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Single-Channel Characterization of the Rabbit Recombinant RyR2 Reveals a Novel Inactivation Property of Physiological Concentrations of ATP

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Abstract Ryanodine receptor 2 (RyR2) cDNA has been available for more than 15 years; however, due to the complex nature of ligand gating in this channel, many aspects of recombinant RyR2 function have been unresearched. We established a stable, inducible HEK 293 cell line expressing full-length rabbit RyR2 cDNA and assessed the single-channel properties of the recombinant RyR2, with particular reference to ligand regulation with Ca^{2+} as the permeant ion. We found that the single-channel conductances of recombinant RyR2 and RyR2 isolated from cardiac muscle are essentially identical, as is irreversible modification by ryanodine. Although it is known that RyR2 expressed in HEK 293 cells is not associated with FKBP12.6, we demonstrate that these channels do not exhibit any discernable disorganized gating characteristics or subconductance states. We also show that the gating of recombinant RyR2 is indistinguishable from that of channels isolated from cardiac muscle when activated by cytosolic Ca^{2+}, caffeine or suramin. The mechanisms underlying ATP activation are also similar; however, the experiments highlighted a novel effect of ATP at physiologically relevant concentrations of 5–10 mM. With Ca^{2+} as permeant ion, 5–10 mM ATP consistently inactivated recombinant channels (15/16 experiments). Such inactivation was rarely observed with native RyR2 isolated from cardiac muscle (1 in 16 experiments). However, if the channels were purified, inactivation by ATP was then revealed in all experiments. This action of ATP may be relevant for inactivation of sarcoplasmic reticulum Ca^{2+} release during cardiac excitation–contraction coupling or may represent unnatural behavior that is revealed when RyR2 is purified or expressed in noncardiac systems.

Keywords Ryanodine receptor · Cardiac · ATP · Excitation–contraction coupling · Ca^{2+} release · Sarcoplasmic reticulum

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Introduction

The cardiac ryanodine receptor (RyR2) plays a crucial role in myocardial contractility by releasing sarcoplasmic reticulum (SR) Ca^{2+} in response to Ca^{2+} entry into the cell during the action potential (Bers 1991). Understanding how RyR2 is regulated is important, therefore, for a full appreciation of normal excitation–contraction (EC) coupling and the changes that can occur in heart disease. The ability to work with recombinant RyR2 proteins provides a greater dimension to the study of RyR2 regulation, enabling links between structure and function to be made. We have therefore established a stable inducible HEK 293

cell line expressing the full-length rabbit RyR2 cDNA and, in the present study, have characterized the basic single-channel properties of this recombinant protein, with particular reference to ligand regulation with Ca^{2+} as the permeant ion. Numerous studies have described features of recombinant RyR2 with monovalent cations as permeant ions (Bhat et al. 1999; Du et al. 2001; Jiang et al. 2002; Kong et al. 2007; Stange et al. 2003; Xiao et al. 2007), but little is known about recombinant channel function where luminal Ca^{2+} is the permeant ion, flowing in the luminal to cytosolic direction and at a holding potential of 0 m*V*, as would be expected in a cardiac cell.

It is important to emphasize that the single-channel properties of recombinant RyR2 channels expressed in noncardiac cells may not be identical to those of channels expressed in normal cardiac cells. This is partly because RyR2 gating is modulated by such a wide range of intracellular factors (Coronado et al. 1994; Meissner 1994). RyR channels tend to act as scaffolding proteins, binding numerous other proteins; thus, it is thought that RyR2, in the cardiac setting, performs as a functional unit, operating and cooperating with a particular set of accessory proteins (Györke et al. 2004; Zhang et al. 1997). The levels and variety of proteins associated with RyR2 are expected to vary depending on the type of cell in which it is expressed. For example, FKBP12.6 is tightly associated with RyR2 in cardiac cells (Lam et al. 1995; Timerman et al. 1996), but Xiao et al. (2004, 2005) clearly demonstrated that recombinant RyR2 proteins expressed in HEK 293 cells are not associated with FKBP12.6. Calsequestrin, another protein reported to markedly affect RyR2 function (Györke et al. 2004; Knollmann et al. 2006) which is tightly bound even to purified RyR2 channels isolated from cardiac muscle (West et al. 2002), is also not expressed in HEK 293 cells (Kong et al. 2007). Thus, recombinant RyR2 channels expressed in noncardiac cells, such as HEK 293 cells, will not be bound to the same set of associated proteins as RyR2 isolated from cardiac muscle, and this could significantly influence single-channel behavior. Our aim in the present study, therefore, was to compare the gating and conducting properties of recombinant RyR2 channels with those of channels isolated from cardiac muscle, using identical recording conditions.

With Ca^{2+} as the permeant ion, we now demonstrate that the recombinant rabbit RyR2 channel expressed in HEK 293 cells displays single-channel behavior that is indistinguishable in many respects from that of RyR2 isolated from sheep cardiac muscle. Since FKBP12.6 is not associated with RyR2 channels isolated from HEK 293 cells (Xiao et al. 2004, 2005), our results show that the presence of FKBP12.6 is not required to prevent the destabilization of RyR2 channel function into disorganized subconductance gating modes. Our results also show, for the first time, that ATP ($\geq$5 mM), a physiological regulator of RyR2, is capable of inactivating RyR2 channels at a holding potential of 0 m*V* and with Ca^{2+} as the permeant ion flowing in the luminal to cytosolic direction. The physiological significance of this inactivation is unclear as yet, but the inactivation may be modulated by accessory proteins binding to RyR2.

Methods

Generation of Stable Inducible HEK 293 Cell Line Expressing Rabbit RyR2

A stable inducible HEK 293 cell line expressing rabbit RyR2 was generated using the Flp-In T-REx system (Invitrogen, Paisley, UK). The full-length cDNA encoding the rabbit RyR2 (Nakai et al. 1990) was subcloned into the inducible expression vector pcDNA5/FRT/TO to create the construct pcDNA5/FRT/TO/RyR2. Flp-In T-Rex-293 cells were cotransfected with pcDNA5/FRT/TO/RyR2 and the pOG44 vector encoding the Flp recombinase in a 1:2 ratio using Lipofectamine 2000 (Invitrogen). Transfected cells were subsequently grown in a selective medium containing 200 μg/ml hygromycin. The hygromycin-resistant cells were pooled to form the stable inducible cell line RyR2-HEK 293. Expression was induced for $\sim$24 h following addition of 2 μg/ml tetracycline.

Isolation of Microsomal Membrane Vesicles and Purification of RyR2

HEK 293 cells expressing full-length rabbit RyR2 were harvested and washed twice with ice-cold phosphate-buffered saline. The cell pellet (800 × *g*, 5 min) was resuspended in ice-cold hypotonic lysis buffer (1 mM EDTA, 10 mM HEPES, pH 7.4) and incubated on ice for 20 min. Cells were then homogenized on ice with 10 strokes in a tight-fitting glass Dounce homogenizer, followed by 15 strokes after the addition of an equal volume of restoration buffer (500 mM sucrose, 10 mM HEPES, pH 7.2). Microsomes were collected by centrifugation of postnuclear supernatant (10,000 × *g*, 20 min) at 100,000 × *g* for 1 h. The pellet was resuspended in buffer containing 250 mM sucrose, 10 mM HEPES-Tris (pH 7.2). The membrane vesicles were snap-frozen in liquid N_2 and stored at −80°C. All buffers were supplemented with protease inhibitors (Complete; Roche Diagnostic Crop., Indianapolis, IN). Heavy SR membrane vesicles containing native RyR2 and 3-([3-cholamidopropyl]dimethyl-ammonio)1-propane sulfonate (CHAPS)–purified RyR2 were prepared from sheep hearts as described previously (Sitsapesan et al. 1991; Sitsapesan and Williams 1994a), snap-frozen in liquid N_2 and stored at −80°C.

