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The Role of Calsequestrin, Triadin, and Junctin in Conferring Cardiac Ryanodine Receptor Responsiveness to Luminal Calcium

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ABSTRACT The level of Ca inside the sarcoplasmic reticulum (SR) is an important determinant of functional activity of the Ca release channel/ryanodine receptor (RyR) in cardiac muscle. However, the molecular basis of RyR regulation by luminal Ca remains largely unknown. In the present study, we investigated the potential role of the cardiac SR luminal auxiliary proteins calsequestrin (CSQ), triadin 1, and junctin in forming the luminal calcium sensor for the cardiac RyR. Recordings of single RyR channels incorporated into lipid bilayers, from either SR vesicle or purified RyR preparations, were performed in the presence of MgATP using Cs^+ as the charge carrier. Raising luminal [Ca] from 20 μM to 5 mM increased the open channel probability (P_o) of native RyRs in SR vesicles, but not of purified RyRs. Adding CSQ to the luminal side of the purified channels produced no significant changes in P_o, nor did it restore the ability of RyRs to respond to luminal Ca. When triadin 1 and junctin were added to the luminal side of purified channels, RyR P_o increased significantly; however, the channels still remained unresponsive to changes in luminal [Ca]. In RyRs reassociated with triadin 1 and junctin, adding luminal CSQ produced a significant decrease in activity. After reassociation with all three proteins, RyRs responded to rises of luminal [Ca] by increasing their P_o. These results suggest that a complex of CSQ, triadin 1, and junctin confer RyR luminal Ca sensitivity. CSQ apparently serves as a luminal Ca sensor that inhibits the channel at low luminal [Ca], whereas triadin 1 and/or junctin may be required to mediate interactions of CSQ with RyR.

INTRODUCTION

The cardiac sarcoplasmic reticulum (SR) Ca release channel/ryanodine receptor (RyR) is a multimolecular signaling complex that plays a key role in excitation-contraction coupling. During excitation-contraction coupling, the Ca that enters the cytosol via plasmalemmal voltage-dependent Ca channels binds to and activates RyRs at high-affinity cytosolic Ca activation sites (Meissner et al., 1997; Li and Chen, 2001). When RyR channels open, a much larger amount of Ca is released to the myoplasm, leading to activation of contractile proteins. This mechanism is known as Ca-induced Ca release (CICR) (Fabiato, 1985; Bers, 2002). Besides the high-affinity cytosolic activation sites, the RyR channel is positively controlled by low-affinity Ca sensing sites accessible from the luminal side of the channel (Lukyanenko et al., 1996; Györke and Györke, 1998; Ching et al., 2000). Dissociation of Ca from these sites on decline of intra-SR [Ca] ($[Ca]_{SR}$) leads to RyR deactivation and robust termination of CICR, permitting cardiac relaxation (Terentyev et al., 2002). RyRs, sensitive to levels of Ca in the SR, also render a luminal Ca-dependent leak pathway that plays an important role in setting the SR Ca content and in shaping the response of cardiac cells to various inotropic influences that affect the Ca release or uptake mechanisms (Lukyanenko et al., 2001; Shannon, et al., 2002). Abnormal regulation of RyRs by luminal Ca has been implicated in certain pathological states, including Ca overload, arrhythmia, and heart failure (Györke et al., 2002; Terentyev et al., 2003).

Although significant progress has been made in elucidating the molecular basis of RyR activation by cytosolic Ca (Meissner et al., 1997; Li and Chen, 2001), the molecular mechanisms of luminal Ca sensing remain unknown. One possibility is that the luminal calcium sensor is a part of the RyR protein itself. Alternatively, auxiliary proteins in the heart with luminal localization, such as calsequestrin (CSQ), triadin 1, and junctin, could mediate the effects of luminal Ca on RyR (Ikemoto et al., 1989; Donoso et al., 1995; Zhang et al., 1997; Shin et al., 2000). Cardiac CSQ is a major Ca binding protein in the SR lumen (Scott et al., 1988; Jones et al., 1998) that undergoes conformational changes upon Ca binding (Mitchell et al., 1988) and interacts with RyR either directly (Herzog et al., 2000) or via triadin 1 and junctin (Zhang et al., 1997). Functional studies of RyR modulation by CSQ, mostly conducted with the skeletal muscle isoform, have yielded controversial results. In lipid bilayer studies, adding skeletal muscle CSQ to the luminal side of RyR has been reported to either increase (Kawasaki and Kasai, 1994; Ohkura et al., 1998; Szegedi et al., 1999) or decrease skeletal muscle RyR activity (Beard et al., 2002). Understanding of the role of CSQ as the potential luminal Ca sensor for RyR is further complicated by the fact that high luminal Ca concentrations that lead to maximal RyR potentiation are also known to dissociate CSQ from the RyR complex (Zhang et al., 1997).

