Single-Channel Properties of the Recombinant Skeletal Muscle Ca^{2+} Release Channel (Ryanodine Receptor)

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ABSTRACT We report transient expression of a full-length cDNA encoding the Ca^{2+} release channel of rabbit skeletal muscle sarcoplasmic reticulum (ryanodine receptor) in HEK-293 cells. The single-channel properties of the 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate-solubilized and sucrose gradient-purified recombinant Ca^{2+} release channels were investigated by using single-channel recordings in planar lipid bilayers. The recombinant Ca^{2+} release channel exhibited a K^+ conductance of 780 pS when symmetrical 250 mM KCl was used as the conducting ion and a Ca^{2+} conductance of 116 pS in 50 mM luminal Ca^{2+}. Opening events of the recombinant channels were brief, with an open time constant of ~0.22 ms. The recombinant Ca^{2+} release channel was more permeable to Ca^{2+} than to K^+, with a ${}_PCa^{2+}/{}_PK^+$ ratio of 6.8. The response of the recombinant Ca^{2+} release channel to various concentrations of Ca^{2+} was biphasic, with the channel being activated by micromolar Ca^{2+} and inhibited by millimolar Ca^{2+}. The recombinant channels were activated by ATP and caffeine, inhibited by Mg^{2+} and ruthenium red, and modified by ryanodine. Most recombinant channels were asymmetrically blocked, conducting current unidirectionally from the luminal to the cytoplasmic side of the channel. These data demonstrate that the properties of recombinant Ca^{2+} release channel expressed in HEK-293 cells are very similar, if not identical, to those of the native channel.

INTRODUCTION

The Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor) is located in the junctional terminal cisternae, in close apposition to the transverse tubular membrane (Inui et al., 1987). It plays a key role in the release of Ca^{2+} from the sarcoplasmic reticulum, after depolarization of the transverse tubular membrane. The single-channel properties of ryanodine receptors have been characterized extensively after their incorporation into planar lipid bilayers (Coronado et al., 1994). They form a high-conductance, Ca^{2+}-selective channel that is activated by micromolar Ca^{2+} and millimolar ATP and inhibited by millimolar Ca^{2+} and Mg^{2+} and micromolar ruthenium red. Ryanodine, a plant alkaloid, decreases the maximum conductance of the channel and shifts the channel into states with long-lived openings and brief closings.

Skeletal muscle ryanodine receptor cDNAs have been cloned and sequenced (Takeshima et al., 1989; Zorzato et al., 1990). They encode a protein of up to 5,038 amino acids, depending on alternative splicing, with a mass of ~563,584 Da (Phillips et al., 1996). The ryanodine receptor protein has been expressed from its cDNA in several expression systems. Penner et al. (1989) reported its expression in CHO cells and demonstrated that transfected CHO cells, but not nontransfected cells, release Ca^{2+} from intracellular organelles after the addition of caffeine or ryanodine. Similar observations have been made in measurements of whole-cell Ca^{2+} transients of C2C12 mouse myoblast cells (Otsu et al., 1994) and COS-7 cells (Treves et al., 1994) transfected with skeletal muscle ryanodine receptor cDNA, or by measuring the Ca^{2+}-activated Cl^- current in *Xenopus* oocytes injected with ryanodine receptor RNA (Nakai et al., 1990). These studies indicate that recombinant ryanodine receptors form functional Ca^{2+} release channels in whole cells, but single-channel properties of these recombinant channels have not been reported.

We have expressed rabbit skeletal muscle ryanodine receptor cDNA in COS-1 cells and demonstrated that single recombinant channels respond to modulators such as Ca^{2+}, ATP, Mg^{2+}, ruthenium red, and ryanodine. However, the channels expressed in COS-1 cells displayed multiple, anomalous conductances (Chen et al., 1993). Multiple subconductance states were also detected in single Ca^{2+} release channels expressed in Sf9 cells (Brillantes et al., 1994). The conductance and stability of the recombinant channels expressed in Sf9 cells were improved by coexpressing FK506-binding protein 12 (FKBP12). The occurrence of multiple conductance states made it difficult to contemplate further studies of kinetics, conductance, and ligand gating properties of the Ca^{2+} release channel. Therefore, the development of an alternative expression system for the functional ryanodine receptor was required.

In this report we describe the expression of the ryanodine receptor cDNA in HEK-293 cells and the detailed charac-

Received for publication 3 April 1997 and in final form 7 July 1997.

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0006-3495/97/10/1904/09 $2.00

terization of the single-channel properties of the expressed protein. The recombinant channels expressed in HEK-293 cells have conductance, kinetics of opening, current-voltage relationship, Ca^{2+} permeability, and modulation by physiological and pharmacological ligands identical to those of the native rabbit skeletal muscle Ca^{2+} release channel. The establishment of the HEK-293 cell expression system is a significant advance in the studies of structure-function relationships of the ryanodine receptor, opening the potential for understanding the molecular mechanism of the channel.

MATERIALS AND METHODS

Materials

Ryanodine was obtained from AgriSysems International (Wind Gap, PA). Brain phosphatidylethanolamine and brain phosphatidylserine were obtained from Avanti Polar Lipids. The affinity-purified anti-13c2 antibody against the rabbit skeletal muscle ryanodine receptor was generated as described previously (Chen et al., 1992). The construct for the expression of SERCA1 cDNA was obtained from Toyofuku et al. (1994) and has been characterized previously. Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Promega Biotech. 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and other chemicals were purchased from Sigma. The rabbit ryanodine receptor cDNA has been described previously (Zorzato et al., 1990; Chen et al., 1993).

Cell culture and DNA transfection

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1 mM minimum Eagle's medium nonessential amino acids, 4 mM L-glutamine, 100 units of penicillin/ml, 100 mg of streptomycin/ml, 4.5 g of glucose/liter, and 10% fetal calf serum, at 37°C under 5% CO_2. DNA transfection was carried out using calcium phosphate (Sambrook et al., 1989). Cells were plated in 100-mm tissue culture dishes 18–22 h before transfection and were transfected with 12 μg of skeletal muscle ryanodine receptor cDNA and/or 5 μg of SERCA1 cDNA per dish. Control cells were treated in the same way, with no DNA or with expression vector DNA alone. Microsomal membranes were isolated from cells harvested 19–21 h after transfection.