Caffeine-Induced Release of Ca^{2+} in HEK 293 Cells

Changes in $[Ca^{2+}]_i$ were measured in cells (10^6/ml) resuspended in Krebs-Ringer-HEPES buffer and loaded with 3 μM Fura-2AM. Cells were maintained at 37°C under continuous stirring in a cuvette using a Hitachi (Hitachi High Technologies, Tokyo, Japan) F-4500 spectrofluorometer with fluorescence excitation at 340 and 380 nm and emission at 510 nm.

Single-Channel Experiments

Channels were reconstituted into planar phosphatidylethanolamine lipid bilayers as described previously (Sitsapesan et al. 1991). Channels fused in a fixed orientation such that the *cis*-chamber corresponded to the cytosolic space and the *trans*-chamber to the SR lumen (Sitsapesan and Williams 1994). The *trans*-chamber was held at ground and the *cis*-chamber held at potentials relative to ground. After fusion, the *cis*-chamber was perfused with 250 mM HEPES, 80 mM Tris and 10 μM free Ca^{2+} (pH 7.2), unless stated otherwise. Free $[Ca^{2+}]$ was increased to 100 μmol/l by addition of $CaCl_2$ solution. The *trans*-chamber was perfused with 250 mM glutamic acid and 10 mM HEPES (pH to 7.2) with $Ca(OH)_2$ (free $[Ca^{2+}]$, approximately 50 mM). Experiments were carried out at room temperature (22 ± 2°C) under voltage-clamp conditions with a holding potential of 0 m*V*. The free $[Ca^{2+}]$ and pH of the solutions were determined using a Ca^{2+} electrode (Orion 93–20; Thermo Orion, Boston, MA) and a Ross-type pH electrode (Orion 81-55) as previously described (Chan et al. 2000; Sitsapesan et al. 1991). This was especially important for the ATP experiments since ATP chelates Ca^{2+}, thus altering the pH and free $[Ca^{2+}]$ of the solutions. The free cytosolic $[Ca^{2+}]$ and pH were therefore measured for all concentrations of ATP and maintained at pH 7.2 and 10 μM, respectively, throughout the experiments by the addition of $CaCl_2$ and EGTA as previously described (Chan et al. 2000).

Data Acquisition and Analysis

Channel recordings were displayed on an oscilloscope and recorded on digital audiotape. Current recordings were filtered at 1 kHz (−3 dB) and digitized at 20 kHz using Pulse (HEKA Elektronik, Lambrecht/Pfalz, Germany). Channel open probability (P_o) was determined over 3 min of continuous recording, unless otherwise stated, using the method of 50% threshold analysis (Colquhoun and Sigworth 1983). Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events <1 ms in duration were not fully resolved and were excluded from lifetime analysis. Individual lifetimes were fitted to a probability density function by the method of maximum likelihood according to the equation

$$g(x) = \sum_{i=1}^{N} a_i g_o(x - \ln \tau_i)$$

where $\ln\tau_i$ is the logarithm of the *i*th time constant and a_i is the fraction of the total events represented by that component (Sigworth and Sine 1987). Measurements of current amplitude were made using the WinEDR program (John Dempster, Strathclyde University) http://www.bio-logic.info/electrophysiology/Winwcpandwinedr.html by (1) manually assessing the closed and open current levels using cursors and (2) construction of amplitude histograms.

Statistics

Where appropriate, Student's *t*-test, a paired *t*-test or analysis of variance followed by a modified *t*-test was used to assess the difference between treatments. A *P* value of 0.05 was taken as significant.

Materials

The RyR2 antibody (ARP 106), a polyclonal antibody raised against the C terminus of human RyR2 (residues 4957–4967) (Williams et al. 2001), was a gift from A. J. Williams (Wales Heart Research Institute, School of Medicine Cardiff University, Heath Park). Fura-2AM was obtained from Invitrogen. Other chemicals were analytical grade (AnalaR) or the best equivalent grade from BDH (Poole, UK) or Sigma-Aldrich (Dorset, UK). All solutions were made in deionized water, and those for use in bilayer experiments were filtered through a Millipore (Bedford, MA) membrane filter (0.45-μm pore).

Results

Induction of RyR2 Expression in HEK 293 Cells

To characterize the functional properties of recombinant RyR2, we generated a stable inducible HEK 293 cell line (RyR2HEK 293) expressing rabbit RyR2. RyR2 expression was induced in RyR2HEK 293 cells by addition of tetracycline. Twenty-four hours postinduction, the cells were fixed with paraformaldehyde and stained with an anti-RyR2 primary and a fluorescence-tagged secondary antibody. The immunostained cells were visualized under a confocal microscope for subcellular localization of expressed protein. As shown in Fig. 1A, control, untransfected HEK 293 cells and uninduced RyR2HEK 293 cells showed no significant fluorescence signal whereas tetracycline-induced RyR2HEK 293 cells exhibited strong

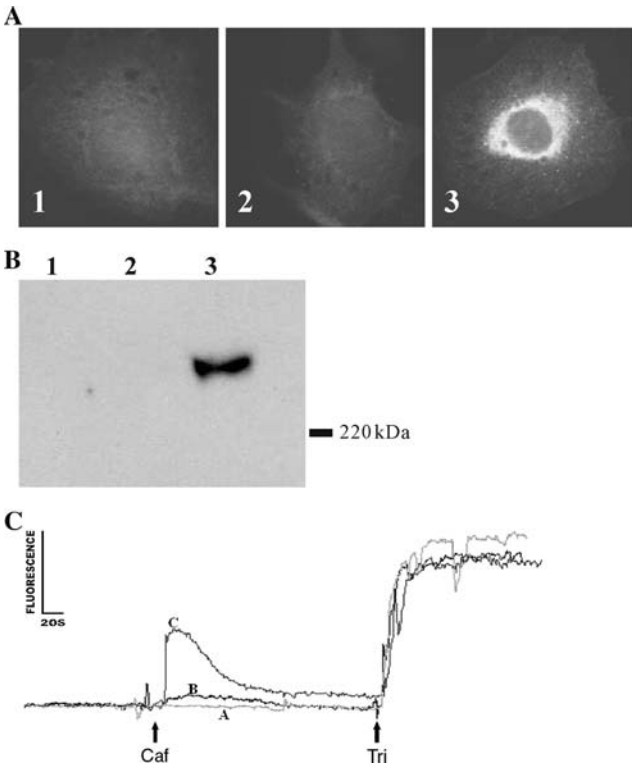

Fig. 1 Expression of rabbit recombinant RyR2 in HEK 293 cells. (**A**) Immunofluorescent staining of HEK 293 cells using an anti-RyR antibody. Confocal imaging of (1) a single, untransfected Flp-In T-Rex-293 cell, (2) an uninduced RyR2HEK 293 cell and (3) a tetracycline-induced RyR2HEK 293 cell. The induced stable cells exhibit strong fluorescence localized to the perinuclear region with a weaker reticular fluorescence, whereas the untransfected and uninduced stable cells show negligible fluorescence. (**B**) Immunoblot of microsomes (40 μg) derived from (lane 1) untransfected Flp-In T-Rex-293 cells, (lane 2) uninduced RyR2HEK 293 cells and (lane 3) tetracycline-induced RyR2HEK 293 cells. Expression of full-length RyR2 in HEK 293 cells is detectable only in those microsomal fractions obtained from induced RyR2 HEK 293 cells. (**C**) Caffeine-induced release of Ca^{2+} in HEK 293 cells. Application of 10 mM caffeine (Caf) does not induce significant release of intracellular Ca^{2+} in either untransfected Flp-In T-Rex-293 cells (A) or uninduced RyR2HEK 293 cells (B). However, a robust response is obtained from tetracycline-induced RyR2HEK 293 cells (C), suggesting the presence of functional ryanodine receptors in these cells. Application of Triton X-100 (Tri) solubilizes the cell membranes and releases the total pool of calcium

