# Expression and Functional Characterization of the Cardiac Muscle Ryanodine Receptor $Ca^{2+}$ Release Channel in Chinese Hamster Ovary Cells

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ABSTRACT To study the function and regulation of the cardiac ryanodine receptor (RyR2) $Ca^{2+}$ release channel, we expressed the RyR2 proteins in a Chinese hamster ovary (CHO) cell line, and assayed its function by single channel current recording and confocal imaging of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$). The 16-kb cDNA encoding the full-length RyR2 was introduced into CHO cells using lipofectAmine and electroporation methods. Incorporation of microsomal membrane vesicles isolated from these transfected cells into lipid bilayer membrane resulted in single $Ca^{2+}$ release channel activities similar to those of the native $Ca^{2+}$ release channels from rabbit cardiac muscle SR membranes, both in terms of gating kinetics, conductance, and ryanodine modification. The expressed RyR2 channels were found to exhibit more frequent transitions to subconductance states than the native RyR2 channels and RyR1 expressed in CHO cells. Caffeine, an exogenous activator of RyR, induced release of $[Ca^{2+}]_i$ from these cells. Confocal imaging of cells expressing RyR2 did not detect spontaneous or caffeine-induced local $Ca^{2+}$ release events (i.e., "$Ca^{2+}$ sparks") typically seen in cardiac muscle. Our data show that the RyR2 expressed in CHO cells forms functional $Ca^{2+}$ release channels. Furthermore, the lack of localized $Ca^{2+}$ release events in these cells suggests that $Ca^{2+}$ sparks observed in cardiac muscle may involve cooperative gating of a group of $Ca^{2+}$ release channels and/or their interaction with muscle-specific proteins.

## INTRODUCTION

In cardiac muscle, excitation-contraction (E-C) coupling involves entry of extracellular $Ca^{2+}$ through voltage-sensitive $Ca^{2+}$ channels, which in turn triggers release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR), via a $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mechanism. This phenomenon is mediated by ryanodine receptor (RyR) which functions as $Ca^{2+}$ release channel (Fleischer and Inui, 1989; McPherson and Campbell, 1993; Sutko and Airey, 1996). RyR is a single polypeptide of ~560 kDa, and exists in a homotetrameric structure with at least two functional domains: a carboxyl-terminal hydrophobic domain containing the conduction pore of the $Ca^{2+}$ release channel (Takeshima et al., 1989; Zorzato et al., 1990; Bhat et al., 1997b), and a large amino-terminal cytoplasmic domain referred to as the "foot structure" (Block et al., 1988; Lai et al., 1989; Sorrentino and Volpe, 1993; Franzini-Armstrong and Jorgensen, 1994). The cardiac (RyR2) and skeletal (RyR1) $Ca^{2+}$ release channels are encoded by different genes, and share a high degree (~66%) of amino acid sequence identity, especially in the carboxyl-terminal region, which contains several putative transmembrane segments (Takeshima et al., 1989; Zorzato et al., 1990; Nakai et al., 1990; Otsu et al., 1990; Wagenknecht et al., 1989; Takeshima, 1993). The carboxyl-terminal region of the protein also contains putative binding site(s) for $Ca^{2+}$ and ryanodine (Callaway et al., 1994; Witcher et al., 1994). In recent studies, we have successfully used a heterologous expression system to study the structure-function relationship of the skeletal $Ca^{2+}$ release channel (Bhat et al., 1997a–c). Full-length RyR1 expressed in Chinese hamster ovary (CHO) cells exhibits single channel properties similar to those of RyR from skeletal muscle SR. The carboxyl-terminal ~20% of the RyR1 (RyR-C) was found to contain structures sufficient to form a functional $Ca^{2+}$ release channel (Bhat et al., 1997b). The amino-terminal foot structure appears to participate in the ion-conduction, $Ca^{2+}$-dependent regulation, and caffeine-induced activation of the $Ca^{2+}$ release channel (Bhat et al., 1997a–c).

Compared with RyR1, it has been difficult to study the structure-function relationship of RyR2. First, the cDNA for RyR2 is intrinsically unstable, which frequently undergoes large deletions and/or recombination during its propagation in *Escherichia coli* strains making the DNA preparation difficult. Second, it is not easy to select stable mammalian cell clones expressing RyR2 proteins. Nakai et al. (1990) expressed and indirectly studied the function of the RyR2 channel in *Xenopus* oocytes by measuring $Ca^{2+}$-dependent chloride current in response to stimulation with caffeine. Caffeine-induced $Ca^{2+}$ release as well as $Ca^{2+}$-dependent [$^3$H]ryanodine binding were studied by Imagawa et al. (1992) in CHO cells expressing RyR2. But, no single channel studies with expressed RyR2 have been reported thus far. In the present study, we have successfully overcome the problem of RyR2 cDNA instability in *E. coli* cells and

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expressed the full-length RyR2 protein in CHO cells. The $Ca^{2+}$ release channel activity of the expressed RyR2 was studied using single channel current measurements and by intracellular $Ca^{2+}$ imaging in single cells using laser scanning confocal microscopy. The single channel properties of RyR2 expressed in CHO cells were similar to those of native $Ca^{2+}$ release channels from the rabbit cardiac muscle SR. RyR2 channels expressed in CHO cells were found to exhibit multiple conductance states more frequently than the native $Ca^{2+}$ release channels from the cardiac muscle SR. Caffeine, an exogenous activator of RyR, induced release of $[Ca^{2+}]_i$ from cells expressing RyR2. Confocal imaging of single CHO cells expressing RyR2 did not detect any spontaneous or caffeine-induced local $Ca^{2+}$ release events (viz., "$Ca^{2+}$ sparks") typically seen in cardiac muscle cells.