Partial purification of the recombinant ryanodine receptor

Microsomal membranes were prepared from transfected and nontransfected HEK-293 cells as described previously (Chen et al., 1993). They were stored frozen in small aliquots and thawed only once before use. Heavy sarcoplasmic reticulum was isolated from rabbit fast-twitch skeletal muscle as described by Campbell and MacLennan (1981). Solubilization and purification of ryanodine receptors from heavy sarcoplasmic reticulum and from microsomal membranes of the transfected and nontransfected HEK-293 cells were carried out as described previously (Chen et al., 1993), except that the solubilized ryanodine receptor was not concentrated 30-fold in volume in Centricon-100 microconcentrators (Amicon filtration). Immunocytochemical staining of transfected and nontransfected HEK-293 cells and enzyme-linked immunosorbent assays for the detection of ryanodine receptor proteins in the sucrose gradient fractions were carried out as described previously (Chen et al., 1993).

Single-channel recordings in planar lipid bilayers

Single-channel recordings were obtained after incorporation into an artificial lipid bilayer of sucrose density gradient-purified ryanodine receptors from either rabbit skeletal muscle heavy sarcoplasmic reticulum or from transfected HEK-293 cells, as described previously (Chen et al., 1993). Brain phosphatidylserine and brain phosphatidylethanolamine, dissolved in chloroform, were combined in a 3:5 ratio (w/w), dried under nitrogen gas, and suspended in 30 μl of *n*-decane at a concentration of 35 mg of lipid/ml. Bilayers were formed across a 250-μm hole in a Delrin partition separating two chambers. The *trans* chamber (400 μl) was connected to the headstage input of a model EPC-7 amplifier (List-Electronics, Darmstadt, Germany). The *cis* chamber (1 ml) was held at virtual ground. Unless indicated otherwise, the luminal (*trans*) potentials were varied and reported. A symmetrical solution containing 250 mM KCl and 25 mM HEPES (pH 7.4) was used for recordings. A 0.5–1.0-μl aliquot of sucrose density gradient-purified native ryanodine receptor or a 2–4-μl aliquot of sucrose density gradient-purified recombinant ryanodine receptor was added to the *cis* chamber. Spontaneous channel activities were each tested for sensitivity to EGTA and/or Ca^{2+}, thereby providing information about Ca^{2+} sensitivity, orientation in the bilayer, and stability of the incorporated channel. Unless indicated otherwise, all further additions were made to that chamber in which the addition of EGTA inhibited the activity of the incorporated channel. This chamber presumably corresponds to the cytoplasmic side of the Ca^{2+} release channel. In the experiments in which the *trans* chamber required perfusion, the configuration of electrodes was reversed so that the *cis* chamber was connected to the input of the amplifier and the *trans* chamber was grounded to avoid a current surge. In this case, the cytoplasmic (*cis*) potentials were varied and reported. Recordings were filtered at 10,000 Hz digitized at 44.1 kHz (PCM-2 A/D VCR Adapter; Medical Systems Corp., Greenvale, NY) and recorded on VHS videotapes. Upon playback, the data were filtered at 1000 Hz, acquired at 5 kHz, and analyzed with pClamp 5.5 software (Axon Instruments). A 50% threshold was applied to detect open events.

RESULTS

Expression in HEK-293 cells

In a previous paper (Chen et al., 1993), we reported the expression of rabbit skeletal muscle ryanodine receptor cDNA in COS-1 cells (Chen et al., 1993). We found that the transfection efficiency of the COS-1 cell expression system was less than 1%. In searching for a better expression system, we examined the transfection efficiency of several other cell lines, including COS-7, Rat-2, National Institutes of Health-3T3, and HEK-293 cells. Of these cell lines, transfection of HEK-293 cells using a Ca^{2+} phosphate-mediated transient transfection method gave the highest transfection efficiency. Fig. 1 shows the immunocytochemical staining with anti-13C2 antibody raised against a short

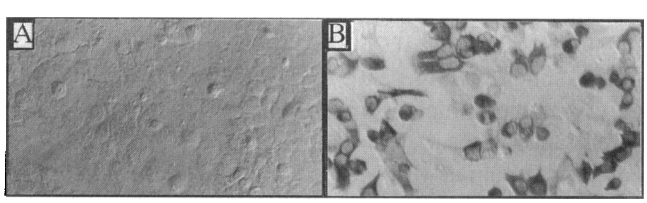

FIGURE 1 Immunocytochemical staining of control and transfected HEK-293 cells. HEK-293 cells were transfected without (*A*) or with (*B*) the full-length rabbit skeletal muscle ryanodine receptor cDNA by a Ca^{2+} phosphate-mediated transfection method. Cells were fixed and permeabilized 19–21 h after transfection. Expressed ryanodine receptor proteins were detected by immunocytochemical staining, using an anti-ryanodine receptor antibody, anti-13C2 (Chen et al., 1992), with horseradish-conjugated anti-rabbit IgG as a secondary antibody.

amino acid sequence of the rabbit skeletal muscle ryanodine receptor (Chen et al., 1992) of HEK-293 cells transfected with or without the full-length cDNA encoding the rabbit skeletal muscle ryanodine receptor. Expression of ryanodine receptor proteins could be detected in more than 25% of the transfected HEK-293 cells, whereas no specific staining was observed in nontransfected cells. Thus the ryanodine receptor cDNA can be transiently expressed in HEK-293 cells with a transfection efficiency 25-fold higher than that of the COS-1 cell system.

HEK-293 cells express virtually no excitable membrane currents and are readily transfected (Ukomadu et al., 1992). Endoplasmic reticulum isolated from HEK-293 cells is not leaky to Ca^{2+} under conditions (μM free Ca^{2+} and mM ATP) that activate Ca^{2+} release channels (Toyofuku et al., 1994). Using immunoblotting and enzyme-linked immunosorbent assay (ELISA), we were unable to detect any ryanodine receptor-like immunoreactivity in microsomal membranes of untransfected HEK-293 cells (unpublished observations). These findings, along with our inability to observe either ryanodine- or caffeine-sensitive Ca^{2+} release from internal stores of untransfected HEK-293 cells or the formation of ryanodine- or caffeine-sensitive Ca^{2+}-release channels in hundreds of bilayers exposed to extracts from untransfected HEK-293 cells, over a period of several years, suggests that this cell line contains, at most, trace levels of intrinsic ryanodine- or caffeine-sensitive Ca^{2+} release channels. Thus the HEK-293 cell line is an excellent mammalian expression system in which to study the structural and functional properties of Ca^{2+} release channels.