fluorescence in the perinuclear region together with weaker reticular fluorescence, indicating that the protein is localized in the endoplasmic reticulum (ER) membrane.

The expression of RyR2 in HEK 293 cells was further examined by Western blot analysis. Fig. 1B demonstrates that a single high–molecular weight protein band was obtained from microsomes derived from tetracycline-induced RyR2HEK 293 cells. No band was observed from microsomal fractions obtained from uninduced RyR2 HEK 293 cells or control, untransfected HEK 293 cells. The functionality of RyR2 was assessed by monitoring the changes in $[Ca^{2+}]_i$ after application of caffeine (Fig. 1C). No significant changes in $[Ca^{2+}]_i$ were observed in either control, untransfected HEK 293 cells or uninduced RyR2HEK 293 cells following caffeine addition, whereas a dramatic increase in $[Ca^{2+}]_i$ was observed from tetracycline-induced RyR2HEK 293 cells, strongly indicating the presence of functional RyR2. Thus, functional calcium-release channels were exclusively expressed and correctly localized in those RyR2HEK 293 cells exposed to the inducing agent tetracycline.

Single-Channel Experiments

Figure 2A illustrates typical current fluctuations through a recombinant RyR2 channel incorporated into bilayers in the presence of 10 μM cytosolic Ca^{2+}. Similar recordings were obtained in 50 experiments from eight different membrane preparations. The average P_o under these conditions was 0.068 ± 0.007 (SEM, $n = 50$). This value corresponds extremely closely to the P_o values that we consistently (under similar recording conditions) obtain with RyR2 channels isolated from sheep cardiac muscle (e.g., Carter et al. 2006; Chan et al. 2000).

Removal of FKBP12.6 from RyR2 channels has been reported to induce long-duration subconductance states by some authors (Marx et al. 2000) but to have no effect on channel function by other authors (Barg et al. 1997; Timerman et al. 1996; Xiao et al. 2007). Since recombinant RyR2 proteins expressed in HEK 293 cells are not associated with FKBP12.6 (Xiao et al. 2004 2005), we examined all recombinant channel traces for long-duration subconductance events. We found no evidence for subconductance events in any recombinant channel ($n = 50$). In all cases, gating and conductance were similar to those of channels isolated from cardiac muscle. Figure 2B compares amplitude histograms from a typical recombinant RyR2 channel and a typical RyR2 channel isolated from cardiac muscle, illustrating that there is no suggestion of subconductance state gating in either case.

Lifetime analysis (Fig. 3A) also confirms that the gating of recombinant RyR2 channels, where cytosolic Ca^{2+} is the sole channel activator, shows no observable differences from RyR2 channels isolated from cardiac muscle (e.g., Kermode et al. 1998; Carter et al. 2006; Hill et al. 2004). The channels are characterized by brief opening events that, for the most part, cannot be fully resolved. Mean open time is close to the minimum resolvable event duration (1.18 ± 0.08 ms), while mean closed time is of longer duration (13.43 ± 3.69 ms). As observed for channels isolated from cardiac muscle, in the presence of 10 μM cytosolic Ca^{2+}, the best fit to the open and closed lifetime histograms derived from recombinant RyR2 is obtained with at least two open and three closed states. The

