Luminal Ca²⁺ controls activation of the cardiac ryanodine receptor by ATP

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The synergic effect of luminal Ca²⁺, cytosolic Ca²⁺, and cytosolic adenosine triphosphate (ATP) on activation of cardiac ryanodine receptor (RYR2) channels was examined in planar lipid bilayers. The dose–response of RYR2 gating activity to ATP was characterized at a diastolic cytosolic Ca²⁺ concentration of 100 nM over a range of luminal Ca²⁺ concentrations and, vice versa, at a diastolic luminal Ca²⁺ concentration of 1 mM over a range of cytosolic Ca²⁺ concentrations. Low level of luminal Ca²⁺ (1 mM) significantly increased the affinity of the RYR2 channel for ATP but without substantial activation of the channel. Higher levels of luminal Ca²⁺ (8–53 mM) markedly amplified the effects of ATP on the RYR2 activity by selectively increasing the maximal RYR2 activation by ATP, without affecting the affinity of the channel to ATP. Near-diastolic cytosolic Ca²⁺ levels (<500 nM) greatly amplified the effects of luminal Ca²⁺. Fractional inhibition by cytosolic Mg²⁺ was not affected by luminal Ca²⁺. In models, the effects of luminal and cytosolic Ca²⁺ could be explained by modulation of the allosteric effect of ATP on the RYR2 channel. Our results suggest that luminal Ca²⁺ ions potentiate the RYR2 gating activity in the presence of ATP predominantly by binding to a luminal site with an apparent affinity in the millimolar range, over which local luminal Ca²⁺ likely varies in cardiac myocytes.

INTRODUCTION

The cardiac RYR2 channels provide the fundamental pathway for release of Ca²⁺ ions from the SR into the cytosol. In cardiac myocytes, cytosolic Ca²⁺ is generally considered to be the main physiological activator of the RYR2 channel (Fabiato, 1985; Ashley and Williams, 1990; Stern, 1992b), whereas luminal Ca²⁺ (Fabiato and Fabiato, 1975, 1978; Sitsapesan and Williams, 1994; Györke and Györke, 1998; Xu and Meissner, 1998; Shannon et al., 2000; Laver, 2007a,b; Györke and Terentyev, 2008; Laver and Honen, 2008; Qin et al., 2008) and cytosolic ATP (Meissner, 1984; Rousseau et al., 1986; Meissner and Henderson, 1987; Copello et al., 2002) together with cytosolic Mg²⁺ (Rousseau et al., 1986; Meissner and Henderson, 1987; Ashley and Williams, 1990; Laver et al., 1997; Copello et al., 2002; Zahradníková et al., 2003; Laver and Honen, 2008; Zahradníková et al., 2010) are considered to play a regulatory role.

At the single-channel level, the regulation of the RYR2 channel by luminal Ca²⁺ has been extensively studied since the late 1990s. However, the precise mechanism underlying this process is still not fully understood because the reported effects of luminal Ca²⁺ are diverse and sometimes even confusing. Currently, there is no consensus about how luminal Ca²⁺ regulates the RYR2 channel. The relative importance of Ca²⁺

binding to a luminal site (Györke and Györke, 1998) and Ca²⁺ binding to cytosolic sites, after having permeated the channel (Xu and Meissner, 1998), is intensively debated (Györke et al., 2002; Laver, 2007b; Liu et al., 2010; Diaz-Sylvester et al., 2011). Likewise, the relative roles of the luminal Ca²⁺ regulatory sites located directly on the channel (Sitsapesan and Williams, 1997; Jiang et al., 2007) and those mediated by the associated luminal protein calsequestrin (CSQ2) (Györke et al., 2004; Qin et al., 2008) are also not clear. Luminal regulation of the RYR2 channel in vivo could involve Ca²⁺-sensing mechanisms on both the luminal (CSQ2-dependent and CSQ2-independent) and cytosolic face of the channel (Sitsapesan and Williams, 1997; Györke et al., 2002; Laver, 2007a,b; Knollmann, 2009), and the prominence of one or the other mechanisms could depend on experimental conditions. Specifically, diastolic activation of RYR2 channels at low cytosolic Ca²⁺ may be regulated differently from activation during Ca²⁺-induced Ca²⁺ release, when local cytosolic Ca²⁺ is elevated to >10 µM (Acsai et al., 2011), as the presence of activating cytosolic Ca²⁺ concentration may compete with Ca²⁺ feedthrough (Györke and Györke, 1998; Ching et al., 1999). In addition, when extrapolating from in vitro data on reconstituted purified or recombinant RYR2 channels,

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it has to be noted that modification or loss of RYR2 sensitivity to luminal Ca^{2+} may occur as a result of dissociation or absence of CSQ2 (Xu and Meissner, 1998; Liu et al., 2010).

Although luminal Ca^{2+} regulates fractional Ca^{2+} release during excitation–contraction coupling (Shannon et al., 2000), its role in modulation of diastolic Ca^{2+} leak (Díaz et al., 1997; Györke et al., 1997; Lukyanenko et al., 2000) and spontaneous Ca^{2+} release (Lukyanenko et al., 1996, 1999) may be considered more important, as it has been shown to be altered in several pathological states such as heart failure (Hobai and O'Rourke, 2001; Piacentino et al., 2003) and Ca^{2+}-induced arrhythmias (Jiang et al., 2005; Terentyev et al., 2006). Hence, in this paper, we were interested in diastolic regulation of the native RYR2 channel. We therefore investigated RYR2 activity in planar lipid bilayers at a diastolic level of cytosolic Ca^{2+} (100 nM) at a wide range of ATP and luminal Ca^{2+}, and at a diastolic level of luminal Ca^{2+} (1 mM) at a wide range of ATP and cytosolic Ca^{2+}. Furthermore, we investigated the inhibition of ATP-activated RYR2 channels by cytosolic Mg^{2+} at a wide range of luminal Ca^{2+}. The data were interpreted in the framework of an allosteric model of RYR2 activation by ATP. We show that luminal Ca^{2+} potentiates the ATP-activated RYR2 channel by binding to a luminal site with an apparent affinity in the millimolar range, over which local Ca^{2+} in the lumen of the SR likely varies in cardiac myocytes. In addition, we show that the inhibitory effect of cytosolic Mg^{2+} on ATP-activated RYR2 channels is not affected by luminal Ca^{2+}, which is consistent with the action of Mg^{2+} at the cytosolic activation site of the RYR2 channel.

MATERIALS AND METHODS

Preparation of SR membrane vesicles
The rats were anesthetized with sodium pentobarbital (100 mg kg^{-1}, intraperitoneally). All anesthetic and surgical procedures were approved by the State veterinary and food administration of the Slovak Republic (Ro-3820/05-221, Ro-2821/09-221, and Ro-2672/08-221). Cardiac SR microsomes were isolated by differential centrifugation from the ventricles of rat hearts, as described previously (Gaburjakova and Gaburjakova, 2006).

Single RYR2 channel measurements
RYR2 channels were incorporated into a bilayer lipid membrane (BLM), and single-channel currents were recorded under voltage-clamp conditions. Cardiac SR microsomes were added to the cis chamber near the BLM formed from a 3:1 mixture of phosphatidyl ethanolamine with phosphatidyl serine (Avanti Polar Lipids, Inc.) across a 20–50-µm aperture in the wall of a polystyrene cup. The cis chamber (corresponding to the cytosol) was filled with 1 ml of (in mM): 250 HEPES, 125 Tris, 50 KCl, 1 EGTA, and 0.5–0.754 CaCl$_2$, pH 7.35. Cytosolic Mg^{2+} and ATP were added as MgSO$_4$ and ATPNa$_2$, respectively. The channels responded to the addition of Mg^{2+} and ATP to their cytosolic face within a few seconds. The concentration of EGTA was determined by potentiometric titration with a standard 100-mM CaCl$_2$ solution (Fluka) at a constant pH of 7.35, using a Ca^{2+}-selective electrode (type 25–20+; Elektrochemické Detektory, Ltd.),

and free $[Ca^{2+}]_C$ and free $[Mg^{2+}]_C$ were calculated with WinMaxc32 (version 2.50; Bers et al., 1994).

The trans chamber (corresponding to the lumen) was filled with 1 ml of (in mM): 50 KCl, 250 HEPES, and 0.5–53 Ca(OH)$_2$. When 0.5 and 1 mM Ca(OH)$_2$ was present, 7.5 and 7.0 mM Ba(OH)$_2$, respectively, were added into the solution to increase the current amplitude to a level optimal for recording. In this case, both ions served as charge carriers. Experiments with 8 mM $[Ba^{2+}]_L$ as the sole permeant ion mimicked the situation in which no Ca^{2+} is present at the luminal face of the RYR2 channel (Gaburjakova and Gaburjakova, 2006). The level of contaminating Ca^{2+} in the trans solution with 8 mM $[Ba^{2+}]_L$ was 5 µM, as determined potentiometrically using a Ca^{2+}-selective electrode. To minimize the changes in both ionic strength and osmolality, which have been shown to affect the basal activity of the RYR2 channel (Jiang et al., 2002, 2004, 2007), we kept the concentration of HEPES in the trans chamber constant and Tris (50–140 mM) was used to buffer pH to 7.35. The ionic strength of the trans solutions increased with luminal Ca^{2+} and ranged from 216 mM at 0.005–8 mM $[Ca^{2+}]_L$ to 290 mM at 53 mM $[Ca^{2+}]_L$, and their osmolality decreased with luminal Ca^{2+} and ranged from 542 mM at 0.005–8 mM $[Ca^{2+}]_L$ to 430 mM at 53 mM $[Ca^{2+}]_L$.

The trans chamber was connected to the head-stage input of an amplifier (MultiClamp 700B; Molecular Devices), and the cis chamber was held at ground. The holding potential was 0 mV. Electrical signals were filtered at 1 kHz, digitized at 4 kHz by an A/D converter (Digidata 1440A; Molecular Devices), and analyzed. Data acquisition and analysis were performed with pCLAMP 10 (Molecular Devices). Channel activity was continuously recorded for >2 min when only a single channel was incorporated into a BLM. To analyze gating behavior, the records were divided into 30-s intervals, and the open probability (P_O) and channel gating parameters were determined for each interval. The average open (t_O) and closed times (t_C) were calculated on these intervals as a standard arithmetic average, and for the calculation of t_O, at least 40 opening events (from several single channels) were collected, as suggested by Laver (2007b).