## MATERIALS AND METHODS

### Cells and expression system

The entire cDNA sequence (~16.5 kb) of the rabbit cardiac muscle RyR was cloned into the pHRRS1 expression vector and the transcription occurs under the control of the SV40 promoter (Nakai et al., 1990). This DNA was transformed into a competent HB101 strain of *E. coli* cells and grown in LB medium at 30°C. The bacteria were harvested for DNA isolation mid-to-late in the logarithmic period of growth. CHO cells were grown at 37°C and 5% $CO_2$ in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. The expression plasmids were introduced into the cells (60–70% confluent) using lipofectAmine reagent (Life Technologies, Inc., Gaithersburg, MD) following manufacturer's instructions, or by electroporation methods (Imagawa et al., 1992). Stable transfectant cells were selected with G418 (0.5 mg/ml, Calbiochem, La Jolla, CA) ~48 h after transfection. The level of RyR2 protein expression was tested using Western blot analysis.

### Western blot analyses

Control and transfected CHO cells were harvested and washed twice with ice-cold PBS and lysed with ice-cold modified RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate) in the presence of protease inhibitors (0.5 mM Pefabloc, 1 $\mu$M pepstatin, 1 $\mu$M leupeptin, 1 $\mu$g/ml aprotinin, and 1 mM benzamidine). The proteins in the whole cell lysate were mixed with the 2X sample buffer (200 mM Tris-Cl, pH 6.7, 9% SDS, 6% $\beta$-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue) and separated on a 3–12% linear gradient SDS-PAGE gel after heating the samples at 37°C for ~15 min. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane and blotted with C3-33 monoclonal antibody raised against the RyR2 protein (Affinity BioReagents, Golden, CO), and horseradish peroxidase-linked secondary antibody. The proteins were visualized using the enhanced chemiluminescence detection system (Amersham Corp., Piscataway, NJ).

### Confocal imaging of intracellular $[Ca^{2+}]$

Single rat cardiac ventricular cells were obtained from two-month-old Sprague-Dawley rats by an enzymatic technique described in detail previously (Lopez-Lopez et al., 1995). Both cardiac myocytes and CHO cells expressing RyR2 were loaded with the $Ca^{2+}$ indicator Fluo-3 by incubation for 30 min or longer in Tyrode's solution to which 10 $\mu$M Fluo-3 AM was added (Molecular Probes Inc., Eugene, OR). Recordings of $[Ca^{2+}]$ were made in normal Tyrode's solution (composition in mM: NaCl, 140; dextrose, 10; Hepes, 10; KCl, 4.0; $MgCl_2$, 1; $CaCl_2$, 1; pH adjusted to 7.3–7.4 with NaOH) at room temperature, as described (Bhat et al., 1997c). For "x-y" or "full-frame" imaging of calcium in the CHO cells a Bio-Rad MRC 600 confocal microscope (Bio-Rad Laboratories, Inc., Hercules, CA) was used. Fluo-3 fluorescence line-scan images were acquired with the homemade confocal microscope attached to the camera port of a Nikon Diaphot inverted microscope equipped with a 60× plan-apo oil-immersion objective (numerical aperture 1.4), with a resolution of 3 ms per scan line (Parker et al., 1997). The fluorescence is expressed as normalized increases in fluorescence compared to "resting" level ($F/F_0$).

### Isolation of SR membranes from rabbit cardiac muscle

Junctional SR membranes were isolated from rabbit cardiac muscle following the procedure similar to that used to prepare the skeletal muscle SR membranes (Ma et al., 1995). Briefly, cardiac muscle tissues were homogenized in 100 mM NaCl, 2 mM EDTA, 0.1 mM EGTA, and 5 mM Tris-Maleate (pH 6.8). Microsome vesicles obtained after sequential centrifugation at 2600 × *g* and 35,000 × *g* were loaded onto discontinuous sucrose gradients. The junctional SR membranes were recovered from the 40–45% region of the gradients. The junctional SR membrane vesicles were stored at −75°C at a concentration of 3–5 mg protein/ml; 1–3 $\mu$l of the vesicles were used for recording of single channel currents in the lipid bilayer.

### Isolation of microsomal membrane vesicles from CHO cells

Microsomal membrane vesicles were isolated from transfected CHO cells as described (Bhat et al., 1997b). Briefly, the cells were homogenized on ice in hypotonic lysis buffer (10 mM Hepes-Tris, pH 7.4, 1 mM EDTA) containing protease inhibitors (0.5 mM Pefabloc-SC, 1 $\mu$M pepstatin, 1 $\mu$M leupeptin, 1 $\mu$g/ml aprotinin, and 1 mM benzamidine) using nitrogen cavitation (300 Psi for 15 min on ice) and with 10 strokes in a tight-fitting Dounce homogenizer, followed by 15 strokes after addition of an equal volume of sucrose buffer (500 mM sucrose, 10 mM Hepes-Tris, pH 7.4, 1 mM EDTA). Microsome vesicles were collected by centrifugation of post-nuclear supernatant (10,000 × *g*, 15 min) at 100,000 × *g* for 45 min at 4°C. The pellet was resuspended in a buffer containing 250 mM sucrose, 10 mM Hepes-Tris, pH 7.2. The membrane vesicles were stored at a protein concentration of 2–6 mg/ml at −75°C until use. Usually, 1–3 $\mu$l of microsomal membrane vesicles was used for reconstitution of $Ca^{2+}$ release channels in the lipid bilayer system.

### Reconstitution of $Ca^{2+}$ release channels in lipid bilayer membrane

Lipid bilayer membranes were formed across an aperture of ~200 $\mu$m diameter using the Muller-Rudin method with a mixture of phosphatidylethanolamine/phosphatidylserine/cholesterol (6:6:1); the lipids were dissolved in decane at a concentration of 40 mg/ml. Incorporation of the $Ca^{2+}$ release channel in bilayer was achieved by addition of membrane vesicles containing RyR2 proteins to the *cis* solution, under a concentration gradient of 200 mM (*cis*)/50 mM (*trans*) cesium gluconate. After incorporation of a single $Ca^{2+}$ release channel, the concentration of cesium gluconate in the *trans* solution was adjusted to 200 mM. The pH in both *cis* and *trans* solutions was maintained throughout the experiment at 7.4 with 10 mM Hepes-Tris. The free $Ca^{2+}$ concentration in both solutions was buffered with 1 mM EGTA, and measured using a $Ca^{2+}$-sensitive electrode (Orion, Boston, MA). Orientation of the $Ca^{2+}$ release channel in the lipid bilayer, usually in the *cis*-cytoplasmic *trans*-luminal SR manner, was determined by the sensitivity of the channel to cytoplasmic $Ca^{2+}$ (Bhat et al., 1997b). To maintain stability of the bilayer membrane and channel activity, designed pulse protocols were used to measure currents through the single $Ca^{2+}$ release channels. The bilayer membrane was kept at a

holding potential of 0 mV, and pulsed to different test potentials of 0.5–1-s durations. Single channel currents were recorded with an Axopatch 200A patch clamp unit (Axon Instruments, Inc., Foster City, CA). Data acquisition and pulse generation were performed with a 486 computer and 1200 Digidata A/D-D/A convertor (Axon Instruments). The currents were sampled at 0.05 ms/point and filtered at 1 kHz through an 8-pole Bessel filter. Single channel data analyses were performed with the pClamp program.