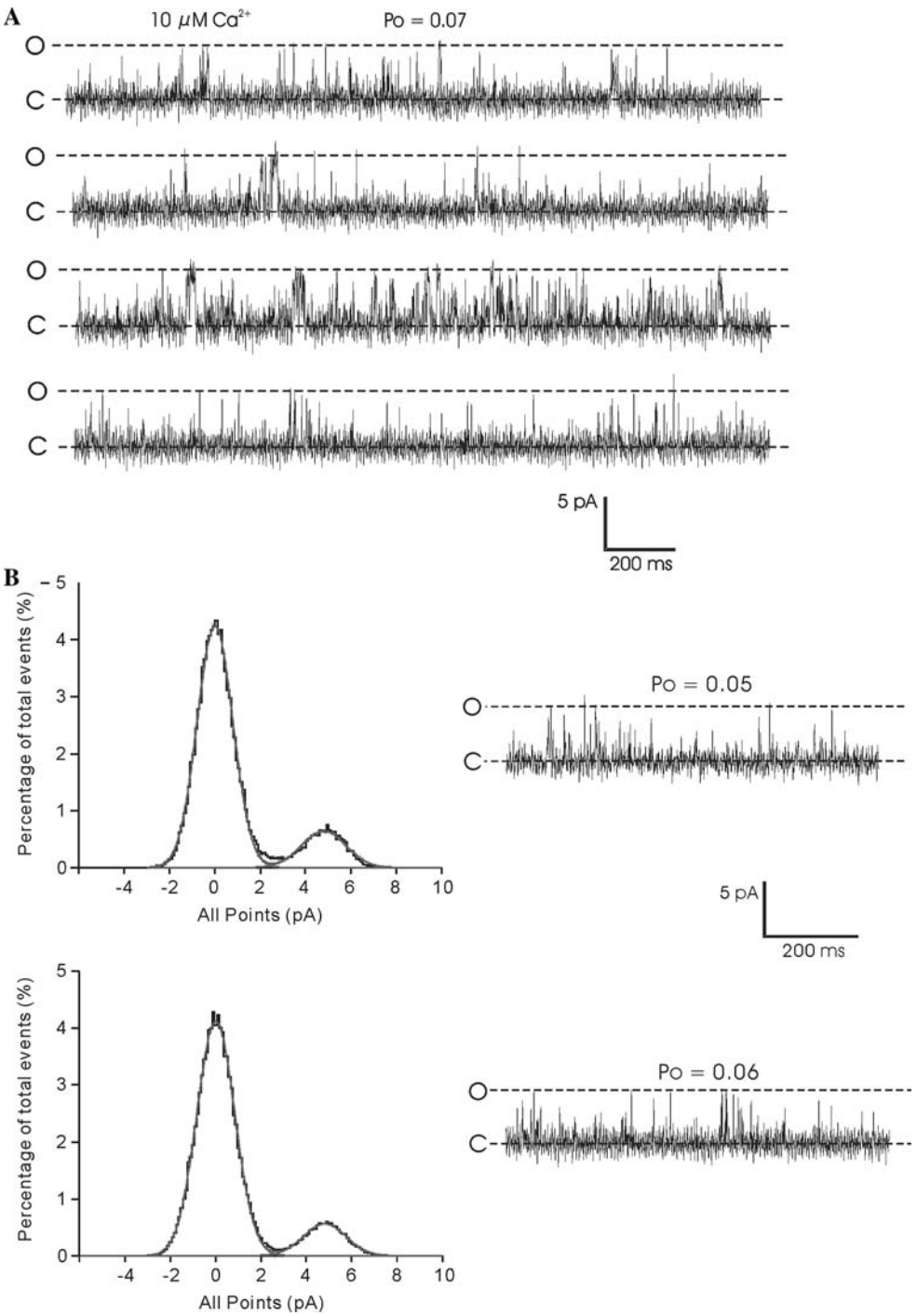

Fig. 2 Gating of the recombinant RyR2 channel in the presence of 10 μM cytosolic Ca^{2+}. (**A**) Traces illustrate consecutive single-channel current fluctuations through a representative channel. The average P_o for 3 min consecutive recording is indicated above the traces. Holding potential was 0 m*V*, and *O* and *C* indicate the fully open and closed channel levels, respectively. (**B**) Amplitude histograms of single-channel data from a representative channel isolated from cardiac muscle (*top*) and a recombinant RyR2 (*lower*). *Inset* illustrates the channel recordings used

similarity of the lifetime distributions that we observe from recombinant RyR2 and RyR2 isolated from cardiac muscle under identical recording conditions is conspicuous (e.g., Carter et al. 2006; Hill et al. 2004).

In general, multiple channels incorporated into the bilayer; and Fig. 3B illustrates a typical example where at least five channels are gating. Lowering the free cytosolic [Ca^{2+}] to subnanomolar levels abolished all channel openings, demonstrating the absolute requirement for cytosolic Ca^{2+}, again a characteristic of nonrecombinant RyR2 channels that we have previously described (e.g., Kermode et al. 1998; Hill et al. 2004). It has also previously been shown that cytosolic Ca^{2+}, as sole activating ligand, causes very variable increases in P_o (Copello et al. 1997; Sitsapesan and Williams 1994a), acts like a partial agonist under the conditions of the current experiments and is unable to activate RyR2 to a high P_o level (Ashley and Williams 1990). This was also the case with recombinant

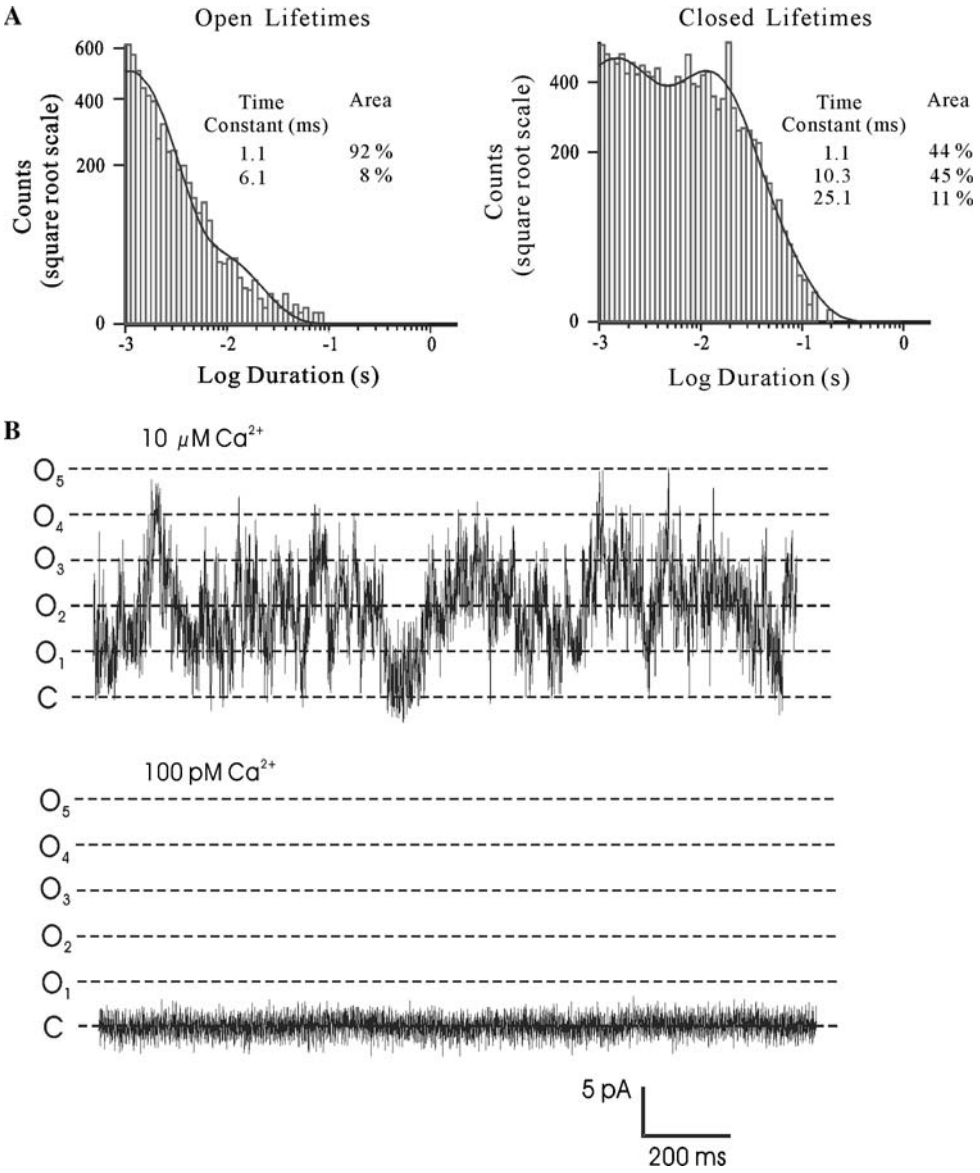

Fig. 3 Ca^{2+}-activated channel gating. (**A**) Open and closed lifetime distributions and probability density functions from the channel shown in Fig. 2a activated by 10 μM Ca^{2+} are shown. The best fits to the data were obtained by maximum likelihood fitting. The resulting time constants and percentage areas are shown. (**B**) Gating of multiple Ca^{2+}-sensitive channels in a bilayer. *Top* At least five recombinant RyR2 channels gate simultaneously in the presence of 10 μM cytosolic Ca^{2+}. *Bottom* All channels completely shut after lowering the cytosolic [Ca^{2+}] to subnanomolar levels. Holding potential was 0 m*V*, and *O* and *C* indicate the fully open and closed channel levels, respectively

RyR2. In the presence of 50 μM Ca^{2+}, P_o was only increased to 0.265 ± 0.190 ($n = 3$, sd), the approximate P_o expected for this level of Ca^{2+} (Ashley and Williams 1990; Carter et al. 2006).

Figure 4A illustrates that we also observed no difference in single-channel conductance when comparing the current–voltage relationships of recombinant RyR2 (squares) and RyR2 isolated from cardiac muscle (circles) using the same recording solutions. Linear regression of the measurements between −40 and +20 m*V* gives conductance values of 117 ± 4.6 pS ($n = 3$, sd) for recombinant channels and 120 ± 6.6 pS ($n = 4$, sd) for RyR2 channels isolated from cardiac muscle. Similarly, ryanodine irreversibly modified the gating of recombinant RyR2 channels to an open state 40.1 ± 1.6% ($n = 3$, sd) of the fully open state. The same fractional conductance level is observed with RyR2 isolated from cardiac muscle under these recording conditions (Sitsapesan and Williams 1996). This is demonstrated in Fig. 4B and shows a single-channel gating with a high P_o in the presence of caffeine (10 μM) before the ryanodine modification takes place.