All chemicals were from Sigma-Aldrich, if not stated otherwise.

Data analysis
The dependence of P_O on ATP concentration was characterized by fitting the values of open probability by the Hill function,

$$P_O = P_O^{\min} + \left(P_O^{\max} - P_O^{\min}\right)\frac{[ATP]^{n_H}}{[ATP]^{n_H} + EC_{50}^{n_H}}, \qquad (1)$$

where $P_O^{\min}$ is the open probability in the absence of ATP, $P_O^{\max}$ is the maximum achievable open probability induced by ATP, EC_{50} is the ATP concentration inducing half-maximal activation, and n_H is the Hill coefficient. The values of $P_O^{\min}$ could not be reliably fitted with Eq. 1; therefore, they were set to the average P_O at the given $[Ca^{2+}]_L$ and $[Ca^{2+}]_C$ in the absence of ATP.

The dependence of normalized P_O on Mg^{2+} concentration was characterized by fitting the values of relative open probability by the Hill function,

$$\frac{P_O}{P_{O0}} = \frac{IC_{50}^{n_H}}{[Mg^{2+}]^{n_H} + IC_{50}^{n_H}}, \qquad (2)$$

where P_{O0} is the open probability in the absence of Mg^{2+}, IC_{50} is the Mg^{2+} concentration leading to half-maximal inactivation, and n_H is the Hill coefficient.

The action of ATP was further quantified within the framework of the allosteric model of RYR2 activation (Monod et al., 1965; Zahradník et al., 2005). The model is depicted in Fig. 1. Because $P_O^{\max}$ in the presence of luminal Ca^{2+} and ATP reached 1, the low
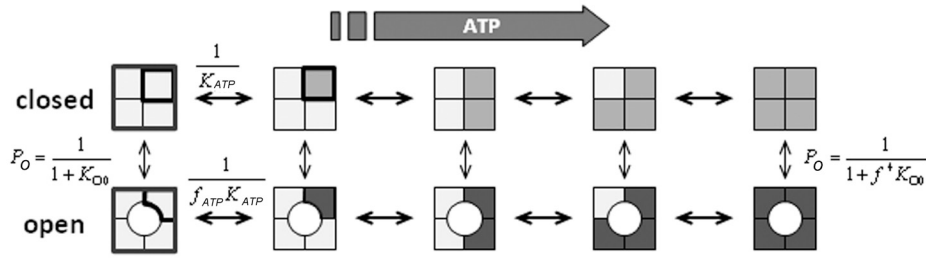
Figure 1. The allosteric gating model of RYR2 activation by ATP. All channel transitions are reversible and obey the principle of detailed balance. From left to right, the states of the channel differ by fractional ATP occupancy (ATP-free monomers, ATP-bound monomers of the closed state [top row], and ATP-bound monomers of the open state [bottom row] are depicted in light gray, gray, and dark gray, respectively). The equilibrium constants of the microscopic ATP-binding reaction (i.e., that related to a single monomer and not to the whole tetramer) to the first monomer of the closed and open channel are given above the respective arrows. The open probability of the fully ATP-free and ATP-bound channels is given at the left and right margin, respectively.

activity and inactivated modes were not accounted for. The model has five closed (Fig. 1, top row) and five open states (Fig. 1, bottom row) in which the channel tetramers contain 0–4 ATP-bound monomers (from left to right). The opening probability in the absence of ATP is determined by the equilibrium constant of the opening transition of the ATP-free tetramer, $K_{O0} = [C_0]/[O_0]$, where $[C_0]$ and $[O_0]$ are the probabilities of ATP-free closed and open states, respectively. Binding of ATP to the closed channel is defined by the microscopic ATP dissociation constant of the RYR2 monomer in the closed state, K_{ATP}. ATP exerts its effect by binding more strongly to RYR2 monomers in the open than to those in the closed states of the channel, and this property is defined by the allosteric factor f_{ATP}. Because of the principle of detailed balance, the equilibrium constants of the opening transitions of ATP-bound tetramers are affected as well (Monod et al., 1965; Zahradník et al., 2005). As a result, the propensity for opening of the whole channel tetramer is allosterically increased by the binding of ATP to each monomer.

The ATP dependence of RYR2 P_O has the following form:

$$P_O = \frac{\left([ATP] + f_{ATP} K_{ATP}\right)^4}{\left([ATP] + f_{ATP} K_{ATP}\right)^4 + K_{O0} f_{ATP}^4 \left([ATP] + K_{ATP}\right)^4}, \quad (3)$$

where [ATP] is ATP concentration, and the remaining parameters have been defined previously.

The effect of Ca^{2+} feed-through on the local cytosolic Ca^{2+} concentrations at a distance r from the RYR2 pore, $[Ca^{2+}]_C$, was calculated according to Stern (1992a) using the equation

$$\left[Ca^{2+}\right]_C (r) = e^{-\frac{r}{\sqrt{\frac{D_{Ca}}{k[B]}}}} \frac{i_{Ca}}{4\pi F D_{Ca} r}, \quad (4)$$

where i_{Ca} is the Ca^{2+} current amplitude, $D_{Ca} = 3 \times 10^{-10}$ m^2 s^{-1} is the Ca^{2+} diffusion coefficient, F = 96,500 C mol^{-1} is the Faraday constant, and [B] = 1 mM is the concentration of the Ca^{2+} buffer (EGTA or ATP) at the cytosolic side of the channel. The rates of Ca^{2+} binding, k, were 1.5×10^6 M^{-1} s^{-1} for EGTA (Stern, 1992a) and 2.25×10^8 M^{-1} s^{-1} for ATP (Michailova and McCulloch, 2001).

In all fitting procedures, if a fitted parameter did not change significantly for a set of tested conditions ($[Ca^{2+}]_L$, $[Ca^{2+}]_C$, [ATP], $[Mg^{2+}]_C$), fitting was repeated with a model in which this parameter was independent of these conditions. The quality of the resulting fits was compared using the F-test at a significance level of P = 0.05. When two models did not show a significant difference, the model with fewer parameters was chosen.

Calculations and fitting were performed in Origin (version 8E; OriginLab). Eq. 3 was derived in Mathematica (version 8; Wolfram Research). The results are reported as average ± SEM. The fitted parameters are reported with the standard error of the fit. Statistical comparisons (P < 0.05) of differences were made by either the Student's t test or ANOVA.

Online supplemental material

Fig. S1 documents the presence of CSQ2 in the cardiac SR microsomes used for reconstitution of the RYR2 channel into the BLM. Standard Western blotting method was used to detect CSQ2 (see legend to Fig. S1). Fig. S2 displays the theoretical dependence of local $[Ca^{2+}]_C$ at a distance r from the channel pore for 1, 8, 26, and 53 mM $[Ca^{2+}]_L$, calculated using Eq. 4. Parameters for calculation were taken from Stern (1992a). Figs. S1 and S2 are available at http://www.jgp.org/cgi/content/full/jgp.201110708/DC1.

RESULTS

Rat cardiac SR microsomes were fused into a BLM, and single-channel currents were recorded under asymmetric conditions with luminal Ca^{2+} and/or Ba^{2+} as charge carriers at a membrane potential of 0 mV.

Regulation of ATP-activated RYR2 channels by luminal Ca^{2+}

The activity of three to seven channels was recorded at 0.005, 1, 8, 15, 26, and 53 mM $[Ca^{2+}]_L$; 100 nM $[Ca^{2+}]_C$; and 0–30 mM ATP (27 single channels). Typical current traces of a single RYR2 channel in the presence of 100 nM $[Ca^{2+}]_C$ at 0.005, 8, and 53 mM $[Ca^{2+}]_L$ are illustrated in Fig. 2 A, and the effect of luminal Ca^{2+} on the dose–response of the RYR2 channel to ATP is illustrated in Fig. 2 B (top). Solid lines are the best fits by the Hill equation (Eq. 1). The fitted parameters are

TABLE 1

Effect of $[Ca^{2+}]_L$ on activation of the RYR2 channel by ATP

$[Ca^{2+}]_L$	P_O^{max}	EC_{50} for ATP	n_H
mM		*mM*	
0.005	0.0048 ± 0.0007	9.9 ± 1.8	2.51 ± 0.82
1	0.051 ± 0.017	0.549 ± 0.034	1.90 ± 0.17
8	0.290 ± 0.027	0.549 ± 0.034	1.90 ± 0.17
15	0.574 ± 0.024	0.549 ± 0.034	1.90 ± 0.17
26	0.662 ± 0.029	0.549 ± 0.034	1.90 ± 0.17
53	1.000 ± 0.049	0.549 ± 0.034	1.90 ± 0.17

$[Ca^{2+}]_C$ was 100 nM.

summarized in Table 1. Substantial activation of the RYR2 channel could be achieved only at high levels of luminal Ca^{2+} ($P_O = 0.267 \pm 0.047$ at 2.5 mM ATP for 8 mM [Ca^{2+}]$_L$ and 0.955 ± 0.015 at 2 mM ATP for [Ca^{2+}]$_L$ = 53 mM), whereas at 5 µM [Ca^{2+}]$_L$, the increase of activity, although over 10-fold, led to only a marginal open probability (0.0041 ± 0.0009 at 30 mM ATP).