Triadin 1 (Kobayashi and Jones, 1999) and junctin (Jones et al., 1995) are transmembrane proteins that form quaternary

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complexes between themselves, RyR, and CSQ (Zhang et al., 1997; Kobayashi and Jones, 1999; Jones et al., 1995; Kobayashi et al., 2000). The structures of both triadin 1 and junctin have luminal domains with areas of highly dense, charged amino acid residues that could form the putative luminal Ca receptor (Kobayashi and Jones, 1999; Jones et al., 1995; Kobayashi et al., 2000). Alternatively, these proteins could be involved in luminal Ca sensing by mediating interactions between RyR and CSQ (Zhang et al., 1997). In this study, we examined the impact of purification of canine cardiac RyR and the effects of subsequent reassociation of the purified channel with canine cardiac CSQ, triadin 1, and junctin on the functional activity of the RyR channel and its ability to sense luminal Ca. The results show that a complex of CSQ, triadin 1, and/or junctin confers luminal Ca sensitivity to RyR. CSQ apparently serves as a luminal Ca sensor that inhibits RyR channel activity at low luminal [Ca]; furthermore, triadin 1 and/or junctin are stimulatory by themselves and are required to mediate the inhibitory interactions between CSQ and the RyR channel.

MATERIALS AND METHODS

Single RyRs were reconstituted by fusing canine heavy SR microsomes into planar lipid bilayers as described previously (Györke and Györke, 1998). Bilayers were composed of 80% phosphatidylethanolamine and 20% phosphatidylcholine dissolved in decane at a final concentration of 50 mg/ml. Channel incorporation was performed in solutions containing (in mM) 350 $CsCH_3SO_3$, 0.02 $CaCl_2$, and 20 Hepes (pH 7.4), on the *cis* (cytosolic) side of the bilayer, and 20 $CsCH_3SO_3$, 0.02 $CaCl_2$, and 20 Hepes (pH 7.4) on the *trans* (luminal) side of the bilayer. The experimental solutions contained (in mM) 350 $CsCH_3SO_3$, 3 MgATP, 0.6 $MgCl_2$ (free [Mg^{2+}] ~0.9 mM), 0.02 $CaCl_2$, and 20 Hepes, pH 7.4 (*cis*), and 350 $CsCH_3SO_3$, 0.02 $CaCl_2$, and 20 Hepes, pH 7.4 (*trans*). The holding potential in all experiments was +40 mV. Single channel currents were recorded with an Axopatch 200A (Axon Instruments, Foster City, CA) patch-clamp amplifier. Acquisition and analysis of data were performed using pClamp 6.01 software (Axon Instruments). Data are presented as mean ± SE. Paired or unpaired data sets were statistically evaluated using a Student's *t*-test or a Mann-Whitney U test ($P < 0.05$), as appropriate. Canine cardiac RyRs were purified from SR vesicles using methods of Lai et al. (1988) and Zhang et al. (1997). These methods yield samples of purified RyRs that do not contain junctin, triadin, and calsequestrin (Zhang et al., 1997; Beard et al., 2002). Recombinant purified canine cardiac calsequestrin (Kobayashi et al., 2000), junctin (Zhang et al., 1997), and triadin 1 (Kobayashi and Jones, 1999) were obtained as described. To test the effects of calsequestrin, junctin, and triadin 1 on the RyR channel activity, the purified proteins were added to the *trans* chamber at concentrations of 5–20 μg/ml. CSQ was stored and added from a buffer solution containing 20 mM Mops, 150 mM NaCl (pH 7.0), and ~3 mg/ml of the protein; the solutions containing triadin 1 and junctin (~–1.0 mg/ml) included 1% CHAPS, 0.8 M NaCl, and 20 mM Mops (pH 7.2).

RESULTS

Canine cardiac SR microsomes and purified RyRs reconstituted into proteoliposomes were fused into planar lipid bilayers and single-channel currents were recorded with Cs^+ as the charge carrier in the presence of cytosolic Mg^{2+} and ATP. We considered two possibilities for the luminal Ca sensor location: 1), Ca binds either directly to the luminal aspect of the RyR protein; or 2), Ca binds to an auxiliary protein with a luminal location. First, to investigate the possibility that the luminal Ca sensor is located on the RyR protein itself, we compared the abilities to respond to changes in luminal [Ca] of native RyRs in SR vesicles and of purified RyRs. Channel activity was recorded at *cis* [Ca] levels of 6 μM, and the luminal (*trans*) [Ca] was increased from 20 μM to 5 mM. Consistent with previous studies (Györke and Györke, 1998), the native RyRs responded with a dramatic increase in P_o; however, the purified RyRs showed negligible difference in channel activity upon increasing luminal Ca concentration. Traces of native and