Comparison of conductance and open time constants

Ryanodine receptors from rabbit skeletal muscle heavy sarcoplasmic reticulum membranes and from microsomal membranes of transfected HEK-293 cells were solubilized in CHAPS and partially purified by sucrose density gradient centrifugation. ELISA was used to localize ryanodine receptors in sucrose gradients. Gradient fractions containing the peak of immunoreactivity were pooled and used for single-channel recordings in planar lipid bilayers.

Single-channel currents of both native and recombinant ryanodine receptor channels were recorded in a solution containing symmetrical 250 mM KCl and 25 mM HEPES (pH 7.4) and in the presence of 2.5 μM free Ca^{2+} added to the *cis* (cytoplasmic) chamber (Fig. 2). Maximum single current amplitudes were ~23 pA at a holding potential of +30 mV (luminal) (Fig. 2, *A* and *B*). A large number of open events with submaximum single-current amplitudes were found in the current records of both native and recombinant channels (Fig. 2, *C* and *D*). They are most likely events that are too brief to be completely resolved by our bilayer system. On the other hand, long-lived open events with submaximum current amplitudes were seldom observed, indicating that well-resolved and long-lived subconductance states were rare in these channels. Open dwell-time histograms of recombinant and native channels are shown in Fig. 2, *E* and *F*. Both recombinant and native Ca^{2+} release channels exhibited very fast kinetics. Fittings of open dwell-time histograms yielded an open time constant of 0.224 ± 0.027 ms (n = 4) for the recombinant channels and 0.220 ± 0.022 ms (mean ± SD) (n = 4) for the native channels. The mean closed times of recombinant and native channels under these conditions are 7.94 ± 0.80 (n = 3) and 8.09 ± 1.08 (n =4), respectively. Some incompletely resolved open events may have been missed in our data analysis, which used a 50% discriminator for detecting openings. Thus the open time constants of both recombinant and native channels may have been overestimated, but comparisons between recombinant and native channels are believed to be valid. The current-voltage relationships of native and recombinant channels were very similar. They displayed a linear relationship with slope conductances of 780 ± 12.8 pS (mean ± SD) (n = 3) for the recombinant channels and 778 ± 5.66 pS (n = 2) for the native channels (Fig. 2 *G*). We reported earlier that the slope of the inward current conductance (luminal to cytoplasmic current) of the native skeletal muscle ryanodine receptor channel differed from the slope of the outward current conductance (cytoplasmic to luminal current) in a recording solution containing 250 mM NaCl, 50 mM Tris, and 100 mM HEPES (pH 7.4) (Chen et al., 1994). We found that the difference between the inward and outward current conductance resulted from an asymmetrical blockade of the channel by Tris (data not shown). In the absence of Tris, however, outward and inward current conductances were identical, as shown in Fig. 2 *G*. Therefore, the Ca^{2+} release channel expressed in HEK-293 cells exhibited single-channel conductance and channel kinetics very similar to those of the native channel.

Ca^{2+} permeability of the recombinant single Ca^{2+} release channel

The Ca^{2+} permeability of the recombinant Ca^{2+} release channels was investigated by examining the effect of luminal addition of $CaCl_2$ on the K^+ conductance of the recombinant Ca^{2+} release channels. In this series of experiments, the cytoplasmic (*cis*) potentials were varied and reported. A single recombinant channel current was recorded in symmetrical 250 mM KCl. The addition of 2.5 mM $CaCl_2$ to the luminal side of the channel decreased inward K^+ current conductance (luminal to cytoplasmic current) from 780 pS to 450 ± 44.8 pS (n = 3), as estimated from the current-voltage relationship between −20 and −60 mV (cytoplasmic), with no significant change in outward current conductance (cytoplasmic to luminal current) (Fig. 3). The outward current conductance (cytoplasmic to luminal current) in the presence of 2.5 mM $CaCl_2$ was 761 ± 54.6 (n = 3), as estimated from the current-voltage relationship between +20 and +60 mV (cytoplasmic). The zero current potential, with symmetrical 250 mM KCl and 2.5 mM luminal $CaCl_2$,