The luminal Ca^{2+} dependence of RYR2 open probability in the absence of ATP, P_O^{min}, and of the fitted values of P_O^{max} and EC_{50} is depicted in Fig. 2 B (middle and bottom). Elevation of luminal Ca^{2+} from 5 µM to 1 mM led to an increase in P_O^{max} from 0.0048 ± 0.0007 to 0.051 ± 0.017

(Fig. 2 B, middle, and Table 1). Despite the 10-fold increase in P_O^{max}, the resulting open probability was still in the low range. Further increases of [Ca^{2+}]$_L$ progressively increased maximal activation of the channel with EC_{50} for luminal Ca^{2+} of 14.3 ± 2.0 mM (Fig. 2 B, middle, solid line), and full activation occurred at [Ca^{2+}]$_L$ = 53 mM. Notably, luminal Ca^{2+} also modulated RYR2 activity in the absence of ATP, albeit to a lesser extent. P_O^{min} changed only negligibly for [Ca^{2+}]$_L$ < 26 mM, but it increased significantly at 26 and 53 mM [Ca^{2+}]$_L$ (P < 0.05; Fig. 2 B, middle). Although high luminal Ca^{2+} was necessary to achieve substantial activation of the RYR2 channel by
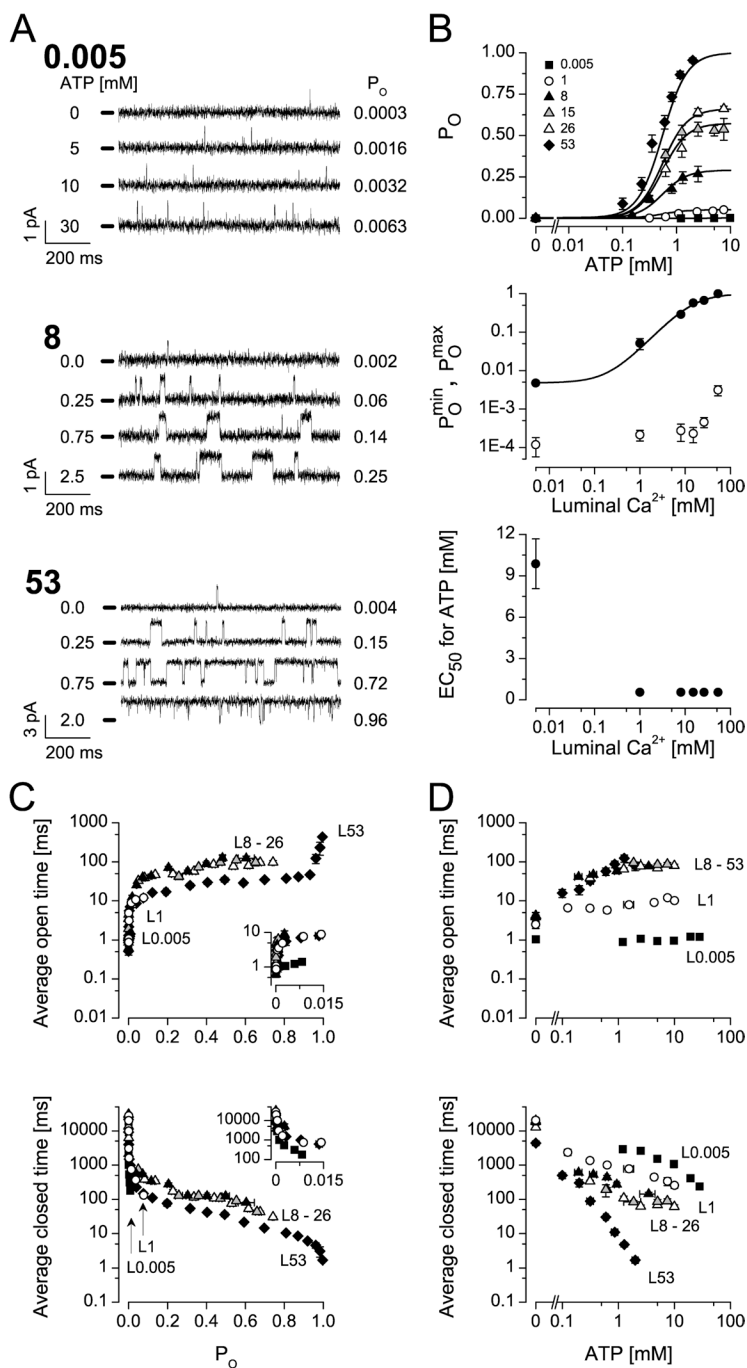

Figure 2. The effects of luminal Ca^{2+} on the response of the RYR2 channel to ATP. (A) Representative current traces of single RYR2 activity at various cytosolic ATP concentrations in the presence of 0.005 (top), 8 (middle), and 53 mM [Ca^{2+}]$_L$ (bottom). In all three datasets, [Ca^{2+}]$_C$ was 100 nM. Channel openings are in the upward direction. Dashes at the left of the traces indicate the closed state of the channel. Please note the different current calibration and different range of ATP concentrations. (B) The ATP dependence of RYR2 P_O (top) in the presence of 0.005, 1, 8, 15, 26, and 53 mM [Ca^{2+}]$_L$ (black squares; open circles; black, gray, and open triangles; and black diamonds, respectively). Solid lines are Hill equation fits. The whole dataset at 1–53 mM Ca^{2+} was fitted simultaneously when n_H and EC_{50} as fitted parameters were shared while P_O^{max} was free and P_O^{min} was set to the average P_O in the absence of ATP. The data in the virtual absence of luminal Ca^{2+} were fitted separately. The luminal Ca^{2+} dependence of P_O (middle) in the absence of ATP (P_O^{min}, open circles) and of the maximum achievable P_O in the presence of ATP (P_O^{max}, black circles). Solid line is the best fit of the relationship between P_O^{max} and [Ca^{2+}]$_L$ by the Hill equation, with apparent affinity for luminal Ca^{2+} of 14.3 ± 2.0 mM. (Bottom) The luminal Ca^{2+} dependence of EC_{50} for ATP. Data are presented as average ± SEM. Asterisks denote a significant increase of P_O^{min} (P < 0.05). (C and D) The average open (t_O) and closed times (t_C) determined on 30-s intervals for 0.005, 1, 8, 15, 26, and 53 mM [Ca^{2+}]$_L$ (black squares; open circles; black, gray, and open triangles; and black diamonds, respectively) are displayed as a function of either P_O (C) or ATP concentration (D). Insets in C show the rising phase of t_O versus P_O and t_C versus P_O plots on an expanded scale (P_O ranged from 0 to 0.02). In C and D, labels next to the points specify the conditions of the experiments (L0.005, L1, L8–26, and L53 for [Ca^{2+}]$_L$ of 0.005, 1, 8–26, and 53 mM, respectively). Error bars in C and D are shown only when the SEM is larger than symbol size.

ATP, 1 mM $[Ca^{2+}]_L$ was sufficient to decrease the value of EC_{50} for ATP from 9.9 ± 1.8 mM to 0.549 ± 0.035 mM. EC_{50} did not further decrease at higher $[Ca^{2+}]_L$ (Fig. 2 B, bottom).

The mechanism by which luminal Ca^{2+} modulated RYR2 activation by ATP was investigated by analyzing the average open (t_O) and closed times (t_C). Channel openings were much shorter at micromolar (Fig. 2 A, top) than at millimolar levels of luminal Ca^{2+} (Fig. 2 A, middle and bottom). In Fig. 2 C, the values of t_O and t_C of RYR2 channels activated by different concentrations of ATP (0–30 mM) are plotted against P_O. Notably, at 5 µM and 1 mM $[Ca^{2+}]_L$, the data are available only for

very low P_O because under these conditions, ATP induced only slight activation of the channel (Fig. 2 C, insets). Nevertheless, in the P_O interval of 0–0.1, a steep increase of t_O was apparent even at 1 mM $[Ca^{2+}]_L$, and a steep decrease of t_C was prominent under all tested conditions. Importantly, both t_O and t_C were larger at millimolar than at micromolar levels of luminal Ca^{2+} for any P_O value (Fig. 2 C, insets). In addition, in the $[Ca^{2+}]_L$ range of 8 to 26 mM, the values of t_O and t_C at a given P_O were independent of $[Ca^{2+}]_L$, although the ATP concentrations at these P_O values were substantially different for different $[Ca^{2+}]_L$. Furthermore, Fig. 2 C demonstrates that at all $[Ca^{2+}]_L$, the values of t_O did not change
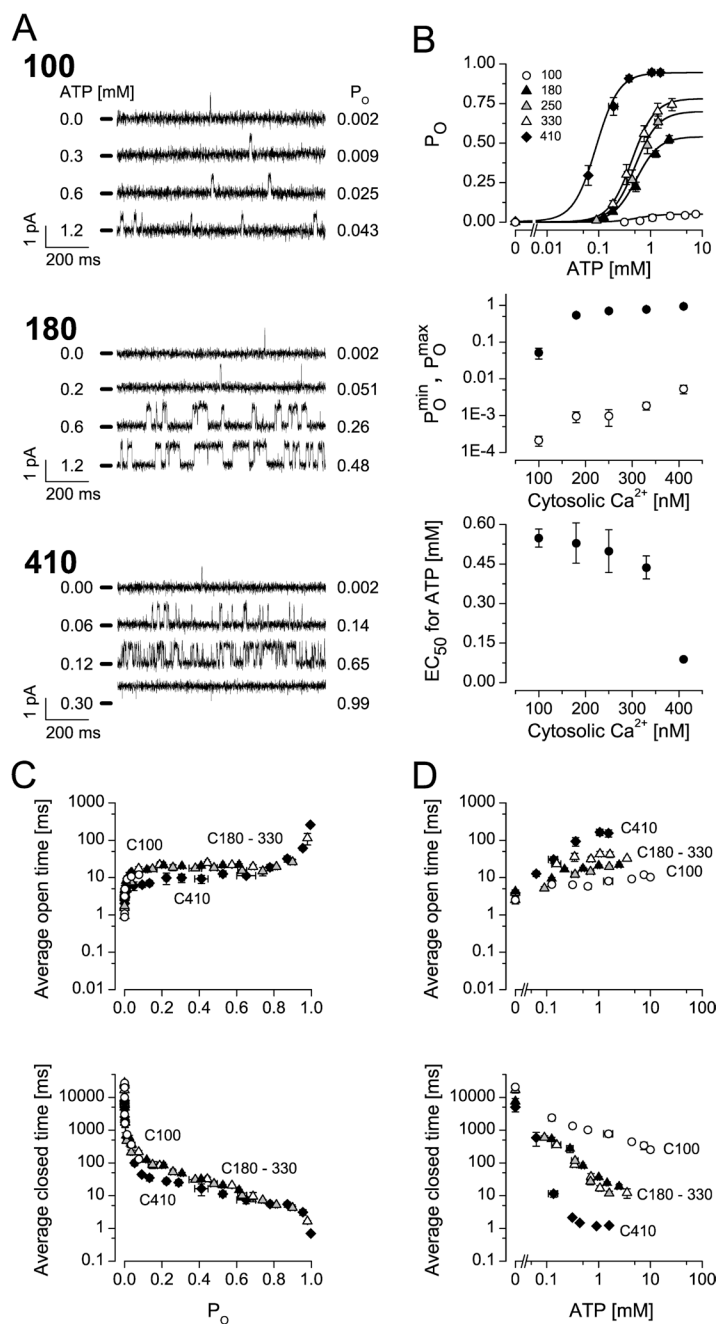