We then investigated the effects of various ligands known to activate RyR2 channels via distinct binding sites on the channel. The recombinant RyR2 channels were very responsive to caffeine, as shown in Fig. 5A. We have previously shown similar effects of caffeine on RyR2 channels isolated from cardiac muscle (Sitsapesan and Williams 1990). Figure 5B illustrates the effects of 500 nM and 100 μM suramin on a typical recombinant RyR2 channel. Little change or, if anything, a reduction in P_o was observed at 500 nM suramin, whereas at 100 μM suramin almost full activation of the channel occurred.

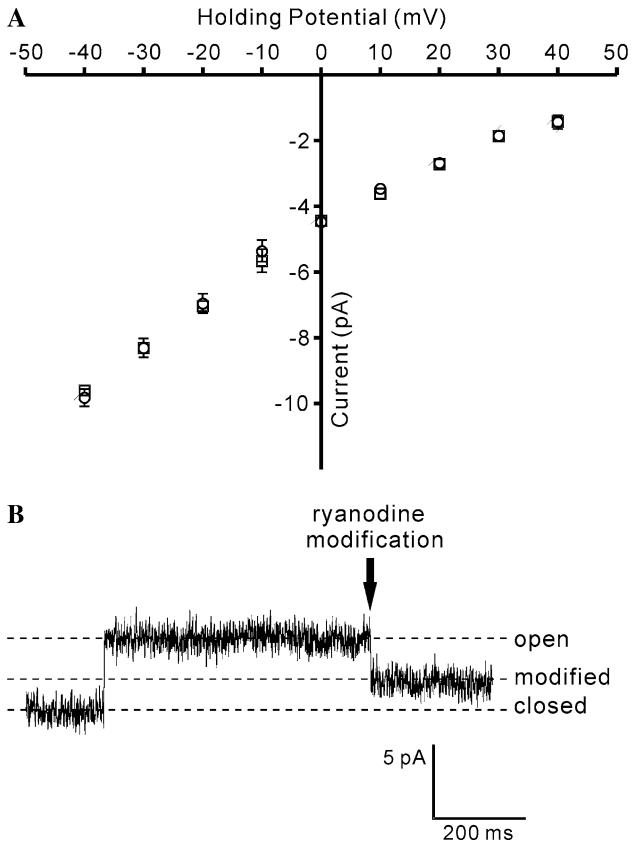

Fig. 4 Single-channel conductance of recombinant RyR2 and ryanodine modification. (**A**) Current–voltage relationship of recombinant RyR2 (*squares*) compared with that of native RyR2 channels isolated from cardiac muscle (*circles*). Values shown are mean ± sd ($n \geq 3$). Where not shown, error bars are within the symbols. (**B**) Example of ryanodine modification of a recombinant RyR2 to a level 41% of the full open state. The channel was activated to a high P_o level by 10 μM cytosolic Ca^{2+} and 10 mM caffeine. Ryanodine (1 μM) was added to the *cis*-chamber. *Arrow* shows time of modification. Holding potential was 0 m*V*

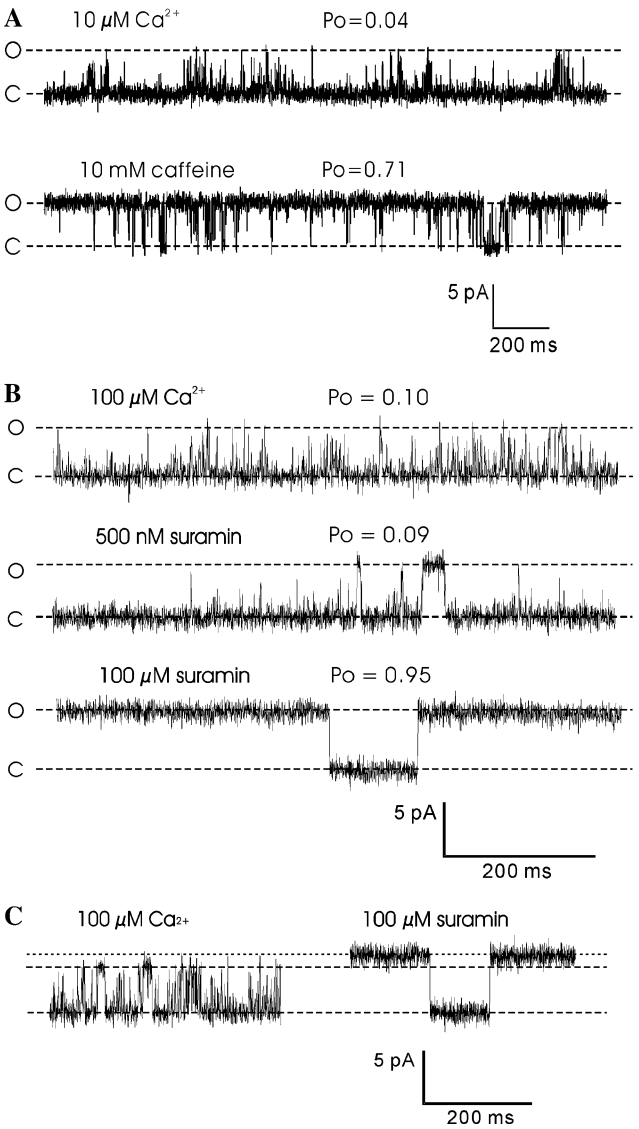

Fig. 5 Activation of recombinant RyR2 channels by ligands caffeine and suramin. (**A**) *Top* A typical channel activated solely by 10 μM cytosolic Ca^{2+}. Subsequent addition of 10 mM caffeine to the cytosolic channel side increased P_o. Holding potential was 0 m*V*, and *O* and *C* indicate the fully open and closed channel levels, respectively. (**B**) *Top* A typical channel activated solely by 10 μM cytosolic Ca^{2+}. Subsequent addition of 500 nm suramin (*middle panel*) caused only a very slight reduction in P_o, whereas 100 μM suramin (*bottom panel*) almost fully activated the channel. Holding potential was 0 m*V*. (**C**) Increase in current amplitude at 0 m*V* caused by adding 100 μM suramin. Control trace is on the *left*. *Right trace* shows the current amplitude after addition of suramin

Similar effects have been reported for RyR2 channels isolated from cardiac muscle (Hill et al. 2004; Sitsapesan and Williams 1996). In addition, suramin is known to increase current amplitude (Hill et al. 2004; Sitsapesan and Williams 1996), and Fig. 5C shows that this effect is also seen in recombinant RyR2 channels.

The results described above convinced us that our methods for expressing the recombinant rabbit RyR2 produced channels with expected RyR2 characteristics and provided no evidence for any loss of function. We therefore proceeded to examine the ATP regulation of recombinant RyR2. Figure 6 shows the effects of ATP in a representative experiment and the dose–response relationship. For doses of ATP up to 2 mM, we observed changes in gating that were broadly similar to those changes observed with RyR2 channels isolated from cardiac muscle (Chan et al. 2000, 2003). At micromolar concentrations of ATP, we observed gating characterized by brief flickery opening and closing events, whereas higher concentrations gave rise to longer opening events (Fig. 6). Thus, low doses of ATP increased P_o by increasing the frequency of channel opening, but with increasing [ATP] (up to 2 mM), increases in the duration of the openings contributed progressively to higher P_o values.