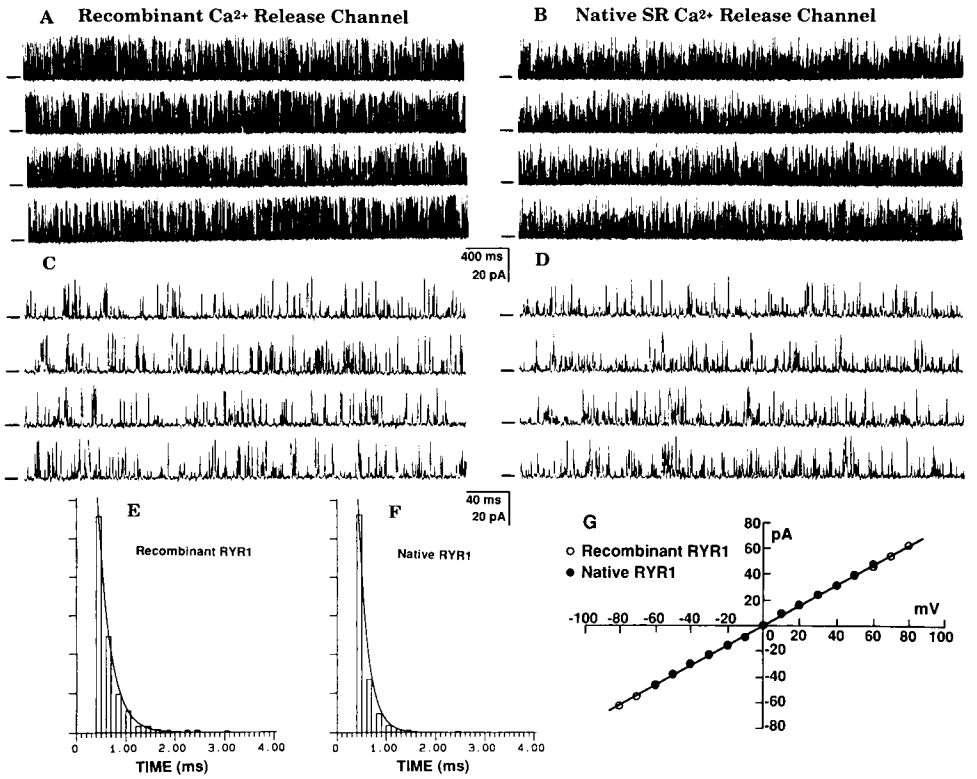

FIGURE 2 Comparison of single-channel properties of recombinant and native single Ca^{2+} release channels. Sucrose density gradient-purified ryanodine receptors from rabbit skeletal muscle heavy sarcoplasmic reticulum vesicles and microsomal membranes of HEK-293 cells transfected with skeletal muscle ryanodine receptor cDNA were incorporated into lipid bilayers as described in Materials and Methods. The *trans* chamber was connected to the headstage input of an amplifier. The *cis* chamber was held at virtual ground. In this experiment, the luminal (*trans*) potentials were varied and reported. A symmetrical recording solution containing 250 mM KCl and 25 mM HEPES (pH 7.4) was used for all recordings. Recordings were filtered at 1 kHz and digitized at 5 kHz. Both native and recombinant single Ca^{2+} channels were first inhibited by 0.1 mM EGTA (*cis*) and then reactivated by 0.1 mM $CaCl_2$ (*cis*), indicating that the cytoplasmic face of the channel was located in the *cis* chamber. Single-channel current fluctuations of the recombinant and native channels in the presence of 2.5 μM free Ca^{2+} (0.1 mM EGTA plus 0.1 mM $CaCl_2$) at +30 mV are shown in *A* and *B*, respectively. Current traces illustrated in *C* and *D* are taken from *A* and *B* and shown in a 10-fold shorter time scale. Baselines are indicated by lines to the left of each current trace. Continuous recordings are shown in each panel. Dwell-time histograms of open events of recombinant and native channels are illustrated in *E* and *F*, respectively. The average open time constant is 0.224 ± 0.027 ms (mean ± SD) (n = 4) for the recombinant channel and 0.220 ± 0.022 ms (mean ± SD) (n = 4) for the native channel. The *y*-axes in the dwell-time histograms *E* and *F* correspond to 52 events/division. (*G*) Current-voltage (*I-V*) relationship of the recombinant channel (○) and native channels (●). Both display a linear relationship with slope conductances of 780 ± 12.8 pS (mean ± SD) (n = 3) for the recombinant channel and 778 ± 5.66 (mean ± SD) (n = 2) for the native channel.

was 2.57 ± 0.45 mV (mean ± SD) (n = 3) (Fig. 4 *C*). The shift in the zero current potential from 0 to 2.57 mV indicated that the recombinant Ca^{2+} release channel was capable of conducting Ca^{2+}.

To determine the Ca^2/K^+ permeability ratio of the recombinant Ca^{2+} release channel, single-channel currents were recorded initially under symmetrical 250 mM KCl. The *trans* chamber (luminal) was then perfused with 50 mM $Ca(OH)_2$, adjusted to pH 7.4 with HCl. Under these biionic conditions, the reversal potential was ~16 mV, and the permeation ratio of pCa^{2+}/pK^+ was estimated to be 6.8 (Fatt and Ginsborg, 1958). The Ca^{2+} conductance of the recombinant channel, estimated from the current-voltage relationship between −20 and −80 mV (cytoplasmic), was 116 ± 7.64 pS under these biionic conditions (n = 3) (Fig. 3 *C*). We also determined the Ca^{2+} conductance under conditions in which the luminal chamber was perfused with 50 mM $Ca(OH)_2$ and the cytoplasmic chamber was subsequently perfused with 125 mM Tris. This measurement yielded a similar Ca^{2+} conductance of 128 pS. These Ca^{2+} permeation and conductance values are very similar to those reported previously for the native Ca^{2+} release channel (Smith et al., 1988).

Activation and inactivation of the recombinant single Ca^{2+} release channel by cytoplasmic Ca^{2+}

The first sign of integration of Ca^{2+} release channels into the bilayer was spontaneous single-channel activity stimulated by the presence of contaminating Ca^{2+} in the recording solution (not shown). The concentration of Ca^{2+} in the bath was then lowered to pCa ~8.15 by the *cis* addition of 0.1 mM EGTA. Under these conditions, the channel was inactivated (Fig. 4 *A*), indicating that it was incorporated into the planar lipid bilayer with its cytoplasmic side facing

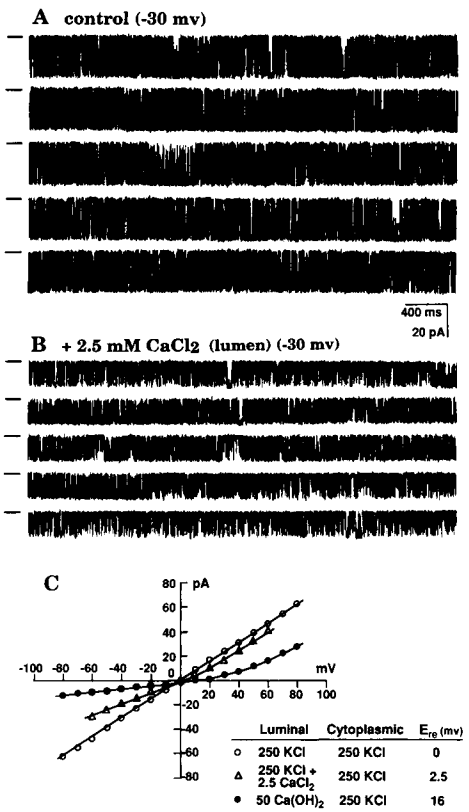

FIGURE 3 Ca^{2+} permeability and Ca^{2+} conductance of recombinant single Ca^{2+} release channels. To facilitate perfusion of the *trans* chamber, the electrode configuration was reversed in this experiment. The *trans* chamber was held at virtual ground, while the *cis* chamber was connected to the headstage input of the amplifier. The cytoplasmic (*cis*) potentials were varied and are reported in this experiment. (*A*) Control single-channel currents, shown as downward deflections, were recorded at −30 mV (cytoplasmic) in symmetrical 250 mM KCl and 25 mM HEPES (pH 7.4), and in the presence of 0.1 mM $CaCl_2$ (*cis*), 0.1 mM EGTA (*cis*), and 2.5 mM ATP (*cis*). The channel was incorporated into the lipid bilayer with its cytoplasmic side facing the *cis* chamber and its luminal side facing the *trans* chamber, because its activity was inhibited by the *cis* addition of EGTA and reactivated by the *cis* addition of $CaCl_2$ (not shown) and further activated by the *cis* addition of ATP. (*B*) Single-channel currents after the subsequent addition of 2.5 mM $CaCl_2$ to the *trans* chamber (the luminal side) of the control channel. Note that the single-channel current amplitude was reduced. Baselines are indicated by a line to the left of each current trace. Recordings (20 s) shown in *A* and *B* are continuous recordings from the same channel. The effects of luminal Ca^{2+} on *I-V* relationships of the recombinant Ca^{2+} release channel are illustrated in *C*. The *I-V* relationship in symmetrical 250 mM KCl (○) is the same as that shown in Fig. 2. In the presence of 2.5 mM luminal $CaCl_2$ and symmetrical 250 mM KCl (△), the unitary conductance was 450 ± 44.8 pS between −60 mV and −10 mV and 761 ± 54.6 pS (mean ± SD) (n = 3) between +10 mV and +60 mV. The reversal potential was 2.5 ± 0.45 mV (mean ± SD) (n = 3). The unitary conductance in the presence of luminal 50 mM $Ca(OH)_2$ and cytoplasmic 250 mM KCl (●) was 116 ± 7.64 pS (mean ± SD) (n = 3) between −10 mV and −80 mV. The reversal potential under this condition was ~16 mV.