Figure 3. ATP dependence of the RYR2 activity at different cytosolic Ca^{2+} concentrations. (A) Representative current traces of single RYR2 channels recorded at 100 (top), 180 (middle) and 410 nM $[Ca^{2+}]_C$ (bottom) in the presence of 1 mM $[Ca^{2+}]_L$ and at ATP concentrations spanning the whole activation range (0–1.2, 0–1.2, and 0–0.30 mM, respectively). Channel openings are in the upward direction. Dashes at the left of the traces indicate the closed state of the channel. (B) The ATP dependence of RYR2 P_O (top) in the presence of 100, 180, 250, 330, and 410 nM $[Ca^{2+}]_C$ (open circles; black, gray, and open triangles; and black diamonds, respectively). Solid lines are Hill equation fits obtained by fitting the whole dataset at 100–410 nM $[Ca^{2+}]_C$ simultaneously when n_H as a fitted parameter was shared while P_O^{max} and EC_{50} were free. The parameters EC_{50} and P_O^{max} for the data at 100 nM $[Ca^{2+}]_C$ were fixed to values obtained by fitting the dataset with variable $[Ca^{2+}]_L$. The cytosolic Ca^{2+} dependence of P_O (middle) in the absence of ATP (P_O^{min}, open circles) and of the maximum achievable P_O in the presence of ATP (P_O^{max}, black circles). (Bottom) The cytosolic Ca^{2+} dependence of EC_{50} for ATP. Data are presented as average $\pm$ SEM. (C and D) The values of t_O and t_C determined on 30-s intervals for 100, 180, 250, 330, and 410 nM $[Ca^{2+}]_C$ (open circles; black, gray, and open triangles; and black diamonds, respectively) are displayed as a function of either P_O (C) or ATP concentration (D). In C and D, labels specify the conditions of the experiments (C100, C180–330, and C410 for $[Ca^{2+}]_C$ of 100, 180–330, and 410 nM, respectively). Error bars in C and D are shown only when the SEM is larger than symbol size.

appreciably with channel open probability in the P_O interval of 0.2–0.9, whereas the values of t_C decreased substantially in this P_O interval. Full activation of the RYR2 channel in the presence of 53 mM $[Ca^{2+}]_L$ was manifested by a further increase in t_O and a decrease in t_C for $P_O > 0.9$. It is possible that these changes of average dwell times were caused by incompletely resolved channel closures under these conditions (see Fig. 2 A, bottom trace in the bottom panel).

Although t_C decreased with increasing [ATP] regardless of the presence of luminal Ca^{2+} (Fig. 2 D, bottom), a different situation was found for t_O. At 5 µM of luminal Ca^{2+}, t_O was not significantly prolonged in the presence of up to 30 mM ATP. In the presence of millimolar levels of luminal Ca^{2+}, the increase in open time saturated at [ATP] < 1 mM. Importantly, under conditions when ATP led to substantial activation of the channel ($[Ca^{2+}]_L$ ≥ 8 mM and [ATP] ≥ 1 mM), the open time was dependent neither on luminal Ca^{2+} concentration nor on ATP concentration (Fig. 2 D, top), despite the large differences in P_O (0.2–1; Fig. 2 B, top). Although ATP modulated both gating parameters, clearly the rise in P_O was predominantly associated with a decrease in the closed time, which spanned over two orders of magnitude, was graded with both luminal Ca^{2+} and ATP concentration, and was the steepest and the largest for 53 mM $[Ca^{2+}]_L$ (Fig. 2 D, bottom). Collectively, luminal Ca^{2+} markedly amplified most effects of ATP on the activity and gating of RYR2 channels at 100 nM $[Ca^{2+}]_C$ by decreasing the closed times at all ATP concentrations.

The effect of cytosolic Ca^{2+} on ATP-activated RYR2 channels in the presence of 1 mM of luminal Ca^{2+}

It was quite surprising that under quasi-physiological conditions, when 1 mM $[Ca^{2+}]_L$ was present, the maximal activity of the RYR2 channel induced by ATP was very low at 100 nM $[Ca^{2+}]_C$. This led us to the question of whether cytosolic Ca^{2+}, which is the main physiological activator, could modulate the effect of luminal Ca^{2+} on the ATP-activated RYR2 channel. To address this question, we performed a series of experiments in which we fixed $[Ca^{2+}]_L$ at the physiological level of 1 mM and examined the whole dose–response of the RYR2 channel to ATP (0–10 mM) at five different sub-activating cytosolic Ca^{2+} concentrations. Activity of three to six channels was recorded at 100, 180, 250, 330, and 410 nM $[Ca^{2+}]_C$ (22 single channels). In the absence of ATP, the RYR2 channel had a P_O of <1% when $[Ca^{2+}]_C$ was varied from 100 to 410 nM. This indicates that cytosolic Ca^{2+} in this range did not activate the RYR2 channel substantially. Fig. 3 A illustrates the activating effect of ATP on representative RYR2 channels in the presence of 100, 180, and 410 nM $[Ca^{2+}]_C$. At 410 nM $[Ca^{2+}]_C$, submillimolar levels of ATP activated RYR2 channels to $P_O > 0.9$ (Fig. 3 A, bottom). Fig. 3 B (top panel) describes the relationship between P_O and ATP concentration at various

$[Ca^{2+}]_C$. Solid lines are fits by Eq. 1, and their parameters are given in Table 2. In Fig. 3 B (middle and bottom), the values of P_o^{min} calculated from the experimental data and the fitted values of P_o^{max} and EC_{50} are plotted as a function of $[Ca^{2+}]_C$. In contrast to the effect of luminal Ca^{2+}, all parameters (P_o^{min}, P_o^{max}, and EC_{50}) were considerably changed by cytosolic Ca^{2+}. P_o^{min} increased by about an order of magnitude, P_o^{max} increased 20 times, and EC_{50} decreased 5 times when $[Ca^{2+}]_C$ was changed from 100 to 410 nM. Our results indicate that a relatively small change in $[Ca^{2+}]_C$, which does not induce substantial activation of the RYR2 channel in the absence of ATP, leads to dramatic changes in the P_o^{max} and EC_{50} of ATP in the presence of 1 mM $[Ca^{2+}]_L$.

Luminal Ca^{2+} considerably affected the gating profile of ATP-activated RYR2 channels (Fig. 2, C and D). In contrast, no noticeable changes in the gating parameters were visible in the current traces that could be generated by the changes in cytosolic Ca^{2+} from 100 to 330 nM (Fig. 3 A). Fig. 3 C shows that the dependence of t_O and t_C on the value of P_O at 100–330 nM $[Ca^{2+}]_C$ is virtually identical. However, at 410 nM $[Ca^{2+}]_C$, both t_O and t_C were slightly shorter at any value of P_O, which resulted in a higher frequency of channel openings (Fig. 3 A, the third trace in the bottom panel). The changes of t_O and t_C at the lower and upper extremes of P_O were similar to those obtained by varying luminal Ca^{2+}. The main factor causing the increase in channel P_O in the range of 0.2 to 0.9 was the decrease in t_C.

The values of t_O gradually increased and those of t_C decreased with increasing [ATP] regardless of $[Ca^{2+}]_C$ (Fig. 3 D). In contrast to luminal Ca^{2+}, cytosolic Ca^{2+} strongly affected the ATP dependence of t_O. The ATP dependence of t_C changed with $[Ca^{2+}]_C$ in a similar manner as it did with $[Ca^{2+}]_L$: the data for intermediate values of $[Ca^{2+}]_C$ (180–330 nM) superimposed, whereas t_C at 100 nM $[Ca^{2+}]_C$ was larger and at 410 nM $[Ca^{2+}]_C$ was smaller. In all cases, the main factor causing the increase in channel P_O was the decrease in t_C accompanied by less extensive changes in t_O. Collectively, our results reveal that Ca^{2+} ions at low cytosolic concentrations, at which they by themselves do not activate the RYR2 channel substantially, do synergistically increase

TABLE 2
Effect of $[Ca^{2+}]_C$ on activation of the RYR2 channel by ATP

$[Ca^{2+}]_C$	P_o^{max}	EC_{50} for ATP	n_H
nM		*mM*	
100	0.051[a]	0.549[a]	1.97 ± 0.21
180	0.541 ± 0.037	0.529 ± 0.076	1.97 ± 0.21
250	0.700 ± 0.061	0.499 ± 0.081	1.97 ± 0.21
330	0.782 ± 0.037	0.437 ± 0.044	1.97 ± 0.21
410	0.946 ± 0.029	0.089 ± 0.006	1.97 ± 0.21

$[Ca^{2+}]_L$ was 1 mM.
[a]Fixed parameter.

the activation effect of ATP on RYR2 channels in the presence of 1 mM $[Ca^{2+}]_L$, predominantly by decreasing the closed times at all ATP concentrations.

Cytosolic Ca^{2+} potentiates the luminal Ca^{2+} regulation of the ATP-activated RYR2 channel

To understand the impact of cytosolic Ca^{2+} on the luminal Ca^{2+} activation of the RYR2 channel, we characterized the dependence of RYR2 open probability on luminal Ca^{2+} in the presence of 10 mM ATP at 180 and 410 nM $[Ca^{2+}]_C$. Activity of three to five channels was recorded at 0.5, 1, 8, and 53 mM $[Ca^{2+}]_L$ (16 single channels).