## RESULTS AND DISCUSSION

### Expression of full-length cardiac RyR in CHO cells

The expression vector pHRRS1 contains the cDNA sequence (~16.5 kb) encoding the full-length RyR2 protein. One of the commonly encountered difficulties in working with large DNA molecules such as pHRRS1 (total size ~24 kb) is their tendency to be unstable in that the cDNA undergoes spontaneous deletions and/or rearrangements during plasmid propagation. This phenomenon appears to be specific for RyR2 cDNA since we encountered no such problems with the RyR1 cDNA (Bhat et al., 1997a–c). We have optimized the procedure to overcome this problem and to stabilize the DNA sequence by growing the host bacterial strain (HB101) at a lower temperature (30°C) and by harvesting the cells for plasmid DNA isolation before the culture grows to saturation. Of the several bacterial strains tested (such as DH5$\alpha$, JM109, SURE, HB101), we found HB101 to be efficient for stable propagation of RyR2 cDNA. A similar technique has been used to reduce the probability of instability of retroviral DNA clones that are otherwise unstable (Kanahan et al., 1991; Joshi and Jeang, 1993).

CHO cells were transfected with pHRRS1 using the cationic lipid lipofectAmine as described by the manufacturer, or using electroporation as described (Imagawa et al., 1992). Transfected cells were isolated ~48 h after transfection, and the expression of RyR2 protein was assayed by Western blot analysis (Fig. 1). CHO cells transfected with pHRRS1 expressed a protein of high $M_r$ (~560 kDa, *lane 7*) that is identical to RyR2 from rabbit cardiac muscle SR (*lane 4*). These proteins were detected with a monoclonal antibody (C3-33) raised against canine cardiac ryanodine receptor, and this antibody also recognizes RyR1 from skeletal muscle SR as well as that expressed in CHO cells (*lanes 2 and 3*). No protein was recognized by the C3-33 antibody in untransfected CHO cells (*lane 1*), indicating that CHO cells do not contain any detectable levels of endogenous RyR1 and RyR2.

To isolate stable clones expressing RyR2, CHO cells expressing RyR2 were cultured by limiting dilution in media containing G418 (0.5 mg/ml). Of the 28 clones analyzed, two (clones C-26 and C-53) were found to express proteins of significantly lower molecular mass than the native RyR2 (Fig. 1, *lane 4*) or RyR2 transiently expressed in CHO cells (*lane 7*), and both these proteins were recognized by the monoclonal antibody C3-33 (Fig. 1, *lanes 5 and 6*). This suggests that the RyR2 cDNA has undergone deletions and/or rearrangements in these stable CHO clones, similar to its instability in bacterial host cells as described above. This result raises the need for caution in the expression of functional RyR2 proteins in heterologous systems. While the reason for this phenomenon is not clearly understood, in this study we have used transiently transfected CHO cells expressing only the high molecular weight (~560 K) RyR2 for functional analysis.

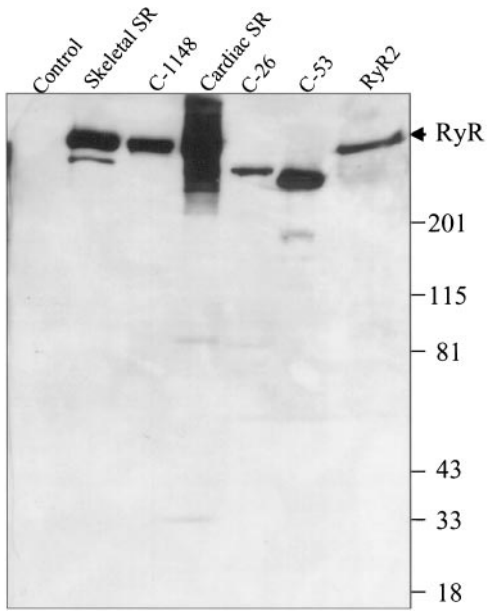


FIGURE 1 Heterologous expression of cardiac muscle RyR (RyR2) in CHO cells. Proteins from whole-cell lysates from control CHO cells or those transfected with RyR2 cDNA were separated on 3–12% SDS gel and probed with C3-33 monoclonal antibody raised against canine cardiac RyR. *Lane 1*, control untransfected CHO cells; *lane 2*, skeletal muscle junctional SR; *lane 3*, RyR1 stably expressed in CHO cells (clone C-1148); *lane 4*, native RyR2 from rabbit cardiac muscle SR; *lane 7*, RyR2 expressed in CHO cells. Two stable CHO cell clones C-26 and C-53 express RyR2 proteins of lower molecular mass (*lanes 5 and 6*), possibly due to rearrangement of the RyR2 cDNA. The C3-33 antibody also recognizes the RyR1 protein from skeletal muscle SR membranes (*lane 2*) and that expressed in CHO cells (*lane 3*). In separate experiments, the RyR2 proteins could not be detected with a monoclonal antibody specific for the carboxyl-terminal portion of RyR1 (data not shown).