the *cis* chamber. All further additions were then made to the *cis* (cytoplasmic) chamber. To determine the effect of cytoplasmic Ca^{2+} on channel activity, Ca^{2+} concentrations in the cytoplasmic chamber were increased stepwise by sequential additions of an aliquot of $CaCl_2$ solution. A few open events were detected when the Ca^{2+} concentration was raised to pCa 7.19. The channel was further activated by increasing the Ca^{2+} concentration. The open probability (P_o) increased from 0.001 to 0.036, 0.086, 0.171, and 0.229 when the Ca^{2+} concentration was raised from pCa 7.19 to 6.59, 5.45, 4.30, and 3.82, respectively. At Ca^{2+} concentrations above 100 μM (pCa ~4), however, Ca^{2+} became inhibitory. P_o decreased from 0.229 to 0.095, 0.017, and 0.002 when Ca^{2+} concentrations were raised from pCa 3.82 to 3.46, 2.73, and 2.55, respectively.

A curve depicting the effect of Ca^{2+} concentration on P_o was bell-shaped (Fig. 4 *J*). P_o increased with increasing Ca^{2+} concentration in the range between 0.1 μM and 100 μM, peaked at ~100 μM, and decreased with increasing Ca^{2+} concentration in the range between 100 μM and 2 mM. Further kinetic analysis of the effect of cytoplasmic Ca^{2+} on P_o revealed that changes in P_o were due mainly to changes in the duration of the mean closed time (Fig. 4 *K*). The duration of the mean open time varied by about twofold, from 0.567 ms to 1.18 ms, whereas the duration of the mean closed time changed more than 100-fold when Ca^{2+} concentrations were changed from pCa 8.15 to 2.55. Similarly, the mean open time varied by about twofold, and the mean closed time varied by ~100-fold for the native Ca^{2+} release channel of rabbit skeletal muscle sarcoplasmic reticulum when pCa was varied from 8.15 to 3.0 (not shown). Ca^{2+}, therefore, activates and inactivates the channel, primarily through modulating the duration of the closed state of the channel.

Effects of modulators on recombinant single-channel activity

Besides Ca^{2+}, the recombinant channel was also modulated by ATP, Mg^{2+}, caffeine, ryanodine, and ruthenium red (Fig. 5). A spontaneous channel was first inhibited by the *cis* (cytoplasmic) addition of 0.1 mM EGTA (not shown) and reactivated by the *cis* addition of 0.1 mM $CaCl_2$ (Fig. 5 *A*). Subsequent addition of 2 mM ATP increased the P_o and mean open time, and decreased the mean closed time (Fig. 5 *B*). In a total of nine similar experiments, the increase in P_o and mean open time, and the decrease in mean closed time after the addition of ATP are statistically significant (paired t-test). After the addition of ATP, the average P_o was increased by 5.63 ± 3.0-fold ($p < 0.0001$), the average mean open time was increased by 2.02 ± 0.4-fold ($p < 0.005$), and the average mean closed time was decreased to 23 ± 13% ($p < 0.002$) compared with values before the addition of ATP. Subsequent addition of 2.2 mM $MgCl_2$ decreased both P_o and mean open time, and increased mean closed time (Fig. 5 *C*). After the addition of $MgCl_2$, the average P_o was decreased to 13 ± 10% ($p < 0.0005$), the average mean open time was decreased to 47 ± 15% ($p < 0.02$), and the average mean closed time was increased by 9.33 ± 6.1-fold ($p < 0.03$) (n = 7) compared with those