The ATP concentration was sufficient to achieve the maximal level of RYR2 activation at all tested $[Ca^{2+}]_L$. Fig. 4 A summarizes this series of experiments where P_O is plotted against $[Ca^{2+}]_L$ for 180 and 410 nM $[Ca^{2+}]_C$. The dashed line is the fit of the data at 100 nM $[Ca^{2+}]_C$ replotted from Fig. 2 B (middle). It is obvious that
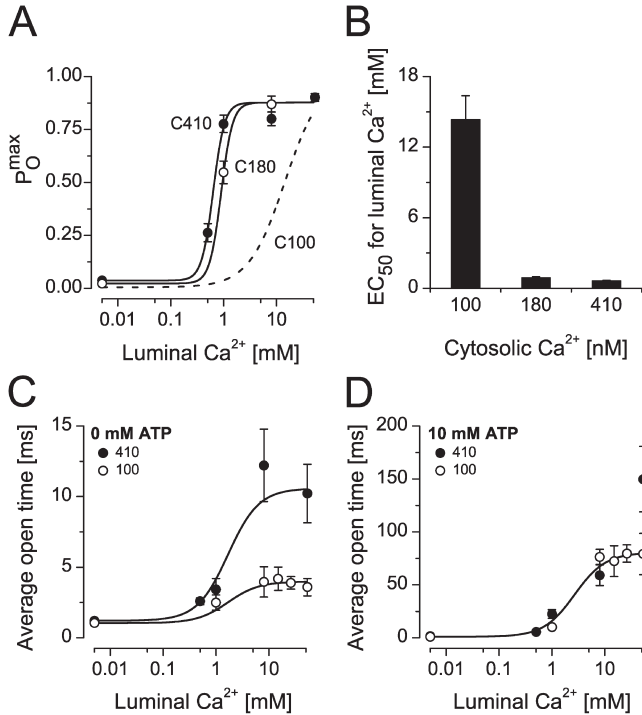
increasing $[Ca^{2+}]_C$ from 100 to 180 nM effectively shifted EC_{50} for luminal Ca^{2+} from 14.3 ± 2.0 mM to 0.897 ± 0.085 mM, which is already a physiologically relevant value (Fig. 4 B). Further, elevation of $[Ca^{2+}]_C$ to 410 nM led to a much smaller decrease in EC_{50} to 0.631 ± 0.049 mM (Fig. 4 B).

The open times in the absence of ATP dose-dependently increased with $[Ca^{2+}]_L$ (Fig. 4 C). Although the extent of t_O prolongation was larger at 410 than at 100 nM $[Ca^{2+}]_C$, the EC_{50} for t_O prolongation by luminal Ca^{2+} did not change with $[Ca^{2+}]_C$ and was 1.68 ± 0.55 mM. At 10 mM ATP, the mean open times did not significantly depend on cytosolic Ca^{2+} (Fig. 4 D). Again, they dose-dependently increased with $[Ca^{2+}]_L$, with $EC_{50} = 2.7 ±$ 1.0 mM. The values at 53 mM $[Ca^{2+}]_L$ were omitted from the fit, as the proportion of unresolved short closures, leading to overestimation of the open time, was very high under these conditions (see Fig. 2 A, bottom trace in the bottom panel). Interestingly, the values of EC_{50} for the prolongation of open times by luminal Ca^{2+} in the absence and presence of ATP were not significantly different.

The effects of luminal and cytosolic Ca^{2+} on the allosteric activation of the RYR2 channel by ATP

Although the exact nature of ATP-binding sites is not certain, it is assumed that these sites reside on individual monomers (Nakai et al., 1990; Otsu et al., 1990). Therefore, we attempted to describe the effect of luminal and cytosolic Ca^{2+} on ATP activation of the RYR2 channel as modulation of ATP binding to individual monomers. The mechanism of channel activation by ATP in the model (Fig. 1) was analogous to that of allosteric activation of the RYR2 channel by cytosolic Ca^{2+} (Zahradník et al., 2005); i.e., the binding of ATP to each monomer was assumed to allosterically increase the propensity for opening of the whole channel tetramer. The open probability in the absence of ATP is determined by the equilibrium constant of the opening transition of the ATP-free tetramer, K_{O0}, which reflects the level of channel activation by luminal and cytosolic Ca^{2+}. Binding of ATP to the closed channel is defined by the microscopic ATP dissociation constant of the RYR2 monomer in the closed state, K_{ATP}. ATP exerts its effect by binding more strongly to RYR2 monomers in the open than to those in the closed states of the channel, and this property is defined by the allosteric factor f_{ATP}. The results of fitting the ATP dependence of RYR2 open probabilities at different concentrations of luminal and cytosolic Ca^{2+} (data from Figs. 2 and 3) by the allosteric model of RYR activation (Eq. 3) are shown in Fig. 5. Optimal fit required the sharing of K_{ATP} for all conditions except at 5 µM of luminal Ca^{2+}. The theoretical prediction of this model was in a very good agreement with the experimental data. The parameters of the best fit are shown in Table 3.

Figure 4. Cytosolic Ca^{2+} regulates the effect of luminal Ca^{2+} on the RYR2 channel. (A) The $[Ca^{2+}]_L$ dependence of P_o^{max} in the presence of 180 and 410 nM $[Ca^{2+}]_C$ (open and black circles, respectively). Solid lines are Hill equation fits obtained by fitting the whole dataset at 180 and 410 nM $[Ca^{2+}]_C$ simultaneously when n_H as a fitted parameter was shared, whereas EC_{50} was free. The dotted line represents the data obtained for 100 nM $[Ca^{2+}]_C$ that are replotted from Fig. 2 B (middle). (B) The cytosolic Ca^{2+} dependence of EC_{50} for luminal Ca^{2+}. Data are presented as average ± SEM. (C) The values of t_{O0} determined in the absence of ATP on 30 s intervals for 100 nM and 410 nM $[Ca^{2+}]_C$ (open and black circles, respectively) are displayed as a function of $[Ca^{2+}]_L$. (D) The values of t_O determined in the presence of 10 mM ATP on 30-s intervals for 100 and 410 nM $[Ca^{2+}]_C$ (open and black circles, respectively) are displayed as a function of $[Ca^{2+}]_L$. Error bars in C and D are shown only when the SEM is larger than symbol size.

At a constant cytosolic Ca^{2+} of 100 nM, the concentration dependence of allosteric activation of the RYR2 channel by ATP at different [Ca^{2+}]$_L$ is shown in Fig. 5 A. The increase of luminal Ca^{2+} from 5 μM to 1 mM led to a decrease of K_{ATP} by over an order of magnitude and was accompanied by an increase of the allosteric effect of ATP (decrease of f_{ATP}). The decrease of K_{O0} was not significant. Further increase of [Ca^{2+}]$_L$ up to 53 mM did not affect ATP binding to the closed RYR2 channel. At lower [Ca^{2+}]$_L$ (≤8 mM), K_{O0} and f_{ATP} decreased in parallel, but for [Ca^{2+}]$_L$ ≥ 8 mM, K_{O0} changed by more than one order of magnitude, whereas f_{ATP} did not change significantly (Fig. 5 C). As a result, there was a marginally
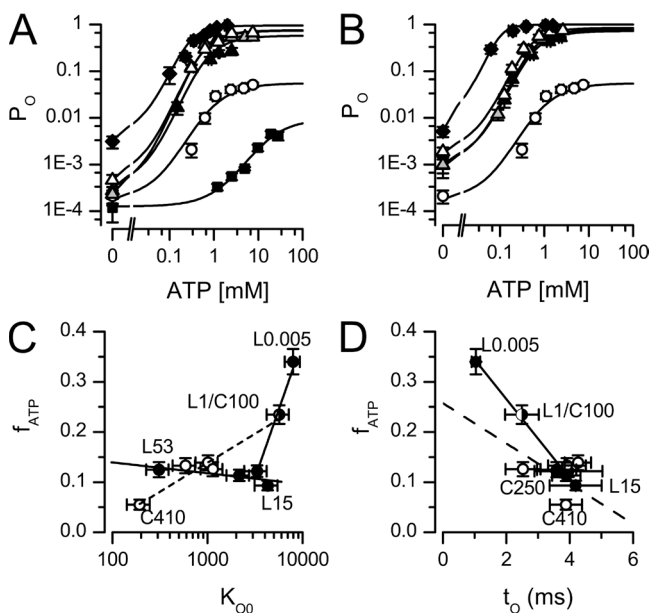

Figure 5. The concentration dependence of activation of the RYR2 channel by ATP analyzed by the allosteric gating model. (A) Values of P_O for 0.005, 1, 8, 15, 26, and 53 mM [Ca^{2+}]$_L$ (black squares; open circles; black, gray, and open triangles; and black diamonds, respectively) are displayed as a function of ATP concentration. Solid lines are the best fits by Eq. 3. (B) Values of Po for [Ca^{2+}]$_C$ of 100, 180, 250, 330, and 410 nM (open circles; black, gray, and open triangles; and black diamonds, respectively) are displayed as a function of ATP concentration. Solid lines are the best fits by Eq. 3. (C) The relationship between K_{O0} and f_{ATP} at different [Ca^{2+}]$_L$ (solid symbols) and [Ca^{2+}]$_C$ (open symbols). Solid lines are the apparent linear fits of the data for variable [Ca^{2+}]$_L$ in the two ranges of 0.005–8 mM (R = 0.99 and P = 0.066) and 8–53 mM (R = −0.62 and P = 0.37). Dashed line is the apparent linear fit of the data for variable [Ca^{2+}]$_C$ (R = 0.97 and P < 0.01). Note that the point representing 1 mM [Ca^{2+}]$_L$ and 100 nM [Ca^{2+}]$_C$ (partially filled circle) was included in both fits. (D) The relationship between t_{O0} and f_{ATP} at different [Ca^{2+}]$_L$ (closed symbols) and [Ca^{2+}]$_C$ (open symbols). Solid lines are the apparent linear fits of the data, with R = 0.99 (P < 0.005) for variable [Ca^{2+}]$_L$ and R = 0.50 (P > 0.35) for variable [Ca^{2+}]$_C$. Note that the point representing 1 mM [Ca^{2+}]$_L$ and 100 nM [Ca^{2+}]$_C$ (partially filled circle) was included in both fits. In C and D, labels next to the points specify the conditions of the experiments (L0.005, L1, L15, and L53 for [Ca^{2+}]$_L$ of 0.005, 1, 15, and 53 mM, respectively, and C100, C250, and C410 for [Ca^{2+}]$_C$ of 100, 250, and 410 nM, respectively).

significant correlation (R = 0.99 and P = 0.066) between log(K_{O0}), proportional to the energy difference between the open and the closed state in the absence of ATP, and f_{ATP} for [Ca^{2+}]$_L$ ≤ 8 mM, and no correlation (R = 0.62 and P = 0.37) between these quantities for [Ca^{2+}]$_L$ ≥ 8 mM. These results explain why EC_{50} for ATP did not change significantly at [Ca^{2+}]$_L$ ≥ 8 mM (Fig. 2 B, bottom). At the same time, there was a significant linear correlation (Fig. 5 D; R = 0.99 and P < 0.005) between t_{O0} and f_{ATP} for all [Ca^{2+}]$_L$, indicating that the prolongation of the open states of the ATP-free RYR2 channel by luminal Ca^{2+} occurred in parallel with the decrease of their microscopic ATP dissociation constant. Of note, t_{O0} and f_{ATP} changed significantly only at [Ca^{2+}]$_L$ ≤ 8 mM, showing that the increase in P_o^{max} at elevated [Ca^{2+}]$_L$ (≥8 mM; Fig. 2 B, middle) was caused solely by the decrease in K_{O0}, i.e., by the increased open probability in the absence of ATP.