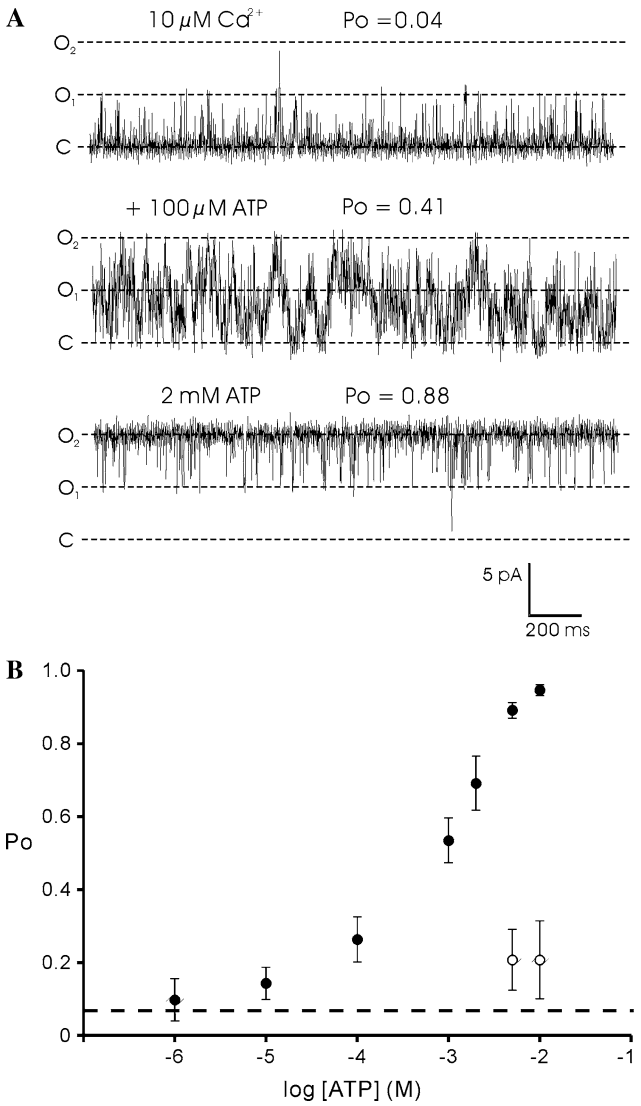

Fig. 6 Activation of recombinant RyR2 channels by ATP. (**A**) Effects of ATP on a typical recombinant RyR2 channel. *Top* A channel activated solely by 10 μM cytosolic Ca^{2+}. Subsequent addition of 100 μM ATP (*middle*) and 2 mM ATP (*bottom*) to the cytosolic channel side increased P_o. Holding potential was 0 m*V*, and *O* and *C* indicate the fully open and closed channel levels, respectively. (**B**) The ATP dose–response relationship for recombinant RyR2. *Filled symbols* show the average P_o measurements without inclusion of inactivation events (at 5 and 10 mM ATP channels inactivated before the end of the 3 min recording period, and therefore, P_o measurements include only the time up to the inactivation event). Open symbols include the average P_o measurement for the whole of each 3 min recording response. Means ± SEM are shown for $n \geq 4$

At the higher and more physiologically relevant levels of ATP (5–10 mM) (Allue et al. 1996; Hohl et al. 1992), however, we unexpectedly observed a novel effect on RyR2 function. Following addition of 5 or 10 mM ATP to the cytosolic channel side, we observed rapid inactivation of the channels, an effect not previously observed with ATP on RyR2 channels isolated from cardiac muscle under these experimental conditions (at a holding potential of 0 m*V* and with luminal Ca^{2+} as the permeant ion) (Kermode et al. 1998). Inactivation of RyR1 and RyR2 (purified or native) has previously been described for various ligands (including ATP) by many groups but *only* with a monovalent cation as the permeant ion and at a holding potential other than 0 m*V*. ATP (or any other activating ligand) has not been shown to inactivate channels in this manner when luminal Ca^{2+} is the permeant ion and the holding potential is 0 m*V*. Figure 7A shows a representative example of the nature of the inactivation. The figure shows that in this case 5 mM ATP produced a high P_o with no inactivation but addition of 10 mM ATP caused the channel to close without further openings within the 3 min recording period. Approximately 70% of the channels could be inactivated by 5 mM ATP; the remaining channels required a higher dose of 10 mM ATP to close them. Figure 6B illustrates the dose–response relationship for ATP activation of recombinant RyR2. The closed symbols show the average P_o before inactivation takes place. Where channels did not inactivate, all P_o values were obtained from 3 min of steady-state recording. At the doses where inactivation occurred (5 and 10 mM), P_o measurements include only measurements before the inactivation event. The open symbols show the average P_o measurement for the full 3-min recording time for 5 and 10 mM ATP (which includes the initial high P_o gating state plus the inactivation state). The inactivation was not instantaneous following addition of ATP to the cytosolic chamber but took several seconds and, in some cases, minutes to occur. The cumulative plot shown in Fig. 7B shows that the time to inactivation after the addition of 5 mM ATP to the cytosolic channel side for the data shown in Fig. 7B is fitted by a double exponential relationship, with half-times to inactivation of 6.9 and 190 s for the fast and slow components, respectively. In three experiments we were successful at perfusing away the ATP from the cytosolic chamber without breaking the bilayer. In two of the experiments the channels remained inactivated for the duration of the recording period (3 min). In the third experiment, the inactivation was reversed. Under the conditions of our experiments, it therefore appears that the effects of ATP are not completely irreversible and may be slowly reversible after washout if it were possible to record for long enough.

In a previous report (Kermode et al. 1998) and with identical recording conditions, we have shown that ATP concentrations ≥5 mM lead to slight reductions in the P_o of native RyR2 channels isolated from cardiac muscle, giving rise to a bell-shaped dose–response curve. Significantly, no inactivation events were observed. The decrease in P_o was brought about by an increase in the duration of