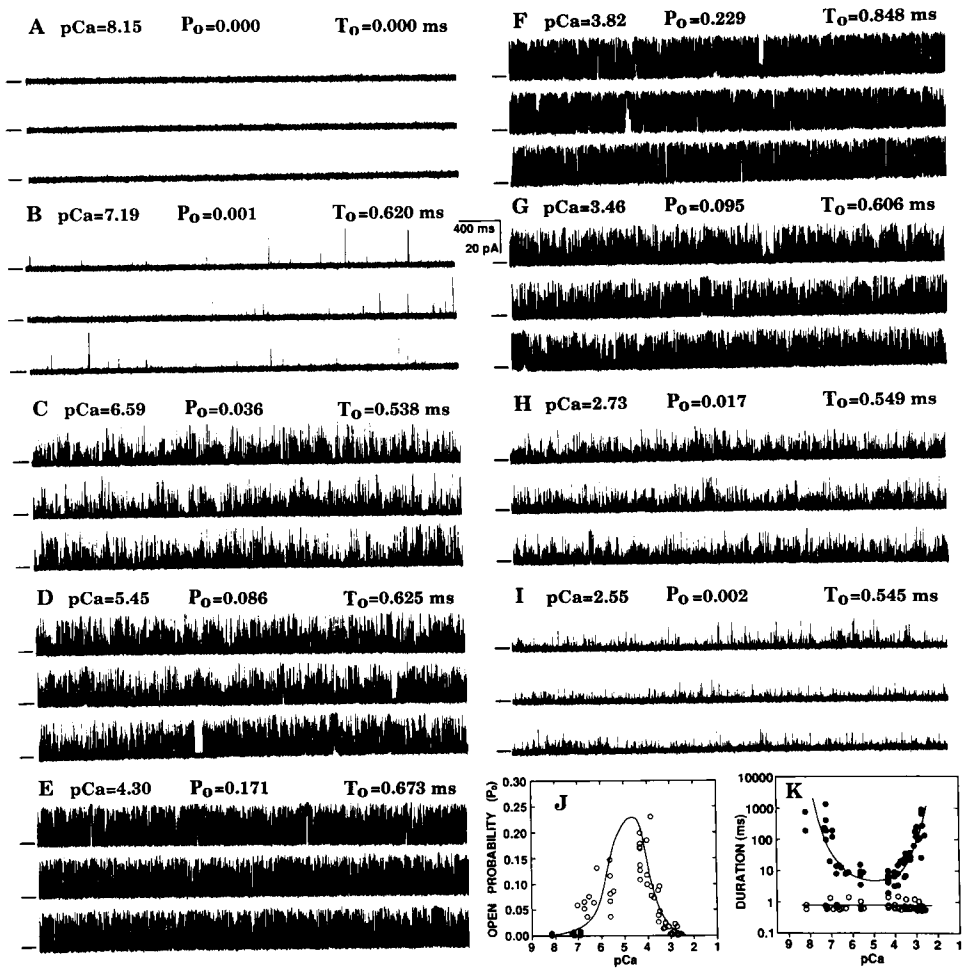

FIGURE 4 The response to cytoplasmic Ca^{2+} of recombinant single Ca^{2+} release channels. Single-channel currents were recorded at +30 mV (luminal) in symmetrical 250 mM KCl, as described in Fig. 2. (*A*) A spontaneous single channel was first blocked by 0.1 mM EGTA (*cis*), indicating that the cytoplasmic side of the channel was facing the *cis* chamber. The Ca^{2+} concentration of the *cis* chamber (cytoplasmic Ca^{2+} level) was then increased gradually from pCa 8.15 to pCa 2.55 by the sequential addition of an aliquot of 10 mM or 100 mM $CaCl_2$ solution. The pCa value, open probability (P_o), and arithmetic mean open time (T_o) at each free cytoplasmic Ca^{2+} concentration are indicated at the top of each panel. A continuous channel recording (12 s) is shown in each of panels *A–I*. Openings are upward and baselines are indicated by a line to the left of each current trace. All recordings are from the same channel. The relationship between the open probability and the Ca^{2+} concentration is shown in *J*. Each point represents the average open probability of an average recording time of 128 s at a Ca^{2+} concentration varying from pCa 8.15 to pCa 2.55. A total of 64 points were obtained from seven separate experiments conducted like that shown in *A–I*. The relationship between the dwell time and the Ca^{2+} concentration is illustrated in *K*. ○, Arithmetic mean open times; ○, arithmetic mean closed times at different Ca^{2+} concentrations. Arithmetic mean open and closed times were obtained from single-channel recordings from which *J* was derived. Note that the arithmetic mean open times remained relatively unchanged when the Ca^{2+} concentration was varied. The average arithmetic mean open time was 0.702 ms.

before the addition of $MgCl_2$. The Mg^{2+}-inhibited channel could be reactivated by the addition of 3 mM caffeine (Fig. 5 *D*). After the addition of caffeine, the average P_o was increased by 12.6 ± 5.7-fold ($p < 0.005$), the average mean open time was increased by 1.94 ± 0.8-fold ($p < 0.02$), and the average mean closed time was decreased to 12 ± 8% ($p < 0.04$) ($n = 6$) compared with those before the addition of caffeine. The channel kinetics and conductance were dramatically altered upon the addition of 10 μM ryanodine (Fig. 5 *E*). Conductance was reduced to 60 ± 2% of the unmodified channel, from 646 ± 60 pS to 385 ± 22 pS. The ryanodine-modified channel was blocked by 30 μM ruthenium red (Fig. 5 *F*). These data clearly demonstrate that the recombinant Ca^{2+} release channels are modulated by various ligands with patterns similar to those observed with the native Ca^{2+} release channel (Coronado et al., 1994).

Blockade of the recombinant single Ca^{2+} release channel

Long closed events were often observed in the current records of most recombinant Ca^{2+} release channels, particularly those in which current flowed from the cytoplasmic to the luminal side of the channel. In experiments described above, the current records show luminal-to-cytoplasmic current, which represents the direction of Ca^{2+} flow during Ca^{2+} release from the sarcoplasmic reticulum. The cyto-