The function of RyR2 expressed in CHO cells was studied by measuring the changes in intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) in response to stimulation with caffeine, which is an activator of the $Ca^{2+}$ release channel (Fig. 2). Application of 10 mM caffeine to CHO cells expressing RyR2 resulted in an increase of $[Ca^{2+}]_i$ in a reversible manner in two of the four cells shown in Fig. 2 *A* (*cells 1 and 2*). No changes in $[Ca^{2+}]_i$ were observed in untransfected CHO cells (not shown). The absence of caffeine response in cells 3 and 4 in Fig. 2 *A* is likely due to the lack of RyR2 expression in these cells. The ability of caffeine to induce $Ca^{2+}$ release suggests that RyR2 expressed in CHO cells is capable of functioning as $Ca^{2+}$ release channels (Imagawa et al., 1992).

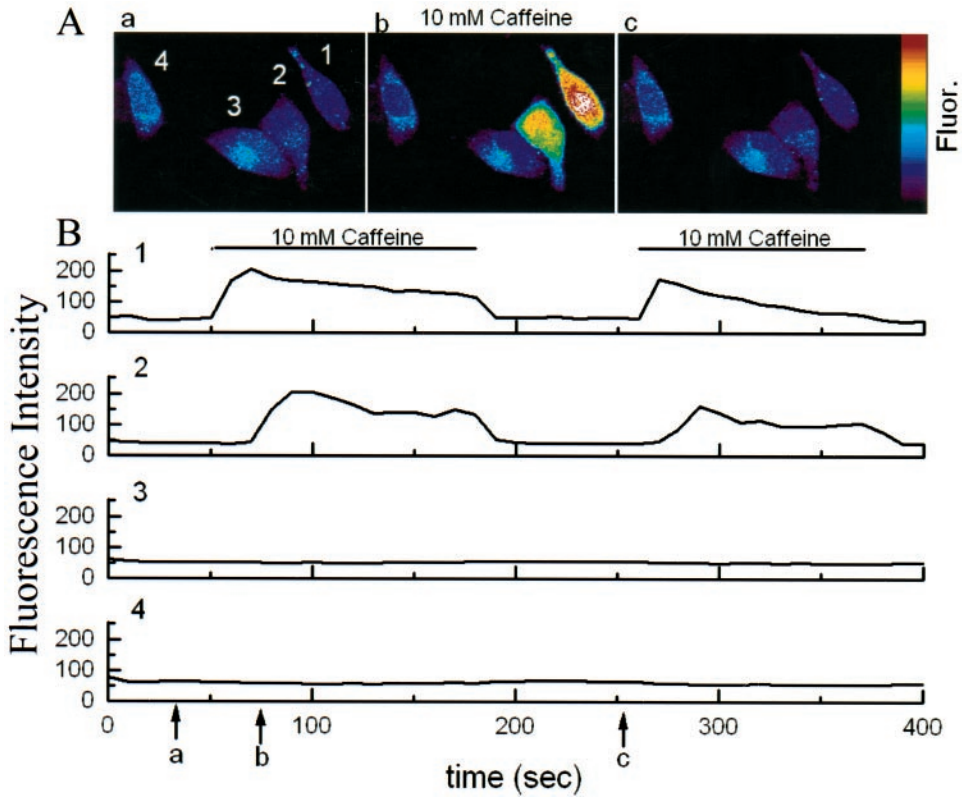


FIGURE 2 Caffeine-induced intracellular $Ca^{2+}$ release in CHO cells expressing RyR2. (*A*) Full-frame confocal image of Fluo-3 fluorescence. Cells maintained at room temperature are superfused with Tyrode's solution with or without caffeine. *a*, Control; *b*, after addition of 10 mM caffeine; *c*, after caffeine washout. (*B*) Time-dependent changes in Fluo-3 fluorescence in four cells shown in the top panel. The images are representative of at least five experiments from three different transfections. Those cells not responding to caffeine (cells 3 and 4) likely lack expression of RyR2 proteins.

## Comparison of single channel properties of RyR1 and RyR2 expressed in CHO cells

The $Ca^{2+}$ release channel functions of native and expressed RyR2 were further studied by using the lipid bilayer reconstitution system. Functional channel activity could be measured by incorporating the microsomal membrane vesicles from CHO cells expressing RyR2 into lipid bilayer using cesium gluconate as current carrier (Bhat et al., 1997b). The single channel currents through expressed RyR2 exhibited fast kinetics of transition between open and closed states (Fig. 3). These properties are comparable to those of the

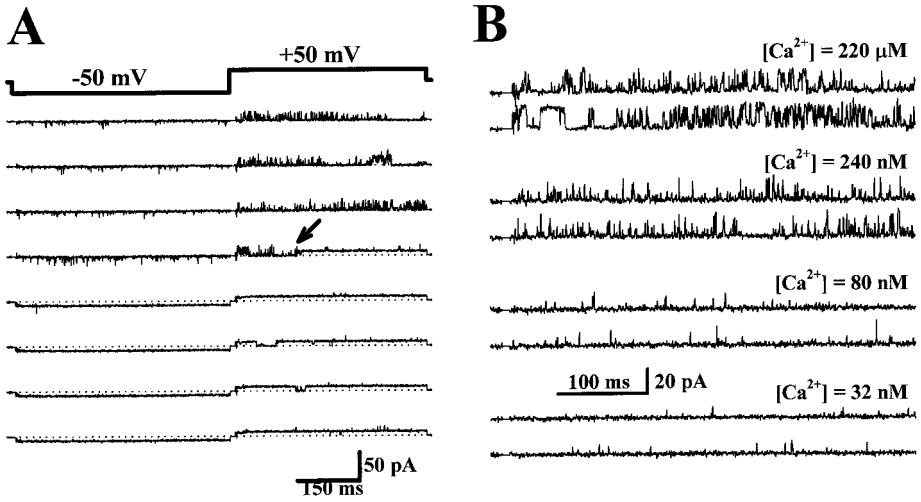


FIGURE 3 Lipid bilayer reconstitution of single RyR2 channels expressed in CHO cells. (*A*) Microsome membrane vesicles from CHO cells expressing RyR2 were reconstituted into planar lipid bilayer and single channel currents were recorded using symmetric 200 mM cesium gluconate as current-carrying ion, as described under Materials and Methods. The free $Ca^{2+}$ concentration in the cytoplasmic solution was maintained at 220 $\mu$M; 5 $\mu$M ryanodine was added to both *cis* (cytoplasmic) and *trans* (luminal) solutions ~3 min before the first trace. Represented are consecutive single channel current traces at test pulses of $-50 \rightarrow +50$ mV. Note the onset of the ryanodine effect (*arrow*). (*B*) The activity of RyR2 channel is dependent on free $Ca^{2+}$ concentration in the *cis* (cytoplasmic) solution, as chelation of $Ca^{2+}$ with EGTA resulted in gradual decrease in open probability of the channel ($P_o$ = 21.47 ± 3.48%, $[Ca^{2+}]$ = 220 $\mu$M; $P_o$ = 1.60 ± 0.36%, $[Ca^{2+}]$ = 240 nM; $P_o$ = 0.70 ± 0.10%, $[Ca^{2+}]$ = 80 nM; $P_o$ = 0, $[Ca^{2+}]$ = 32 nM).