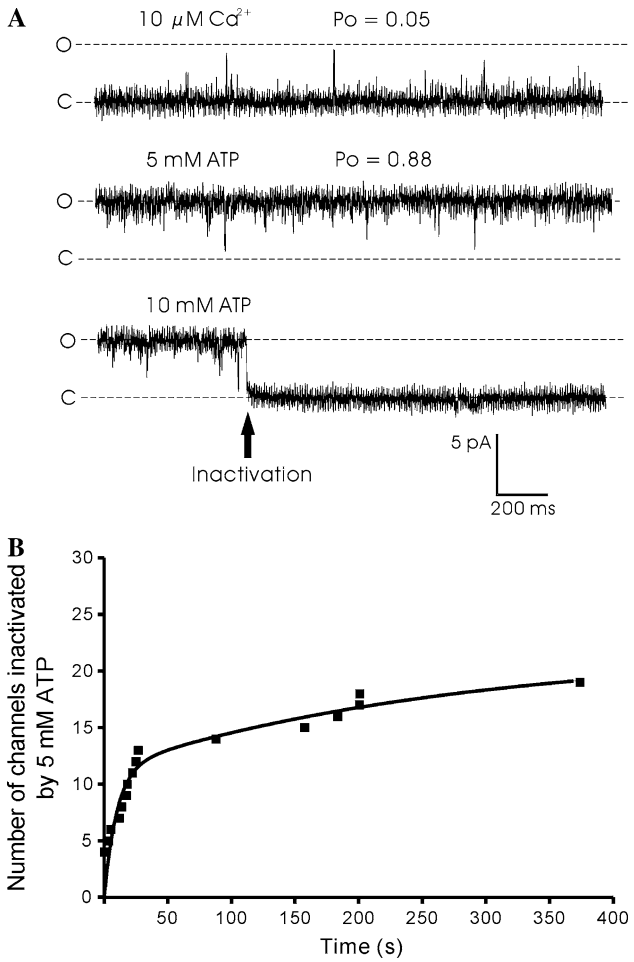

Fig. 7 Inactivation of recombinant RyR2 by ATP. (**A**) *Top* Representative recombinant RyR2 gating in the presence of 10 μM cytosolic Ca^{2+}. Addition of 5 mM ATP (*middle*) increased P_o but did not inactivate the channel. Subsequent addition of a further 5 mM ATP led to inactivation. Holding potential was 0 m*V*, and *O* and *C* indicate the fully open and closed channel levels, respectively. Free cytosolic [Ca^{2+}] was maintained at 10 μM by addition of $CaCl_2$ and EGTA as required. (**B**) Cumulative plot of the number of channels inactivated by 5 mM ATP. Time to inactivation following addition of ATP was measured for each channel, and the exponential fit to the data provides information about the time course of channel inactivation by 5 mM ATP. The curve was fitted according to the equation $y = y_{max1}[1 - \exp(-0.101t)] + y_{max2}[1 - \exp(-0.004t)]$, where y is the number of channels, t is the time in seconds and 0.101 and 0.004 are the rate constants for each exponential component (y_{max1} = 11.35 and y_{max2} = 11.32). The time for half the channels to be inactivated by 5 mM ATP for the fast and slow components was 6.9 and 190 s, respectively

closings and a reduction in the duration of openings. To eliminate the possibility that our earlier channel population was too narrow or unrepresentative of the whole population, we reinvestigated the effects of 5 and 10 mM ATP on the gating of RyR2 isolated from cardiac muscle. Figure 8 illustrates a typical example drawn from 16 new experiments. ATP 5 and 10 mM increased P_o but rarely induced

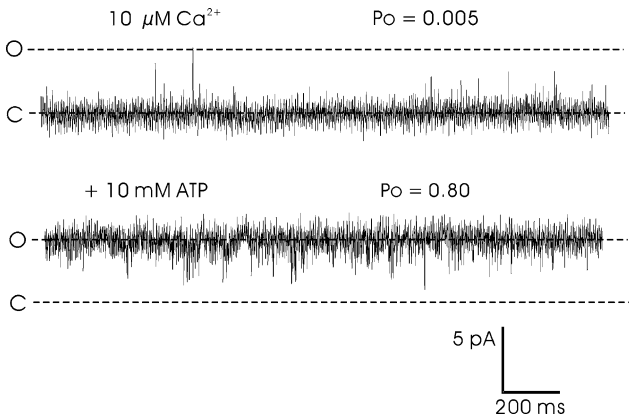

Fig. 8 Effects of ATP on the gating of native RyR2 isolated from cardiac muscle. *Top* Typical channel gating in the presence of 10 μm cytosolic Ca^{2+}. Addition of 10 mM ATP increased P_o but did not inactivate the channel. Holding potential was 0 m*V*, and *O* and *C* indicate the fully open and closed channel levels, respectively. Free cytosolic [Ca^{2+}] was maintained at 10 μm by addition of $CaCl_2$ and EGTA as required

inactivation events. Inactivation was only observed in one of the 16 experiments, thus highlighting an apparent difference between the effects of 5 and 10 mM ATP on recombinant RyR2 and its effects on native RyR2 channels isolated from cardiac muscle.

One obvious difference between the recombinant and nonrecombinant RyR2 channel preparations is the levels of other proteins expressed in HEK 293 cells and mammalian cardiac cells. RyR is known to bind to numerous proteins, forming a functional complex unit (Zhang et al. 1997) that

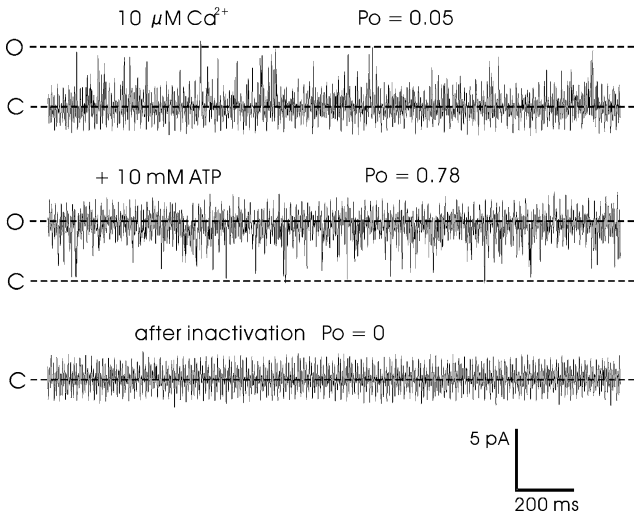

Fig. 9 Effects of ATP on the gating of a typical purified RyR2 channel. *Top* Gating in the presence of 10 μM cytosolic Ca^{2+}. Addition of 10 mM ATP increased P_o to a high level, and this was followed by channel inactivation. Holding potential was 0 m*V*, and *O* and *C* indicate the fully open and closed channel levels, respectively. Free cytosolic [Ca^{2+}] was maintained at 10 μM by addition of $CaCl_2$ and EGTA as required

will vary from cell type to cell type. Possibly, RyR2 channels expressed in HEK 293 cells lack an important accessory protein that interacts with RyR2 in such a way as to prevent inactivation by ATP. To test this hypothesis, we purified RyR2 channels isolated from cardiac muscle and then investigated the effects of 10 mM ATP. Figure 9 shows a representative channel, demonstrating that RyR2 channels isolated from cardiac muscle also show inactivation after purification. This occurred in 100% of the channels ($n = 5$).

Discussion

We have characterized certain key single-channel properties of the rabbit recombinant RyR2 channel under conditions where Ca^{2+} is the permeant ion. Importantly, the channels do not exhibit subconductance states and disorganized channel gating, although it has been shown that RyR2 expressed in HEK 293 cells is not associated with FKBP12.6 (Xiao et al. 2004, 2005). We also demonstrate a novel inactivating property of ATP that can be observed at a holding potential of 0 m*V*. Although much work has already been published on recombinant RyR2 function, most single-channel data have been obtained using a symmetrical monovalent cation as the permeant ion and at membrane potentials other than 0 (e.g., Kong et al. 2007; Xiao et al. 2007). In the present study, we used luminal Ca^{2+} as the permeant ion and membrane potential was held at 0 m*V*. These conditions were chosen because physiologically the potential across the SR is thought to remain close to 0 (Garcia and Miller 1984) and because it is known that luminal Ca^{2+} causes marked effects on the gating of RyR2, particularly affecting the response of the channel to activators (e.g., Sitsapesan and Williams 1994b; Tripathy and Meissner 1996; Györke and Györky 1998; Kong et al. 2007; Laver et al. 2007). We now show that, with luminal Ca^{2+} as permeant ion, most of the fundamental aspects of the rabbit recombinant RyR2 channel function are essentially identical to those of RyR2 channels isolated from sheep cardiac muscle. This includes single-channel conductance and the modifications to conductance induced by ryanodine and suramin. It also includes basic properties of RyR2 channel gating, taking account of the responses of the channel to the ligands Ca^{2+}, caffeine and suramin. These results highlight the very close similarity in single-channel function of the rabbit and sheep RyR2 isoforms. This is probably to be expected since the homology between the primary sequences of known RyR2 isoforms is >95% (Nakai et al. 1990; Otsu et al. 1990; Tunwell et al. 1996; Li and Chen 2001); however, a formal investigation into RyR2 species differences has never been performed.