At a constant luminal Ca^{2+} of 1 mM, the concentration dependence of allosteric activation of the RYR2 channel by ATP at different [Ca^{2+}]$_C$ is shown in Fig. 5 B. The value of K_{ATP} was not affected by [Ca^{2+}]$_C$ (Table 3). This result means that the substantial changes of EC_{50} for ATP that are evoked by changing [Ca^{2+}]$_C$ (Fig. 3 B, bottom) occur in the absence of changes in the microscopic ATP dissociation constant of the closed RYR2 channel. The variation in EC_{50} was fully accounted for by the changes of f_{ATP}, i.e., by the decrease of the microscopic ATP dissociation constant of the RYR2 open state with increasing [Ca^{2+}]$_C$. Under these conditions, the increase in P_o^{max} at elevated [Ca^{2+}]$_C$ (Fig. 3 B, middle) was brought about by changes of both K_{O0} and f_{ATP}. There was a significant linear correlation (Fig. 5 C; R = 0.97 and P < 0.01) between log(K_{O0}) and f_{ATP}. On the other hand, no significant correlation was observed between t_{O0} and f_{ATP} for variation of [Ca^{2+}]$_C$ (R = 0.5 and P = 0.39). The changes of f_{ATP} upon variation of cytoplasmic Ca^{2+} suggest that although ATP binds more strongly to the long open states of the RYR2 channel, induced by low (<8 mM) luminal Ca^{2+}, the stability of the ATP-bound open states is further increased by cytoplasmic Ca^{2+} in the absence of an effect on channel open times.

The inhibitory effect of cytosolic Mg^{2+} on the ATP-activated RYR2 channel in the presence of luminal Ca^{2+}

To this point, the functional profile of the ATP-activated RYR2 channels was examined in the absence of Mg^{2+}. However, in cardiac myocytes, cytoplasmic Mg^{2+} is a potent inhibitor of Ca^{2+} release from the SR; therefore, we assessed how Mg^{2+} would antagonize the potentiating effects of luminal Ca^{2+} on ATP-activated RYR2 channels. Specifically, to determine whether luminal Ca^{2+} affects the inhibitory potency of cytosolic Mg^{2+}, we have examined the effect of cytosolic Mg^{2+} on the activity of RYR2 channels fully activated by 10 mM ATP at 410 nM [Ca^{2+}]$_C$. Under the conditions of these experiments,

TABLE 3
Allosteric effects of ATP on the RYR2 channel

$[Ca^{2+}]_C$	$[Ca^{2+}]_L$	K_{OO}	K_{ATP}	f_{ATP}
nM	*mM*		*mM*	
100	0.005	7,900 ± 1,400	8.9 ± 2.5	0.339 ± 0.025
100	1	5,700 ± 1,500	0.485 ± 0.053	0.235 ± 0.019
100	8	3,310 ± 880	0.485 ± 0.053	0.122 ± 0.012
100	15	4,300 ± 1,200	0.485 ± 0.053	0.093 ± 0.009
100	26	2,160 ± 580	0.485 ± 0.053	0.113 ± 0.011
100	53	308 ± 82	0.485 ± 0.053	0.125 ± 0.015
180	1	1,010 ± 270	0.485 ± 0.053	0.139 ± 0.015
250	1	1,140 ± 300	0.485 ± 0.053	0.126 ± 0.014
330	1	580 ± 150	0.485 ± 0.053	0.133 ± 0.015
410	1	194 ± 52	0.485 ± 0.053	0.055 ± 0.010

the concentration of free $[ATP^{2-}]$ did not decrease below 2.5 mM. Thus, both $[ATP^{2-}]$ and total [ATP] were sufficient to saturate the ATP-binding sites. Activity of three to five channels was recorded at 0.5, 1, 8, and 53 mM $[Ca^{2+}]_L$ (17 single channels).

Typical current traces of a single RYR2 channel at 0.5, 1, and 8 mM $[Ca^{2+}]_L$ are illustrated in Fig. 6 A. In all cases, 0.4–0.5 mM of free $[Mg^{2+}]_C$ caused almost full inhibition of the channel activity. The effect of luminal Ca^{2+} on the dose–response of the RYR2 channel to Mg^{2+} is summarized in Fig. 6 B. Normalized P_O is plotted against free $[Mg^{2+}]_C$ for channels recorded at 0.5, 1, 8, and 53 mM $[Ca^{2+}]_L$. The solid line in Fig. 6 B is the best fit by the Hill equation (Eq. 2). The value of IC_{50} did not depend on $[Ca^{2+}]_L$ and was 61.5 ± 4.2 µM.

The presence of CSQ2

CSQ2 is important in regulation of the RYR2 channel by luminal Ca^{2+}. To ascertain the presence of CSQ2, standard Western blotting was performed, confirming that our preparation contained a detectable amount of CSQ2 (Fig. S1). The stability of CSQ2 binding to the RYR2 channel was further tested by monitoring RYR2 activity after exposure to 8 mM $[Ca^{2+}]_L$. When the channels were negligibly activated by cytosolic Ca^{2+} (100–391 nM) at 8 mM $[Ca^{2+}]_L$, their open probability remained stationary (0.0105 ± 0.0081) over a time period of at least 15 min (not depicted). Furthermore, when the channels were activated by cytosolic Ca^{2+} at concentrations (950 nM to 2 µM) that have been shown to activate CSQ2-bound RYR2 channel considerably but to induce only partial activation of the CSQ2-stripped channel (Qin et al., 2008), the open probability of 50% of RYR2 channels was stable during a 20-min exposure to 8 mM $[Ca^{2+}]_L$, and the activity of the remaining 50% of RYR2 channels significantly and abruptly declined to 37.1 ± 9.2% after 10–20 min. This reduction

of RYR2 open probability may be attributed to CSQ2 dissociation (Qin et al., 2008; Liu et al., 2010; Wei et al., 2009).

DISCUSSION

In this study, the effects of luminal Ca^{2+} on ATP-activated RYR2 channels were investigated. Our main aim was to characterize the regulation of the RYR2 channel relevant to diastolic Ca^{2+} release. To determine the principles by which diastolic RYR2 activation by luminal Ca^{2+} and ATP is governed, we have used a wide range of luminal Ca^{2+} and cytosolic ATP to resolve the shape of the concentration dependence of RYR2 open probability. The experiments revealed strong nonlinearities in the behavior of the RYR2 channel, namely, a strong dependence of activation by ATP on both luminal and cytosolic Ca^{2+} and a strong dependence of activation by luminal Ca^{2+} on cytosolic Ca^{2+}.

Our results clearly show that luminal Ca^{2+} is a potent regulator of ATP-activated RYR2 channels, as documented by a dramatic increase in P_o^{max} from almost zero to almost one when $[Ca^{2+}]_L$ was changed from 1 to 53 mM at 100 nM $[Ca^{2+}]_C$ (Fig. 2, A and B). Three separate effects of luminal Ca^{2+} on the activity of the RYR2 channel could be discerned (Fig. 7). The first effect (Fig. 7, left) is fully developed at 1 mM $[Ca^{2+}]_L$ and 100 nM $[Ca^{2+}]_C$, but it affects neither RYR2 open probability in the absence of ATP nor the maximum attainable open probability in the presence of ATP (P_o^{max}). It transpires as an increase in the sensitivity of the RYR2 channel to ATP (decrease of EC_{50} for ATP) as a result of a decrease of the microscopic ATP dissociation constant of the closed RYR2 channel (K_{ATP}). The EC_{50} values of the cytosolic A-site and the luminal L-site are 1 and 40–60 µM, respectively (Laver, 2007b, 2009; Laver and Honen, 2008). Calculations of local $[Ca^{2+}]_C$ in the vicinity of the channel pore (Fig. S2) predict local $[Ca^{2+}]_C$ of 27–45 µM at $[Ca^{2+}]_L$ of 1 mM, so that both the A-site and L-site should

be saturated under these conditions. This effect is therefore consistent with action at previously observed cytosolic or luminal Ca^{2+}-binding sites.

