 hour before use.

**Results**

*Single-Channel Recordings of Chloride Channel in SR*

Purified cardiac heavy SR vesicles were incorporated into planar lipid bilayers. By using 500 mM CsCl in the cis and 50 mM CsCl in the trans chamber solutions, several kinds of ion channels were identified in the bilayer as reported previously24: At 0 mV, Cs+ current flowed through the ryanoidine-receptor channel in the outward direction, and Cl- channel currents flowed in the inward direction in the same bilayers. When sarcolemmal vesicles, instead of heavy SR vesicles, were incorporated into the bilayer, we could detect neither the ryanoidine-receptor channel currents nor the Cl- channel currents (in nine of nine experiments). Figure 1A shows recordings of Cl- channels reconstituted into the planar lipid bilayer at various holding potentials. The Cl- channel open probability was not affected by 

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[Ca^{2+}]_{\text{los}} \text{ between 0.01 and 10 } \mu \text{M (not shown) and was constant at } \approx 0.9 \text{ between } -40 \text{ and } +100 \text{ mV (Figures 1A and 1C). Thus, like agonist-activated sarcolemmal Cl- channels in airway epithelial cells}^{25} \text{ and cardiac myocytes,}^{26} \text{ openings of this SR Cl- channel were neither Ca2+ dependent or voltage dependent.}
\]

The current–voltage relation of the unitary Cl- channel exhibited slight outward-going rectification (Figure 1B). With 500 mM Cl-, the slope conductance of the inward current was 116 pS (n=22), and the reversal potential was \( +60 \text{ mV} \). With 250 mM Cl-, the slope conductance decreased to 71 pS (n=5), and the reversal potential shifted to \( +42 \text{ mV} \) (Figure 1B). The reversal potential values of this channel current with 500 mM and 250 mM [Cl-]los are approximately equal to the calculated reversal potential for Cl-, which was \( +58 \text{ mV} \) at 500 mM [Cl-]los and \( +41 \text{ mV} \) at 250 mM [Cl-]los. The

**Figure 1.** Tracings and current–voltage relations of chloride channels incorporated into planar lipid bilayers. o, Open channel; c, closed channel. Panel A: Single-channel activities at different holding potentials indicated at the left side of each tracing. Upward direction indicates current flow from cis to trans. Panel B: The plot of unitary current amplitude (mean ± SD) with two different Cl- concentrations in the cis chamber as a function of holding potential. Closed circles indicate current with 500 mM Cl-, and closed squares indicate current with 250 mM Cl-. The slope conductances of 500 mM Cl- and 250 mM Cl- are 116 and 71 pS, respectively. Panel C: Sensitivity of the chloride channel to stilbene derivative distilbene, 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS). Channel activities were blocked by application of 0.2 mM DNDS into the cis side. In these experiments, cis solution contained 5 mM Na2ATP and 2 mM Mg2+ to prevent early rundown. The lower graph is plot of open probabilities (Po) at every 10 seconds versus time. The bottom numbers indicate the time after drug application.
anion channel blocker DNDS (0.1–1 mM), blocked this channel completely when added to the cis solution (Figure 1C, n=4). From these results, it was concluded that the channel recorded here was a Cl⁻ permeable anion channel. A different kind of Cl⁻ channel, whose slope conductance was 75 pS at 500 mM [Cl⁻]₀, could also be detected. However, this 75-pS Cl⁻ channel was not usually observed (in 35 of 292 experiments), and we did not analyze its properties in detail. Thus, we specifically examined the 116-pS Cl⁻ channel in this study.

Inactivation and Reactivation of the Cl⁻ Current

Openings of the 116-pS Cl⁻ channel persisted only for 1.4±1.3 minutes (n=15) after being incorporated into the lipid bilayer (Figure 2a). However, when 5 mM Mg-ATP was added to the cis solution, channel openings were maintained until the experiments were interrupted by the break of bilayer (>8.9±5.4 minutes, n=10, p<0.005 compared with the control condition) (Figure 2b). This observation suggests that adenine nucleotide, Mg²⁺, or phosphorylation might regulate the Cl⁻ channel. Therefore, the following experiments were performed to test these possibilities.

First, we examined the effects of adenine nucleotide on the Cl⁻ channel. The nonhydrolyzable ATP analogue AMP-PNP (2 mM) added to the cis solution, either in the presence or absence of 2 mM Mg²⁺, did not activate the Cl⁻ channel after inactivation (n=9) (Figure 2c). ATP (0.3–5 mM) with 2 mM Mg²⁺ could reactivate the channel (n=6) (Figure 2c), whereas ATP (5 mM) without Mg²⁺ could not maintain or reactivate the channel (Figure 2d). These results indicate that Mg-ATP is sufficient to keep the channel active. Next, we examined the possibility of direct regulation of the channel by Mg²⁺. Addition of 2 mM Mg²⁺ in the presence of ATP remarkably increased channel openings (Figure 2d), whereas Mg²⁺ alone failed to activate the channel in the absence of ATP (Figure 2e). These results indicate that both ATP and Mg²⁺ are necessary to maintain the channel activity and suggest that phosphorylation may be involved in the Mg-ATP-mediated channel activation. Consistent with this notion, the protein kinase inhibitor (20 μM) specific for PKA prevented the activation of this Cl⁻ channel by Mg-ATP (n=4) (Figure 2f).