An important feature of our recombinant RyR2 preparation is the complete absence of subconductance gating and lack of disorganized, unregulated channel gating. It has been reported that loss of FKBP12.6 from RyR2 causes the appearance of disorganized, unregulated channel gating that is characterized by high P_o and subconductance gating states (Kaftan et al. 1996; Marx et al. 2000, 2001). Given the findings of Xiao et al. (2004, 2005) that RyR2 expressed in HEK 293 is not associated with FKBP12.6, it might have been reasonable to expect that recombinant RyR2 isolated from HEK 293 cells would display such uncontrolled channel gating. Indeed, the FKBP12.6–RyR2 interaction is considered so vital for correct recombinant RyR2 channel gating that some groups routinely coexpress FKBP12.6 with RyR2 (Marx et al. 2000). However, we have demonstrated (see Fig. 5) no evidence for subconductance state gating in recombinant RyR2 expressed in HEK 293 cells. In fact, in most respects, the single-channel behavior of recombinant RyR2 and that of RyR2 from cardiac muscle is indistinguishable. Our data support the early observations (Barg et al. 1997; Timerman et al. 1996) that absence of FKBP12.6 does not result in the generation of subconductance gating states, contradicting the hypothesis that FKBP12.6 association is an absolute requirement for tightly controlled RyR2 channel gating (Kaftan et al. 1996; Marx et al. 2001). A recent study by Xiao et al. (2007), using different recording conditions (K^+ as permeant ion and holding potentials other than 0 m*V*), also makes this point very strongly. Our results and those of Xiao et al. (2007) are especially valuable because there is a widely held viewpoint that FKBP12.6 is a therapeutic target for heart disease because of its RyR2-"stabilizing" properties.

A novel finding of the present study is that ATP can inactivate RyR2. At ATP concentrations (5–10 mM) expected to be present in a cardiac cell (Allue et al. 1996; Hohl et al. 1992), the recombinant channels were first activated to a high P_o but then quickly shut down. A crucial aspect of the ATP-dependent inactivation that we describe here is that it takes place at the holding potential of 0 m*V*. This is important from a physiological viewpoint because the SR is not thought to develop any significant electrical potential (Garcia and Miller 1984). Voltage-dependent inactivation of RyR2 isolated from cardiac muscle has previously been reported in the presence of certain activating ligands (e.g., Laver and Lamb 1998; Ma et al. 1995; Sitsapesan et al. 1995), but the physiological significance of this effect has been questioned because of the need for a high positive or negative holding potential. All previous experiments reporting voltage-dependent inactivation were also performed with a monovalent cation as permeant ion. However, since luminal Ca^{2+} has profound effects on RyR2 channel gating (Györke and Györke 1998; Kong et al. 2007; Laver 2007; Sitsapesan and Williams 1994b; Tripathy and

Meissner 1996), it is important that the effects of RyR2 ligands are investigated with Ca^{2+} as the permeant ion.

In previous reports, with a monovalent cation as permeant ion, we observed voltage-dependent inactivation of RyR2 (as did others) in the presence of a variety of activators, which include ATP, caffeine and EMD41000 (a potent caffeine analogue) (e.g., Laver and Lamb 1998; Sitsapesan et al. 1995). This voltage-dependent inactivation of RyR2 tended to occur at high P_o levels, and it was suggested that inactivation followed long open events. This was certainly the case in the present study, as can be seen in Fig. 7. Inactivation did not occur at lower concentrations of ATP, where P_o was lower and gating predominantly consisted of brief opening and closing events.

More difficult to explain is why most of the recombinant channels were inactivated by 5–10 mM ATP whereas the channels isolated from cardiac muscle, in general, were not. A possible explanation for these results is that native RyR2 channels isolated from cardiac muscle are associated with an accessory protein, the binding of which serves to protect against the inherent ability of ATP to cause inactivation of RyR2 channels. The recombinant RyR2 channels expressed in HEK 293 cells may not be associated with this protein, either because it is not expressed or because it is expressed only at low levels in these cells. In line with this theory, after native RyR2 channels were subjected to CHAPS solubilization and purification using a sucrose gradient, inactivation was observed in all channels. Unfortunately, this hypothesis is difficult to test because of the large number of possible accessory proteins that could be involved.

One alternative explanation for the ATP-dependent inactivation is a species difference since we are comparing rabbit recombinant RyR2 with RyR2 isolated from sheep cardiac muscle. We suggest that this is unlikely, however, since the inactivation can be induced in the sheep isoform after purification. Another explanation could be that our methods for expressing recombinant RyR2 channels in HEK 293 cells and/or our isolation procedure somehow damage the channels, as does purification of native cardiac RyR2 by CHAPS solubilization. While we cannot completely rule out this possibility, our thorough evaluation of many of the basic conductance and gating properties of recombinant RyR2 provides no evidence for a generalized change in function. Therefore, any damage to the channel must be very specific and subtle. After all, ATP can still activate recombinant RyR2 and purified RyR2 channels to high P_o levels. In addition, lifetime analysis (not shown) demonstrates that the mechanisms underlying the ATP-induced increases in P_o are the same irrespective of whether the channels are expressed in HEK 293 cells or have been isolated or purified from cardiac muscle. In all cases, the inactivation events are not apparent until the higher, more physiological levels of ATP are reached.

Is there a physiological relevance for this novel inactivation property of ATP? We have shown that the inactivation takes place at a holding potential of 0 m*V* with Ca^{2+} as the permeant ion and that it occurs only at physiological levels of ATP ($\geq$5 mM). It is possible that ATP-dependent inactivation serves to close down RyR2 channels following SR Ca^{2+} release during EC coupling. However, since we observed inactivation only rarely with native RyR2 channels isolated from cardiac muscle, it may be that this response to ATP represents a pathophysiological response that operates when the levels of an RyR2 cofactor/accessory protein are lowered. RyR2 is associated with many accessory proteins that are thought to regulate its function. The levels and activity of some of these proteins are thought to change in cardiac disease. For example, the levels of calmodulin (CaM) immunoprecipitated with RyR2 are altered in heart failure (Ai et al. 2005). The activity of kinases bound to RyR2, e.g., protein kinase A and CaM kinase II, are also reported to change in heart failure (Ai et al. 2005; Guo et al. 2006; Marx et al. 2000). Further experiments are therefore necessary to identify if the binding of a specific protein to RyR2 is responsible for protecting the channel against ATP-dependent inactivation.

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