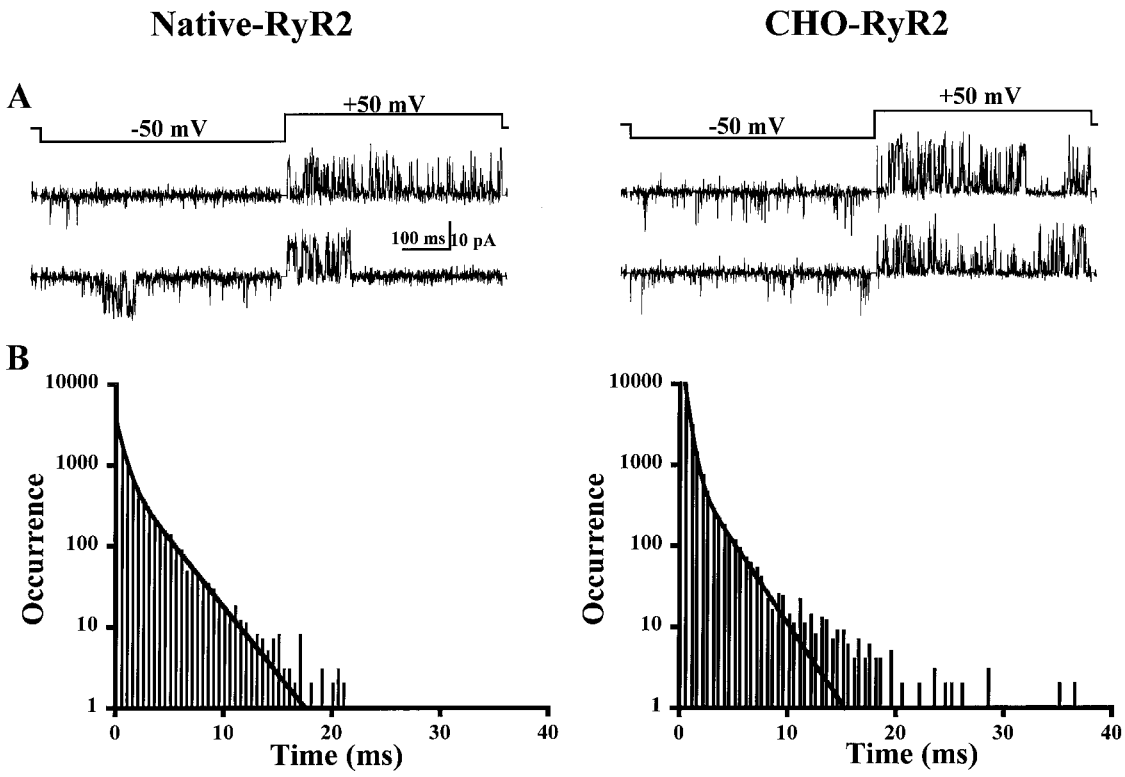


FIGURE 4 Single channel properties of native RyR2 and recombinant RyR2 expressed in CHO cells. (*A*) Representative single channel currents recorded with a pulse protocol of $-50 \rightarrow +50$ mV under the experimental conditions described in Fig. 3, using SR vesicles from rabbit cardiac muscle (Native-RyR2) and microsome membrane vesicles from CHO cells expressing RyR2 (CHO-RyR2). (*B*) Open time histograms were constructed at the test potential of +50 mV (total open events of 6071 for Native-RyR2 and 17101 for CHO-RyR2). Solid lines represent the best fits according to $y = y_{01}/\tau_{01} \exp(-t/\tau_{01}) + y_{02}/\tau_{02} \exp(-t/\tau_{02})$, where $y_{01} = 1872$, $\tau_{01} = 0.65$, $y_{02} = 2093$, $\tau_{02} = 2.63$ (Native-RyR2); $y_{01} = 12{,}222$, $\tau_{01} = 0.44$, $y_{02} = 2346$, $\tau_{02} = 2.19$ (CHO-RyR2).

native RyR2 $Ca^{2+}$ release channel currents recorded using SR membrane vesicles from rabbit cardiac muscle (see Fig. 4 *A*). The RyR2 channels are activated by micromolar concentrations of $[Ca^{2+}]$ in the *cis* (cytoplasmic) solution. As shown in Fig. 3 *B*, chelation of $[Ca^{2+}]$ in the cytoplasmic solution from 220 $\mu$M to 240 nM and 80 nM gradually decreased the channel open probability ($P_o = 21.47 \pm 3.48\%$, 220 $\mu$M; $P_o = 1.60 \pm 0.36\%$, 240 nM; $P_o = 0.70 \pm 0.10\%$, 80 nM), leading to complete inhibition of the channel activity at $[Ca^{2+}] = 32$ nM. This is similar to the