In Figure 3, we examined effects of the catalytic subunit of PKA on the Cl⁻ channel. After the complete inactivation of the Cl⁻ channel, the catalytic subunit (60–148 ng/ml from 3.5 μM PKA) with 0.05 mM Mg-ATP was added to the cis solution. In nine of nine experiments, the Cl⁻ channel openings resumed immediately after application of the catalytic subunit (Figure 3a). The boiled subunit did not activate the channel in four of four experiments (Figure 3b). The protein kinase inhibitor (10 μM) prevented the catalytic subunit–mediated reactivation of the channel in five of five experiments (Figure 3c).

The kinetic properties of the Cl⁻ channel before inactivation and after reactivation by the catalytic subunit of PKA were compared in Figures 3d and 3e. The closed-time and open-time histograms of the channel could be fitted by the sum of two exponentials in both cases. The time constants were the same before inactivation and after reactivation: Before inactivation, the closed time constants were 0.6 and 2.9 msec, and the open time constants were 1.3 and 57 msec. After reactivation, these values were 0.7 and 3.2 msec for the closed time, and 1.3 and 56 msec for the open time. Therefore, we conclude that the same Cl⁻ channel was reactivated by the catalytic subunit of PKA via phosphorylation in these experiments.

Discussion

This study reports a novel finding that one type of cardiac SR Cl⁻ channel is regulated by cAMP-dependent phosphorylation. The regulation of an ion channel of an intracellular membrane by cAMP-dependent phosphorylation has not previously been demonstrated,
with the exception of a report that chloride conductance in endocytic vesicles from the rabbit proximal tubule is activated by phosphorylation through a cAMP-dependent protein kinase.\(^{17}\)

The Cl\(^-\) channel in the present study was consistently detected in the lipid bilayer containing cardiac heavy SR vesicles (in 263 of 292 experiments). This Cl\(^-\) channel was not detected when sarcolemmal vesicles were incorporated into the bilayer (in zero of nine experiments). Furthermore, the sarcolemmal Cl\(^-\) channel incorporated into the lipid bilayer had different kinetic and conductance properties from those examined in this study.\(^ {14}\) Therefore, the 116-pS Cl\(^-\) channel in this report was most likely derived from the cardiac SR membrane. Another type of SR Cl\(^-\) channel, whose conductance was 55 pS with 260 mM Cl\(^-\), was previously reported in cardiac muscle.\(^ {15}\) However, the smaller conductance Cl\(^-\) channel had different kinetic properties from those of the 116-pS Cl\(^-\) channel. The small Cl\(^-\) channel had one open state and three closed states, and its open probability was voltage dependent between -60 and +60 mV. On the other hand, the 116-pS channel may have two open and two closed states, and the open probability was not voltage dependent between -40 and +100 mV. Furthermore, Mg-ATP did not affect the activity of the small Cl\(^-\) channel. Therefore, the small SR Cl\(^-\) channel previously reported by Rousseau\(^ {15}\) is presumably different from the 116-pS Cl\(^-\) channel examined in this study. In rabbit skeletal muscle, a large-conductance anion-selective channel in SR, whose slope conductance was 200 pS with 100 mM Cl\(^-\), was reported.\(^ {26}\) However, the open probability of the skeletal SR Cl\(^-\) channel was voltage dependent, and the regulation of the channel by intracellular second messengers has not yet been examined.

Our results showed that Mg-ATP prevented the spontaneous channel inactivation of this Cl\(^-\) channel and also reactivated this channel after inactivation without adding exogenous kinases (Figure 2). These effects of Mg-ATP on the Cl\(^-\) channel were prevented by an inhibitor for PKA. Therefore, it is speculated that PKA may be contained in the native SR vesicles. Recently, Chung et al\(^ {27}\) reported the protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. The endogenous protein kinase may be intrinsically associated with this Cl\(^-\) channel protein.
Although the physiological role of the monovalent ion permeability of the SR membrane has not yet been clarified, it is postulated that the passive distribution of monovalent ions, such as K\(^+\) and Cl\(^-\), across the SR membrane can neutralize the potential generated by Ca\(^{2+}\) release through the ryanodine-receptor channel from cardiac SR, resulting in the facilitation of Ca\(^{2+}\) uptake by SR during relaxation.\(^1\) For this neutralization, it is expected that K\(^+\) flows into SR and Cl\(^-\) flows out of SR during Ca\(^{2+}\) release. The Cl\(^-\) channel in this study may possibly contribute to the neutralization of the potential across the SR membrane generated by Ca\(^{2+}\) uptake through the calcium pump during relaxation. However, further studies are needed to elucidate exact roles of the SR Cl\(^-\) channel and its modulation by second messengers in the physiological regulation of cardiac function.

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