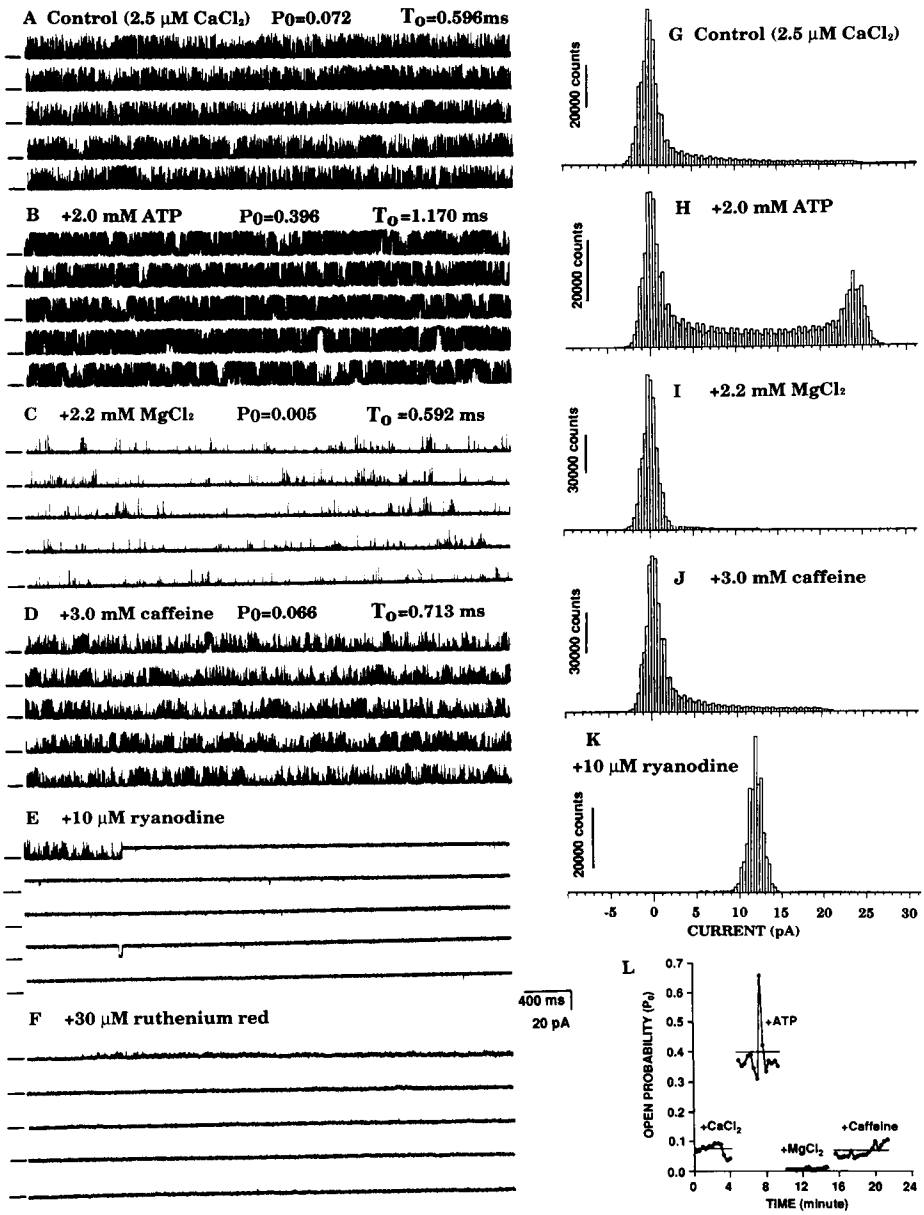

FIGURE 5 Ligand gating properties of recombinant single Ca^{2+} release channels. Single-channel recordings were carried out as described in the legend to Fig. 2. A spontaneous single-channel activity was first inhibited by the addition of 0.1 mM EGTA (*cis*) (not shown) and reactivated by the addition of 0.1 mM $CaCl_2$ (*cis*), as shown in the control recording (*A*), suggesting that the channel was incorporated into the bilayer with its cytoplasmic side facing the *cis* chamber. Single-channel currents before (control) and after sequential additions (*cis*) of 2.0 mM ATP (*B*), 2.2 mM $MgCl_2$ (*C*), 3.0 mM caffeine (*D*), 10 μM ryanodine (*E*), and 30 μM ruthenium red (*F*) were recorded in symmetrical 250 mM KCl at +30 mV (luminal). The average open probability (P_o) of 4–5-min continuous recordings and the arithmetic mean open time (T_o) for each condition are indicated at the top of each panel (*A–D*). Amplitude histograms of 4–5 min of continuous recording of control (*G*), after sequential additions of 2.0 mM ATP (*H*), 2.2 mM $MgCl_2$ (*I*), 3.0 mM caffeine (*J*), and 10 μM ryanodine (*K*) are displayed. All current recordings were from the same channel. A diary plot of open probability is shown in *L*. Each point represents the average open probability of a 20-s recording. The average open probability for each condition is indicated by a horizontal line.

plasmic to luminal current was often blocked in these cases. Fig. 6 illustrates one of these asymmetrically blocked channels. At +30 mV (luminal), the channel was conducting a luminal-to-cytoplasmic current with properties similar to those described earlier (Fig. 6 *A*). At −30 mV (luminal), however, the channel began conducting in the cytoplasmic-to-luminal direction, but closed a few seconds after the direction of voltage was switched (Fig. 6 *B*). The channel reactivated only after the voltage was changed from −30 back to +30 mV (Fig. 6 *C*). Asymmetrical blockade of single-channel currents was observed in most of the single recombinant channels that we detected.

DISCUSSION

In this study we describe the expression of a full-length cDNA encoding the rabbit skeletal muscle sarcoplasmic reticulum Ca^{2+} release channel (the ryanodine receptor) and the functional characterization of the expressed protein. The Ca^{2+} release channel expressed in HEK-293 cells was characterized by immunochemical assays and by single-channel recordings in planar lipid bilayers. The expressed protein was recognized specifically by an anti-rabbit ryanodine receptor antibody (Fig. 1) and exhibited mobilities in sodium dodecyl sulfate-polyacrylamide gels and in sucrose-density gradients that were identical to those of the native channel (not shown). The recombinant ryanodine receptor formed a large conductance channel with a slope conductance of ~780 pS in 250 mM KCl and 116 pS in 50 mM Ca^{2+} and displayed fast gating kinetics with an open time constant of ~0.22 ms (Figs. 2 and 3). Like the native Ca^{2+} release channel, the recombinant Ca^{2+} release channel was more permeable to Ca^{2+} than to K^+. The permeation ratio, $P^{Ca^{2+}}/P^{K^+}$, was ~6.8 (Fig. 3). Furthermore, the open prob-

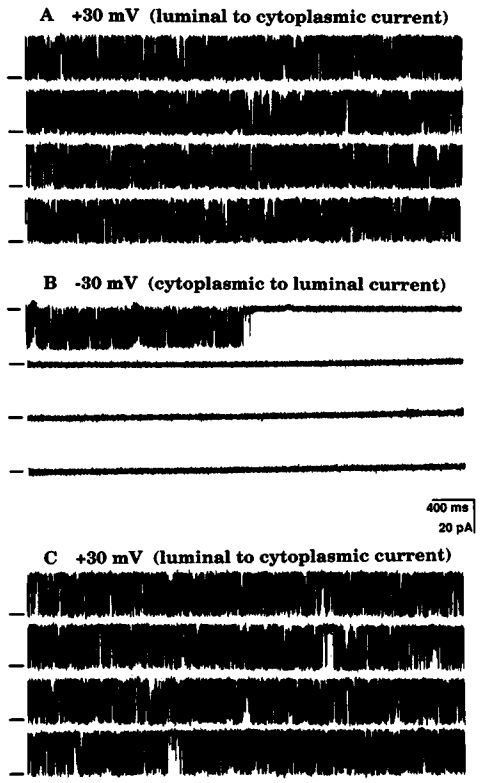

FIGURE 6 Asymmetrical blockade of recombinant single Ca^{2+} release channels. A spontaneous channel activity was inactivated by *cis* 0.21 mM EGTA, reactivated by *cis* 0.21 mM $CaCl_2$, and further activated by *cis* 2.0 mM ATP, indicating that the cytoplasmic face of the channel lies in the *cis* chamber. In this experiment, the luminal (*trans*) potentials were varied and reported. (*A*) Single-channel currents recorded at +30 mV in symmetrical 250 mM KCl. Openings are upward and baselines are indicated by lines on the left of each current trace. Under these conditions, the direction of the current flow was from the luminal to the cytoplasmic side of the channel. Long-lived closed events were rare under these conditions. (*B*) Single-channel currents shortly after the voltage was changed from +30 mV to −30 mV. Openings are downward and baselines are marked. At −30 mV, current flowed from the cytoplasmic to the luminal side. Note that the channel was completely closed after opening for several seconds at −30 mV. Single-channel currents recorded after the voltage was changed from −30 mV back to +30 mV are shown in *C*. Openings are shown as upward deflections. Note that the channel was reactivated at +30 mV. A 16-s continuous recording is shown in each panel; all recordings are from the same channel.