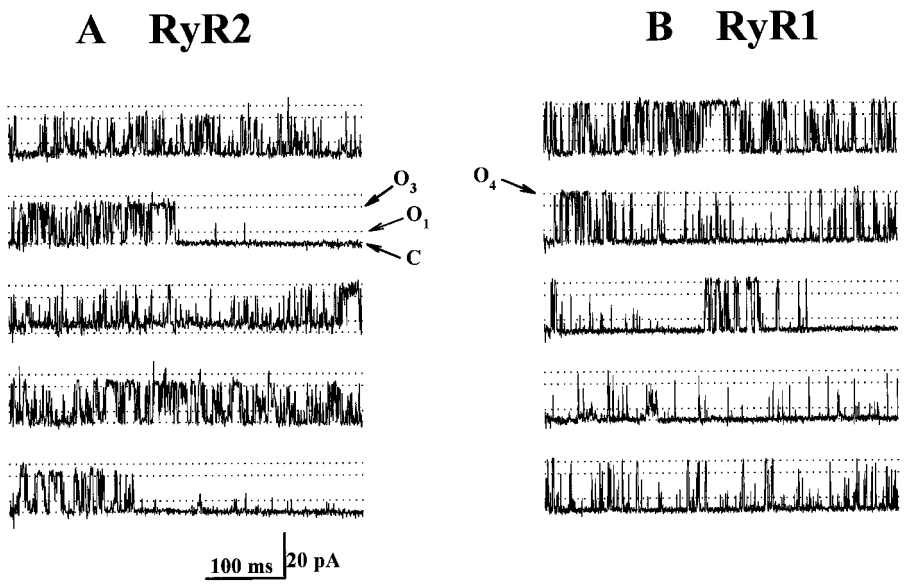


FIGURE 5 Comparison of the single channel properties of RyR1 and RyR2 expressed in CHO cells. Single channel currents were recorded at a holding potential of +50 mV as described in Fig. 3, using microsome membrane vesicles from CHO cells expressing RyR2 (*A*) and RyR1 (*B*). The free $Ca^{2+}$ concentration in the cytoplasmic solution was maintained at 220 $\mu$M. The three major subconductance levels ($O_1$, $O_3$, and $O_4$) are indicated by the dotted lines. Note that RyR2 channels exhibit frequent subconductance open states mainly to $O_1$ and $O_3$ levels with only brief transitions to $O_4$ (*A*), whereas the RyR1 channels open mainly to the full conductance state of $O_4$ (*B*).

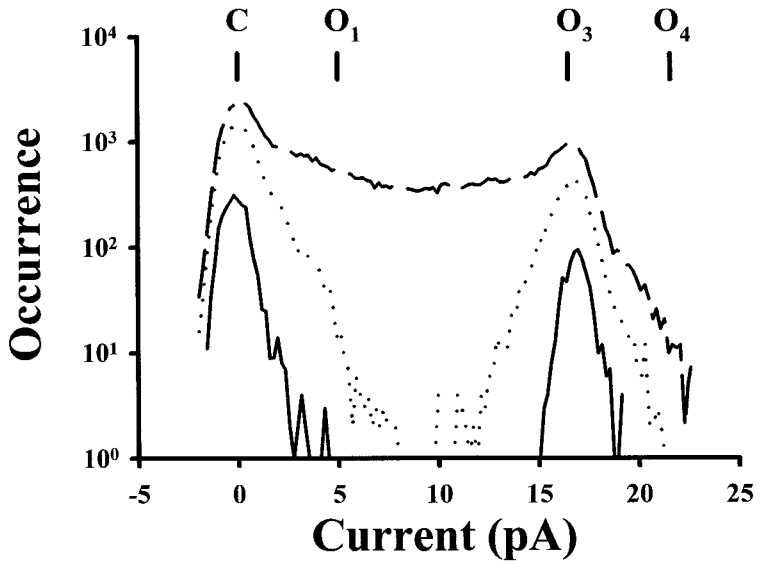


FIGURE 6 Mean variance analysis of subconductance states of RyR2 channels expressed in CHO cells. The amplitude histograms were constructed from five consecutive data files with a total of 80 episodes (each 500 ms, +50 mV test pulse) obtained under experimental conditions similar to Fig. 3 *A*. The dashed line contains all data points, and the dotted and solid lines correspond to data points selected for lower variances. $O_4$, $O_3$, and $O_1$ correspond to full, ¾, and ¼ of the open conductance states, and C represents the closed state. The algorithm for mean variance analysis was written by Dr. Stephen W. Jones.

$Ca^{2+}$-dependent activation of recombinant RyR1 channels expressed in CHO cells (Bhat et al., 1997a–c). Furthermore, the channels formed by the expressed RyR2 are sensitive to modification by ryanodine in that the channel conductance is reduced by ~50% and the open lifetime of the channels is increased dramatically (Fig. 3 *A, bottom four traces*). Open-time histogram analyses of native and expressed RyR2 channels revealed two similar time constants, i.e., $\tau_{01}$ = 0.65 ms and 0.44 ms, and $\tau_{02}$ = 2.63 ms and 2.19 ms for native and expressed RyR2 channels, respectively (Fig. 4 *B*).

We have previously described the functional properties of RyR1 expressed in CHO cells (Bhat et al., 1997a–c). Comparison of the single channel properties of full-length RyR1 and RyR2 expressed in CHO cells is illustrated in Fig. 5. Both RyR1 and RyR2 channels exhibit distinct subconductance states ($O_1$ through $O_4$). However, the RyR2 expressed in CHO cells stays open more frequently at lower conductance levels (i.e., $O_1$ and $O_3$) with rare transitions to half and full conductance levels (i.e., $O_2$ and $O_4$) (Fig. 5 *A*). This is in contrast to RyR1 channels expressed in CHO cells, which mostly open to full conductance state (i.e., $O_4$) when activated (Fig. 5 *B*). The $O_4$ state occurs in the majority of the experiments with the RyR1 channels (~63%, 30 of 48 experiments), whereas $O_3$ is the major conductance state occurring with the RyR2 channels (~80%, 23 of 29 experiments), with only brief transitions to the $O_4$ state. The mean variance analysis of the amplitude histogram for RyR2 channels expressed in CHO cells is presented in Fig. 6, which illustrates the presence of a major peak corresponding to the $O_3$ state with minor peaks at $O_1$ and $O_4$ states (Ma and Zhao, 1994). At +50 mV, the RyR2 channels exhibit a mean outward (cytoplasm → lumen) current amplitude of 14.90 ± 0.68 pA ($n$ = 24), which corresponds to the $O_3$ conductance level. By contrast, the major outward current amplitude for RyR1 was 20.23 ± 1.29 pA ($n$ = 48, Bhat et al., 1997b), which corresponds to the $O_4$ conductance level, and this is similar to the native RyR1 channels from rabbit skeletal muscle SR (Bhat et al., 1997b). The analysis of the current-voltage relationship of the RyR2 channels is presented in Fig. 7. Under the recording conditions of symmetrical 200 mM cesium gluconate, three distinct conductance states could be measured ($O_1$ = ~100 pS, $O_3$ = ~290 pS, and $O_4$ = ~401 pS) (Fig. 7).