The second effect (Fig. 7, middle) has millimolar affinity (1.7 ± 0.6 mM), is independent of cytosolic Ca^{2+}, and transpires as prolongation of the mean open time in the absence of ATP (t_{O0}) and as an increase in the
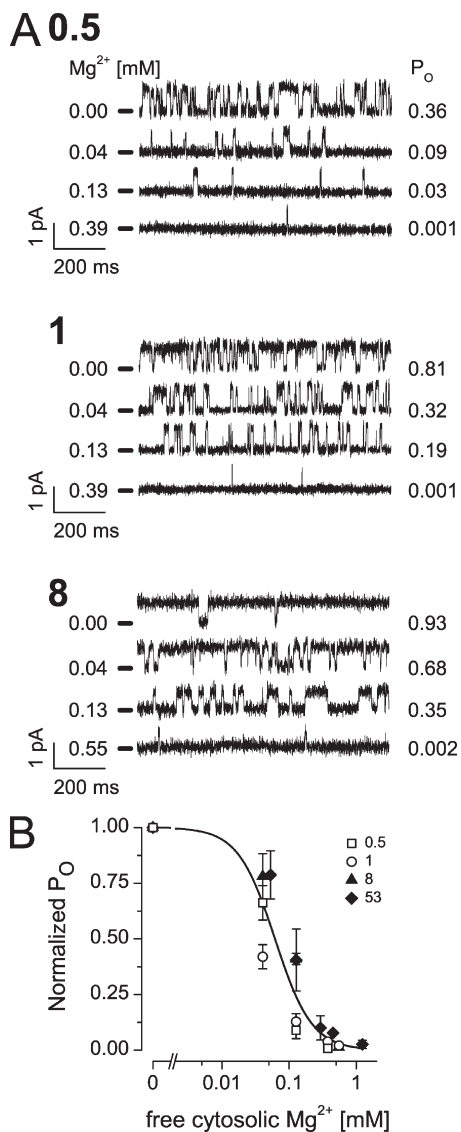

Figure 6. Inhibitory effect of cytosolic Mg^{2+} on the activity of the ATP-activated RYR2 channel. (A) Representative current traces of single RYR2 channels recorded at 0.5 (top), 1 (middle) and 8 mM $[Ca^{2+}]_L$ (bottom) at free $[Mg^{2+}]_C$ spanning the whole inhibitory range (0–0.39, 0–0.39, and 0–0.55 mM, respectively). All channels were activated by 10 mM ATP at 410 nM $[Ca^{2+}]_C$ to reach the maximal level of channel activation. Channel openings are in the upward direction. Dashes at the left of the traces indicate the closed state of the channel. (B) The free $[Mg^{2+}]_C$ dependence of normalized RYR2 P_O in the presence of 0.5, 1, 8, and 53 mM $[Ca^{2+}]_L$ (open squares and circles, and black triangles and diamonds, respectively). Solid lines are Hill equation fits obtained by fitting the whole dataset at 0.5–53 mM $[Ca^{2+}]_L$ simultaneously when n_H and IC_{50} as fitted parameters were shared.

relative stability of the ATP-bound open state (decrease of f_{ATP}) without a change of EC_{50} for ATP. Because even small changes in the cytosolic Ca^{2+} increased P_o^{max} in parallel with decreasing EC_{50} for ATP, and because local $[Ca^{2+}]_C$ during RYR2 openings was sufficient to fully saturate the cytosolic A-site (Fig. S2), neither an increase of t_{O0} nor a decrease of f_{ATP} can be attributed to a feed-through effect of luminal Ca^{2+}. We propose that these effects are mediated by a previously uncharacterized true luminal Ca^{2+} site (L_A-site), different from the L-site described by Laver (2009).

The third effect (Fig. 7, right) is apparent at ≥8 mM $[Ca^{2+}]_L$, transpires as an increase of RYR2 open probability in the absence of ATP (decrease of K_{O0}), and thus indirectly increases activation of the channel in the presence of ATP without affecting ATP binding to either closed (K_{ATP}) or open states (f_{ATP}). The low diastolic open probabilities in the absence of ATP precluded reliable determination of the concentration dependence of this process, and therefore it is not clear whether it is caused by Ca^{2+} binding to the L_A-site or by another mechanism.

Mechanism of the effect of luminal Ca^{2+} on the ATP-activated RYR2 channel

It has been observed previously that activation of RYR2 channel by luminal Ca^{2+} is most prominent in the presence of agonists (Sitsapesan and Williams, 1994, 1997; Lukyanenko et al., 1996; Györke and Györke, 1998; Laver, 2007b). Two competing hypotheses were proposed to explain this phenomenon: that the luminal Ca^{2+} sites are inaccessible in the absence of the agonist (Sitsapesan and Williams, 1997), or that luminal-triggered Ca^{2+} feed-through is necessary to sufficiently amplify the effect of ATP on the relative stability of the open and closed RYR2 conformation (Laver, 2007b). Our experiments showed that the effect of luminal Ca^{2+} is operative in the absence of ATP but that it is strongly amplified by ATP binding to the channel.
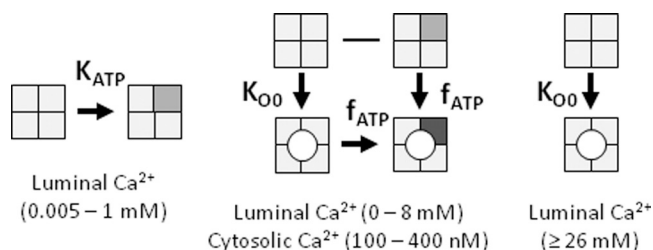

Figure 7. Summary of the effects of luminal and cytosolic Ca^{2+} on activation of the RYR2 channel by ATP. (Left) Luminal Ca^{2+} increases the affinity of the closed RYR2 channel to ATP (decrease of K_{ATP}), with saturation at 1 mM $[Ca^{2+}]_L$. (Middle) Both luminal (0–8 mM) and cytosolic Ca^{2+} (100–400 nM) increase the stability and ATP sensitivity of the open state (decrease of f_{ATP} and K_{O0}). (Right) High luminal Ca^{2+} further activates the channel by increasing the stability of the ATP-free open state (decrease of K_{O0}).

The amplification was not caused by Ca^{2+} feed-through but rather by allosteric interaction between the cytosolic and luminal Ca^{2+}-binding sites and the ATP-binding site (Fig. 7).

The increase of t_O by ATP in the presence of luminal Ca^{2+} was proposed previously to be caused by Ca^{2+} binding to the A-site (Laver, 2007b). In contrast with this supposition, we have observed a significant and prominent increase of the open time in the absence of ATP (t_{O0}) by luminal Ca^{2+} (Fig. 4 C). Moreover, the increase of open time by luminal Ca^{2+} was half-maximal at 1.7–2.7 mM $[Ca^{2+}]_L$ independently of [ATP] and of $[Ca^{2+}]_C$. The steady-state gradient around the open RYR2 channel leads to local $[Ca^{2+}]_C$ >10 µM under these conditions (Fig. S2); thus, binding of the permeating Ca^{2+} ions to the A-site via feed-through could not be responsible for this effect. Luminal Ca^{2+} therefore affects RYR2 open time without acting at the cytosolic site; i.e., occupation of the L_A site allosterically increases the kinetic stability of the open RYR2 conformation regardless of the presence of ATP. This result is important, as prolongation of open times by luminal Ca^{2+} has been considered previously the hallmark of "feed-through" regulation (Xu and Meissner, 1998; Laver, 2007b).

When expressed as the effect on t_{O0} (EC_{50} = 1.7 ± 0.6 mM), the EC_{50} of the L_A site is comparable to that described by Györke and Györke (1998): EC_{50} = 2–3 mM. When expressed as the effect on P_o^{max}, the EC_{50} of the L_A site, although substantially higher than that described by Györke and Györke (1998) at 100 nM $[Ca^{2+}]_C$ (EC_{50} = 14.3 ± 2.0 mM), decreases to a comparable value (0.5–1 mM) at 180–410 nM $[Ca^{2+}]_C$. Because the data of Györke and Györke (1998) were obtained at a cytosolic Ca^{2+} of 1 µM, the concentration dependence of the effects observed by Györke and Györke (1998) is in a good accordance with the luminal Ca^{2+} sensitivity of the L_A site for both prolongation of RYR2 open time and increasing RYR2 open probability.

Analysis of the concentration dependence of ATP activation using an allosteric model of RYR2 activation by ATP has revealed two important differences between the action of luminal and cytosolic Ca^{2+} on the ATP-activated RYR2 channel. First, luminal Ca^{2+} affected f_{ATP} inasmuch as it prolonged the mean open time of the ATP-free RYR2 channel (t_{O0}), whereas cytosolic Ca^{2+}, despite having a stronger effect on f_{ATP}, did not affect t_{O0} significantly. Second, the effect of high luminal Ca^{2+} (≥8 mM) on the open probability of the ATP-free RYR2 channel (a decrease of K_{O0}) did not affect the allosteric action of ATP (f_{ATP}), whereas cytosolic Ca^{2+} affected both parameters in parallel (Fig. 5 C). This implies that ATP-free open states with different properties were induced by variation of $[Ca^{2+}]_L$ and $[Ca^{2+}]_C$. The combined effect of cytosolic Ca^{2+} on K_{O0} and f_{ATP} at $[Ca^{2+}]_L$ = 1 mM indicates that increased occupancy of cytosolic Ca^{2+} sites increases the relative stability of ATP binding

to the open states of the RYR2 channel. These results suggest that the effect of ATP might be mediated by the free energy of the RYR2 open states in the absence of ATP, i.e., indirectly by the allosteric effect of the occupancy of the channel by Ca^{2+} at the cytosolic A-site and at the luminal L_A site.

The presence of CSQ2

CSQ2 is an important dynamic regulator of SR Ca^{2+} release not only because of its Ca^{2+}-buffering function but also as a putative luminal Ca^{2+} sensor for the RYR2 channel (Györke et al., 2004; Liu et al., 2010). CSQ2 presence was confirmed by Western blotting (Fig. S1), indicating that this protein was likely tightly bound to RYR2 channels, as it has been reported that the final crude SR preparation contains only ∼5% of the CSQ2 that had been originally present in the ventricular tissue (Murphy et al., 2011). The presence of CSQ2 in our membrane preparation also implies that triadin and junctin were present as well, as these membrane proteins are localized in the close proximity to RYR2 channels and anchor CSQ2 to these channels (Zhang et al., 1997).

Most of our experiments were performed at high $[Ca^{2+}]_L$ (≥5 mM) that has been used by several laboratories to dissociate CSQ2 from the RYR2 channel complex after a prolonged exposure (Zhang et al., 1997; Beard et al., 2002; Qin et al., 2008, 2009; Wei et al., 2009). Each laboratory used specific conditions defined by $[Ca^{2+}]_L$, luminal ionic strength, duration of high $[Ca^{2+}]_L$ exposure, and the extent of RYR2 activation by cytosolic agonists, the significance of which in the CSQ2 dissociation process is not known. Our data demonstrate that under the conditions of our experiments, 8 mM $[Ca^{2+}]_L$ is not strong enough to release CSQ2 from RYR2 channels within 10 min. Thus, it is most likely that CSQ2 remained bound to the RYR2 channel at 8 mM $[Ca^{2+}]_L$ in the experiments described in our study. At 0.005 and 1 mM $[Ca^{2+}]_L$, CSQ2 can be assumed to be present in the RYR2 channel complex (Györke et al., 2004; Qin et al., 2008, Wei et al., 2009; Liu et al., 2010). For $[Ca^{2+}]_L$ of 15, 26, and 53 mM, CSQ2 may be presumed to have been dissociated from the RYR2 channel, considering the fact that the rise in $[Ca^{2+}]_L$ gradually reduces the ability of CSQ2 to bind to other proteins (Mitchell et al., 1988; Park et al., 2004).