ability of the recombinant channel was regulated by cytoplasmic Ca^{2+} in a typical bell-curve fashion in which the channel was activated by Ca^{2+} at micromolar concentrations and inhibited by millimolar Ca^{2+} (Fig. 4). The recombinant Ca^{2+} release channels were also modulated by ATP, Mg^{2+}, caffeine, ryanodine, and ruthenium red with patterns similar to those observed with the native channels (Fig. 5). These results demonstrate that the Ca^{2+} release channel expressed in HEK-293 cells possesses all of the major characteristics of the native single Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum.

Functional expression of the rabbit skeletal muscle ryanodine receptor cDNA in a heterologous cell culture system has been the subject of previous investigations. Penner et al. (1989), Otsu et al. (1994), and Treves et al. (1994) reported the expression of skeletal muscle ryanodine receptor cDNAs in CHO cells, C2C12 cells, and COS-7 cells, respectively. In these studies, the expressed ryanodine receptor appeared to function as an intracellular Ca^{2+} release channel at the whole-cell level. However, gating kinetics, channel permeation and conductance, and response to cytoplasmic Ca^{2+} and modulation by ATP, Mg^{2+}, and ruthenium red of the recombinant Ca^{2+} release channel have not been reported.

We have described the expression of the ryanodine receptor cDNA in COS-1 cells (Chen et al., 1993). The Ca^{2+} release channel expressed in COS-1 cells displayed anomalous single-channel conductances. The reason for these differences between recombinant and native channels is not clear, but the abnormal conductance states of the expressed channel may have been artifacts induced by channel purification and manipulation. For instance, sucrose gradient fractions containing the CHAPS-solubilized expressed channel were concentrated up to 30-fold to enrich the expressed channel protein, which was expressed at a very low level in COS-1 cells. This concentrating step may have resulted in channel aggregation or partial denaturation. Alternatively, COS-1 cells may have lacked specific components that are essential for the proper function and regulation of the skeletal muscle Ca^{2+} release channel.

Coexpression of FKBP12 cDNA improved the stability and conductance of the Ca^{2+} release channel expressed in insect cells (Brillantes et al., 1994), suggesting that FKBP12 is an important component for the proper function of the Ca^{2+} release channel. Because the Ca^{2+} release channel expressed in HEK-293 cells exhibits properties very similar to those of the native channel, it is likely that FKBP12 is expressed endogenously in HEK-293 cells and associates with the Ca^{2+} release channel. The presence of the FKBP12 message in HEK-293 cells was confirmed by reverse transcriptase polymerase chain reaction and sequence analysis of the amplified cDNA (not shown). The deduced amino acid sequence of the FKBP12 cDNA, expressed in the HEK-293 human cell line, was identical to the sequence previously reported for human FKBP12 (Standaert et al., 1990). The presence of FKBP12 in whole cells was confirmed by immunocytochemical staining, and the presence of FKBP12 in fractions containing the expressed Ca^{2+} release channel was confirmed by immunoblotting. The presence of endogenous FKBP12 in HEK-293 cells may be one of the factors that contributes to the high level of expression and the highly conserved function of the recombinant skeletal muscle Ca^{2+} release channel.

The physiological role of FKBP12 is not fully understood. It may have multiple functions in the regulation of the Ca^{2+} release channel. We found that excess FKBP12 added to the cytoplasmic face of the CHAPS-solubilized native Ca^{2+} release channel blocked the channel asymmetrically, allowing current to flow only from the luminal side to the cytoplasmic side, but not in the reverse direction (Chen et al., 1994). Removal of excess added FKBP12 after the onset

of asymmetrical blockade did not relieve the inhibition, suggesting that the binding of FKBP12 to the Ca^{2+} release channel, which leads to asymmetrical blockade, is tight. In the absence of added FKBP12, asymmetrical blockade was occasionally observed in native Ca^{2+} release channels. By contrast, most recombinant Ca^{2+} release channels from HEK-293 cells displayed asymmetrical blockade, conducting current unidirectionally from the luminal to the cytoplasmic side (Fig. 6). When we observed bidirectional current, the currents in both directions were modulated by the same ligands. The asymmetrical blockade of the Ca^{2+} release channels expressed in HEK-293 cells appeared to be similar to that observed when excess FKBP12 was added to native channels. However, further investigation is required to determine whether the endogeneous FKBP12 in HEK-293 cells is involved in asymmetrical blockade of the recombinant channels. A similar asymmetrical blockade has also been observed in rabbit (Ma et al., 1995) and chicken (Percival et al., 1994) skeletal muscle ryanodine receptor isoforms.

Although most recombinant channels exhibited similar gating kinetics and sensitivity to Ca^{2+} activation and Ca^{2+} inactivation, the extent of maximum activation by Ca^{2+} varied from channel to channel. Of 10 single recombinant channels analyzed, seven showed a maximum P_o of ~0.2, whereas three channels displayed a maximum P_o of ~0.6. Different levels of maximum activation of the α-ryanodine receptor of chicken skeletal muscle by Ca^{2+} have also been reported (Percival et al., 1994). In this system, the difference in maximum P_o was believed to result from gating mode switching between a low- and a high-activity mode. We do not know whether variation in maximum P_o of the recombinant channels results from gating mode switching, because we have not observed channels that switch between low and high activity spontaneously. Considering the high degree of retention of native channel function, the recombinant channel expressed in HEK-293 cells should be useful for studying the effects of mutation on conduction, permeation, gating kinetics, Ca^{2+} activation and inactivation, and modulation by ATP, Mg^{2+}, caffeine, ryanodine, and ruthenium red.

This research was supported by grant MT-3399 from the Medical Research Council of Canada (MRCC) and by a grant from the Canadian Genetic Diseases Network of Centers of Excellence to DHM. SRWC was supported by a postdoctoral fellowship from the MRCC, PL by a studentship from the MRCC, and JPI by a postdoctoral fellowship from the Heart and Stroke Foundation of Canada.

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