The difference in the distribution of the conductance states between RyR2 and RyR1 may reflect the differences in the pore properties of these channels. This may happen because of the differences in the channel structure itself and/or differential interaction with the regulatory proteins.

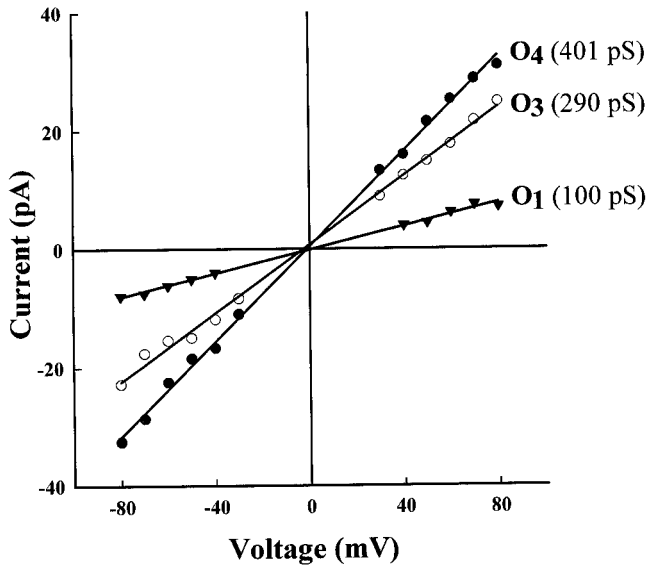


FIGURE 7 Current-voltage relationship of RyR2 channels expressed in CHO cells. The current-voltage relationship of RyR2 channels was measured between −80 and +80 mV, using symmetric 200 mM cesium gluconate as the current carrying ion. The amplitudes of the current levels at $O_1$ (▼, 100 pS), $O_3$ (○, 290 pS), and $O_4$ (●, 401 pS) are plotted against holding potential. The full conductance of the expressed channels was similar to that of the native RyR2 channels from rabbit cardiac muscle.