The only effect of high $[Ca^{2+}]_L$ (26–53 mM) was an increase of RYR2 open probability in the absence of ATP. This P_O increase was fully responsible for the increased ability of ATP to activate the channel. If CSQ2 dissociation causes an increase of RYR2 P_O (Györke et al., 2004), the increased RYR2 open probability at high $[Ca^{2+}]_L$ may be attributed to the absence of CSQ2 from the channel. However, many laboratories report a decrease of RYR2 P_O after dissociation of CSQ2 (Qin et al., 2008, 2009; Wei et al., 2008; Liu et al., 2010). It is possible, as suggested by Qin et al. (2009), that CSQ2 inhibits the

channel under conditions where the luminal Ca^{2+}-dependent RYR2 deactivation (Györke et al., 2004) is operative, whereas it activates the channel otherwise (Qin et al., 2008, 2009). If this is the case, both inhibition of the RYR2 channels after prolonged incubation with high luminal Ca^{2+} under activating conditions and increased RYR2 open probability at high luminal Ca^{2+} under diastolic conditions can be explained by CSQ2 dissociation. On the whole, the observed allosteric interactions between the L_A site and the two cytosolic binding sites (for Ca^{2+} and ATP) suggest that the L_A site resides directly on the RYR2 channel. Nevertheless, if CSQ2 acts by modulating the properties of RYR2 Ca^{2+}-binding sites, as suggested by Qin et al. (2008), it may be involved in the observed effects of luminal Ca^{2+}. However, the role of CSQ2 in the observed effects cannot be decided with certainty.

The identity of the activating ATP species

Currently, it is not clear whether only free ATP^{2-} or also divalent ion-bound ATP activates the RYR2 channel, although the importance of the negative charges on the phosphates of ATP for its effect on RYR2 open probability (Chan et al., 2003) would suggest the first to be the case. In our experiments, a significant decrease of $[ATP^{2-}]$ may have occurred during RYR2 openings caused by the binding of the permeating Ca^{2+} to ATP in the pore vicinity. Using Eq. 4, an up to 60% decrease was predicted at 53 mM $[Ca^{2+}]_L$ and 100 µM of total ATP for a distance of the ATP-binding site from the pore, $r = 12$ nM. However, $[ATP^{2-}]$ changes were <10% for ATP concentrations that evoked significant RYR2 activation; therefore, our data cannot help to resolve this question.

Physiological implications

Our results have significant physiological and patho-physiological implications for joint regulation of Ca^{2+} release by ATP, cytosolic and luminal Ca^{2+}, as well as by cytosolic Mg^{2+}.

Variation of luminal Ca^{2+} in the range of 0.005 to 1 mM at physiological ATP levels (400–600 µM of free [Mak et al., 1999] and $\sim$3 mM of total ATP [Bers, 2001]) does not bring about RYR2 activation to high levels under diastolic conditions (100 nM $[Ca^{2+}]_C$; Fig. 2). However, the relative increase of RYR2 P_O upon increasing $[Ca^{2+}]_L$ is very prominent (48- or 57-fold increase of P_O if, respectively, free or total ATP is considered the activating species; see Fig. 5 A). Because RYR2 inhibition by cytosolic Mg^{2+} is not affected by luminal Ca^{2+} (Fig. 6), the same relative increase of P_O would be observed in its presence. The increase in spark frequency brought about by a 50-fold increase in RYR2 open probability under diastolic conditions can be estimated to be $\sim$20-fold (Fig. 4 of Zahradníková et al., 2010). Using Eq. 3 and Table 3, it can be calculated that

the change of EC_{50} for ATP, evoked by the previously described high affinity processes involving the A-site and the L-site (Laver, 2007b), is responsible for only 18–23% of the increase in RYR2 P_O over the physiological $[Ca^{2+}]_L$ range. Thus, the L_A site, despite having a relatively low affinity, can be considered to significantly contribute to the physiological regulation of diastolic Ca^{2+} leak.

Under physiological conditions, $[ATP^{2-}]$ is close to the EC_{50} for ATP observed under diastolic conditions (100 nM $[Ca^{2+}]_C$ and 1 mM $[Ca^{2+}]_L$), and the total [ATP] is about six times larger. However, in failing human myocardium and in hearts of animal models of severe heart failure, [ATP] is decreased (Ingwall and Weiss, 2004); therefore, diastolic $[ATP^{2-}]$ may drop below the EC_{50}. In the absence of changes in RYR2 gating properties, the resulting decrease of diastolic leak would tend to counteract the parallel decrease of Ca^{2+} ATPase (SERCA2) function. Considering the net reduction of SR Ca^{2+} content observed in heart failure (Belevych et al., 2007), the decrease of SERCA2 function likely exceeds the decrease of diastolic RYR2 activity in the failing heart.

Furthermore, the dramatic increase of the maximal P_O induced by ATP at diastolic $[Ca^{2+}]_C$ by elevated $[Ca^{2+}]_L$ may play a significant role in the Ca^{2+} overload-induced Ca^{2+} release during the diastole (Berlin et al., 1987; Bassani et al., 1995; Lukyanenko et al., 1996, 1999). Increased SR Ca^{2+} content (Ca^{2+} overload) is a characteristic feature of various pathological states such as metabolic inhibition, ischemia/reperfusion, digitalis poisoning, and the early stage of heart failure (Kass et al., 1978; Levi et al., 1993; Pogwizd et al., 2001), and arises because of imbalance between Ca^{2+} entry and efflux (Trafford et al., 1997, 2001). It is well established that Ca^{2+} overload causes certain forms of arrhythmia that are triggered by Ca^{2+} waves propagating along the myocyte (Cheng et al., 1996; Pogwizd and Bers, 2004). Such arrhythmias account for $\sim$70% of the cases of ventricular tachycardia in patients without structural heart disease (Lerman et al., 1986). Ca^{2+} waves occur only under certain conditions, and one of them is enhanced activation of the RYR2 channel in the diastole (Lukyanenko et al., 1999). Collectively, potentiation of the RYR2 channel by $[Ca^{2+}]_L$ that is elevated beyond physiological levels might play a significant role in the generation of Ca^{2+} overload-induced cardiac arrhythmias.

Finally, the increase of P_O^{max} and decrease of EC_{50} for ATP at physiological $[Ca^{2+}]_L$ by small elevations of $[Ca^{2+}]_C$ may increase diastolic Ca^{2+} release, even in the absence of changes of RYR2 gating properties under conditions in which the Ca^{2+} uptake into the SR is compromised, such as during heart failure (Piacentino et al., 2003; Armoundas et al., 2007), or when Ca^{2+} signaling via IP_3R channels, located in the close proximity to the RYR2 channels, is enhanced, such as in hypertrophied hearts (Harzheim et al., 2009).

The elevated Ca^{2+} leak is becoming an increasingly recognized feature of the diseased heart. It is generally accepted that changes in RYR2 gating significantly contribute to the increased diastolic Ca^{2+} leak (Lehnart et al., 2004; Belevych et al., 2007; Terentyev et al., 2008; Tateishi et al., 2009). Relatively small changes in the parameters of RYR2 interaction with ATP (Fig. 5 and Table 3) may result in large changes in the open probability under diastolic conditions. Specifically, the ligand-independent opening transition (K_{OO}) seems to be essential for the joint RYR2 activation by ATP and luminal Ca^{2+}. Our data show that any non-ATP–related open probability increase of the RYR2 channel will be greatly amplified by ATP and luminal Ca^{2+}. In this way it can be explained why mutant RYR2 channels that exhibit increased ryanodine binding in the absence of agonists (Jiang et al., 2002, 2004) show increased luminal Ca^{2+} sensitivity (Jiang et al., 2005) and enhanced store overload–induced Ca^{2+} release (Jiang et al., 2004), and conversely, why RYR2 mutants that show decreased ryanodine binding under these conditions show decreased luminal Ca^{2+} sensitivity and reduced store overload–induced Ca^{2+} release (Jiang et al., 2007). Additionally, allosteric interactions between the occupation of binding sites for cytosolic and luminal Ca^{2+} and for ATP on one hand and the opening transition of the RYR2 channel tetramer on the other hand result in potentiation of the effects of each of these ligands by the remaining ones (Figs. 2, 3, and 5). This property of RYR2 channels might explain some of the apparent discrepancies when different laboratories attributed the causes of activity changes in RYR2 channels containing the same mutation to different mechanisms (V4653F: Lehnart et al., 2004 and Jones et al., 2008; S2246L: George et al., 2003, 2006, Jones et al., 2008, and Suetomi et al., 2011; R4496C: Jiang et al., 2002, 2004, George et al., 2003, 2006, and Fernández-Velasco et al., 2009; R2474S: Laver et al., 2008, Uchinoumi et al., 2010, and Xu et al., 2010).

Our results show that the potentiating effect of ATP on the activity of the RYR2 channel is antagonized by cytosolic Mg^{2+}. This inhibitory effect was not dependent on luminal Ca^{2+} (Fig. 6), and the IC_{50} for Mg^{2+} inhibition was in a very good accordance with the previously reported values for action of cytosolic Mg^{2+} at the A-site (Laver et al., 1997; Zahradníková et al., 2003, 2010; Gusev and Niggli, 2008). In view of our results, the diastolic Ca^{2+} leak is effectively controlled by cytosolic Mg^{2+} in the healthy heart. A different situation may occur under diseased conditions in which either lowered $[Mg^{2+}]_C$ (Haigney et al., 1995; Griffiths, 2000; Tong and Rude, 2005) or the decreased sensitivity of RYR2 channels to cytosolic Mg^{2+} has been reported (Lehnart et al., 2004). In both scenarios, the RYR2 channel is less inhibited by cytosolic Mg^{2+}; thus, the synergetic activation effect of ATP and of cytosolic and luminal Ca^{2+} described in our study can become more prominent and thus may further contribute to cardiac dysfunction.

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