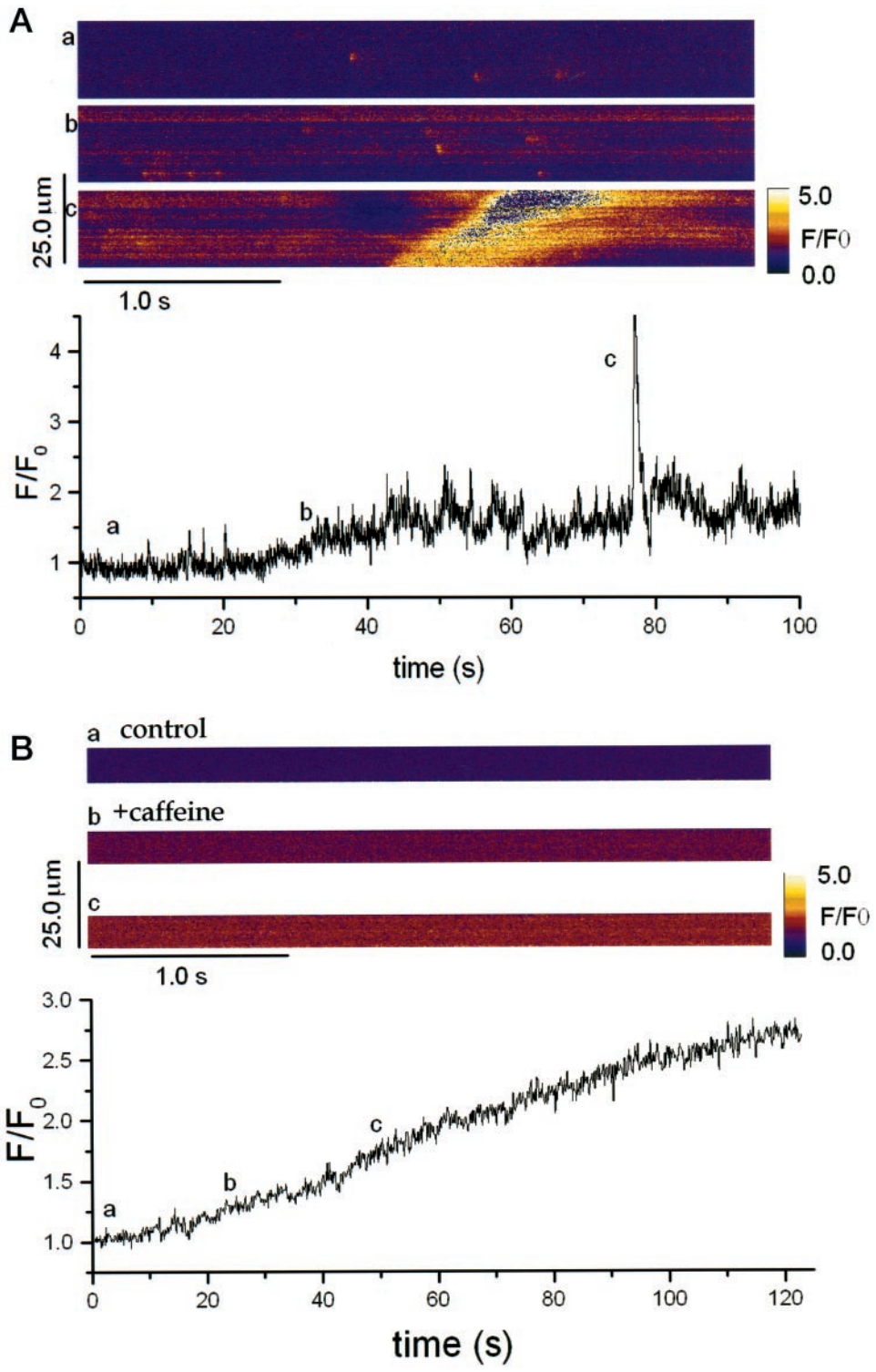


FIGURE 8 Lack of $Ca^{2+}$ sparks in CHO cells expressing RyR2. (*A*) Typical $Ca^{2+}$ sparks in cardiac myocytes. *Top panel:* line-scan image of Fluo-3 fluorescence ratio in rat cardiac myocyte. Under control conditions (*a*) spontaneous local $Ca^{2+}$ transients or sparks could be observed, whose frequency increases in response to application of 0.5 mM caffeine (*b*), leading up to a global increase in the intracellular $Ca^{2+}$ (*c*). The time course of the caffeine effect is illustrated in the bottom panel. (*B*) Lack of $Ca^{2+}$ sparks in CHO cells expressing RyR2. *Top panel:* line-scan image of Fluo-3 fluorescence in CHO cells expressing RyR2 were obtained with the same spatial and temporal resolution as shown in (*A*). No local $Ca^{2+}$ transients or "sparks" are evident under control conditions (*a*), or in the presence of 0.5 mM caffeine (*b*). However, caffeine elicits a gradual increase in $[Ca^{2+}]_i$ in these cells (*c*). The bottom panel shows the time-dependent change in the fluorescence ratio. The results shown in (*B*) represent at least five experiments from three different transfections.

While the RyR1 channel opens to the full conductance state in >60% of the experiments, the RyR2 channel exhibits full conductance state in only <20% of the experiments. Furthermore, the RyR2 channels appear to be unstable, as they always exhibit frequent transitions to subconductance states of $O_1$ and $O_3$ (see Fig. 5 *A*). Subconductance states are characteristic features of the $Ca^{2+}$ release channels from both skeletal and cardiac muscles, which likely reflect the oligomeric structure of the RyR protein complex, although the molecular mechanism(s) is largely unknown. FK506 binding proteins (FKBP) have been shown to associate and regulate the function of the $Ca^{2+}$ release channels (Marks, 1996). While FKBP12 specifically associates with RyR1, RyR2 preferentially interacts with FKBP12.6 (Jayaraman et al., 1992; Timerman et al., 1994, 1996). These proteins are known to regulate the function of RyRs by stabilizing the

conductance state(s) of the $Ca^{2+}$ release channels (Brillantes et al., 1994; Ma et al., 1995; Ahern et al., 1997), and the RyR channels depleted of FKBP12 have been shown to exhibit subconductance states (Ahern et al., 1997; Shou et al., 1998). While we do not know whether CHO cells express any endogenous FKBP12 or FKBP12.6, it will be interesting to examine the properties of RyR2 channels in CHO cells co-transfected with these regulatory proteins.

### Lack of $Ca^{2+}$ sparks in CHO cells expressing RyR2

In cardiac muscle cells, spontaneous local increases in intracellular $Ca^{2+}$, termed $Ca^{2+}$ sparks, have been observed which occur spontaneously (Fig. 8 *A top, panel a*; Cheng et al., 1993), and in response to activation of voltage-gated $Ca^{2+}$ channels (Cannell et al., 1994, 1995; Lopez-Lopez et al., 1994, 1995). Stimulation of cardiac myocytes with caffeine increases the frequency of $Ca^{2+}$ sparks (Fig. 8 *A top, panel b*) leading up to a global increase in the intracellular $Ca^{2+}$ (Fig. 8 *A top, panel c*). Similar elementary $Ca^{2+}$ release events, although smaller in size than the cardiac $Ca^{2+}$ sparks, have also been recorded in skeletal muscle cells (Tsugorka et al., 1995). However, it is not known whether a single or a group of $Ca^{2+}$ release channels acting in concert constitute the "$Ca^{2+}$ release units" underlying the local $Ca^{2+}$ transients in muscle cells. We tested for the presence of spontaneous changes in intracellular $Ca^{2+}$ in CHO cells expressing RyR2 (Fig. 8 *B*). Under resting conditions (Fig. 8 *B top, panel a*), or in response to stimulation with caffeine (Fig. 8 *B top, panel b*) no spontaneous local $Ca^{2+}$ transients were evident in the line-scan images of CHO cells expressing RyR2, although caffeine was capable of inducing $Ca^{2+}$ release in these cells (Fig. 8 *B top, panel c*, and *bottom*).

The lack of local $Ca^{2+}$ transients in CHO cells expressing RyR2 is similar to our recent results where CHO cells expressing RyR1 also did not exhibit spontaneous or caffeine-activated signals typical of $Ca^{2+}$ sparks (Bhat et al., 1997c). These results suggest that ryanodine receptors by themselves are not sufficient to support elementary $Ca^{2+}$ release events, although they are capable of functioning as $Ca^{2+}$ release channels both in vivo (caffeine-induced $Ca^{2+}$ release, Fig. 2) and in vitro (single channel experiments, Figs. 3–5). The absence of muscle-specific spatial environment in CHO cells may not support local cooperative opening of expressed $Ca^{2+}$ release channels which is believed to be responsible for the origin of $Ca^{2+}$ sparks. The absence in heterologous expression systems of muscle-specific accessory protein(s) (as discussed above) that interact with RyR to constitute a "local $Ca^{2+}$ release unit" may also contribute to the lack of spontaneous or caffeine-induced $Ca^{2+}$ sparks in CHO cells. The activity of both skeletal and cardiac $Ca^{2+}$ release channels is controlled by both cytoplasmic and luminal $Ca^{2+}$ (Sitsapesan and Williams, 1997). Furthermore, in cardiac myocytes the fractional SR $Ca^{2+}$ release and the frequency and amplitude of $Ca^{2+}$ sparks are increased by an increase in the SR $Ca^{2+}$ content (Bassani et al., 1995; Lukyanenko et al., 1996). Although the $Ca^{2+}$ content of the intracellular stores of CHO cells is not known, it may not mimic that of muscle cells to support spontaneous opening of the expressed RyR channels.

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