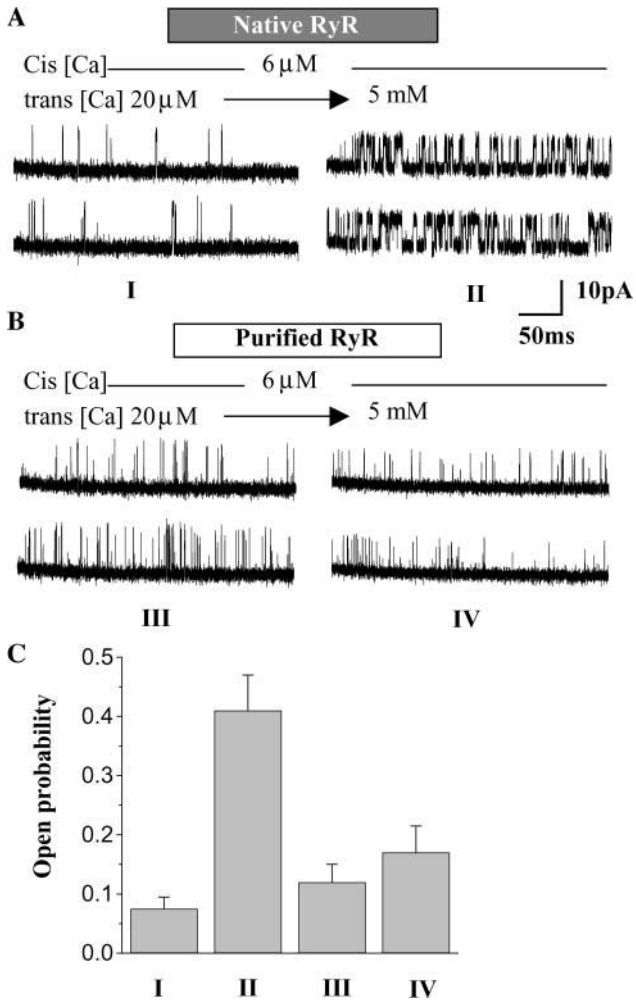

FIGURE 1 Responsiveness of native and purified RyRs to increasing luminal Ca. Representative traces of current recordings from a native (*A*) and a purified (*B*) RyR before and after increasing [Ca] in the *trans* chamber from 20 μM to 5 mM. Current recordings were obtained at +40 mV holding potential in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4. The *cis* [Ca] was 6 μM. Channel openings are upward. (*C*) Pooled P_o for conditions of low and high *trans* [Ca] in native and purified RyRs. Data presented as mean ± SE; $n = 14$ and 8 for native and purified channels, respectively.

purified controls (Fig. 1, *A I* and *B III*, respectively) as well as the activity upon luminal [Ca] increase can be seen in Fig. 1 (native and purified luminal traces in Fig. 1, *A II* and *B IV*, respectively). Pooled data for these conditions show that the native RyR channel activity increased from a P_o of ~0.08 to a P_o of ~0.40, whereas the purified channels remained virtually unaffected from a P_o of ~0.12 to ~0.15 (Fig. 1 *C*). These results suggested that the luminal Ca sensor is not located on the RyR protein itself, but may be part of a complex of luminal proteins that were lost during the purification process.

Cardiac RyRs localized to the junctional SR appear to complex with a number of proteins, including CSQ, triadin 1, and junctin, at the luminal side of the SR membrane (Ikemoto et al., 1989; Zhang et al., 1997). To explore the potential role of these proteins in mediating RyR luminal Ca sensitivity, we examined the regulatory effects of different combinations of purified cardiac triadin 1, junctin, and CSQ on purified RyRs. We began our examination with the *trans* addition of triadin 1 and junctin (5 μg/ml of each) to purified RyR. Both triadin 1 and junctin have well-defined hydrophobic domains (Kobayashi and Jones, 1999; Jones et al., 1995), which should facilitate incorporation of these proteins into bilayer after their addition to the solution in the *trans* chamber. *Cis* [Ca] was 6 μM and *trans* [Ca] was 20 μM for the control and the initial addition of triadin 1 and junctin (Fig. 2 *A*, *I* and *II*). Then, *cis* [Ca] was reduced to 2 μM due to excessive channel activity and the necessity to observe any changes in channel activity once the luminal [Ca] was increased (Fig. 2 *A III*). Next, luminal [Ca] was increased to 5 mM (Fig. 2 *A IV*). Initial addition of triadin 1 and junctin showed a significant increase in channel activity from P_o of 0.11 to 0.54, but addition of 5 mM luminal Ca did not affect channel activity (Fig. 2 *B*). Similar potentiatory effects on channel P_o were observed when either triadin 1 or junctin alone was added to the *trans* side of purified RyRs in several additional experiments (not shown). The increase in RyR open probability observed with these proteins provided a ''functional control'' for their association with the RyR. Addition of the proteins to the *cis* side of the bilayer had no

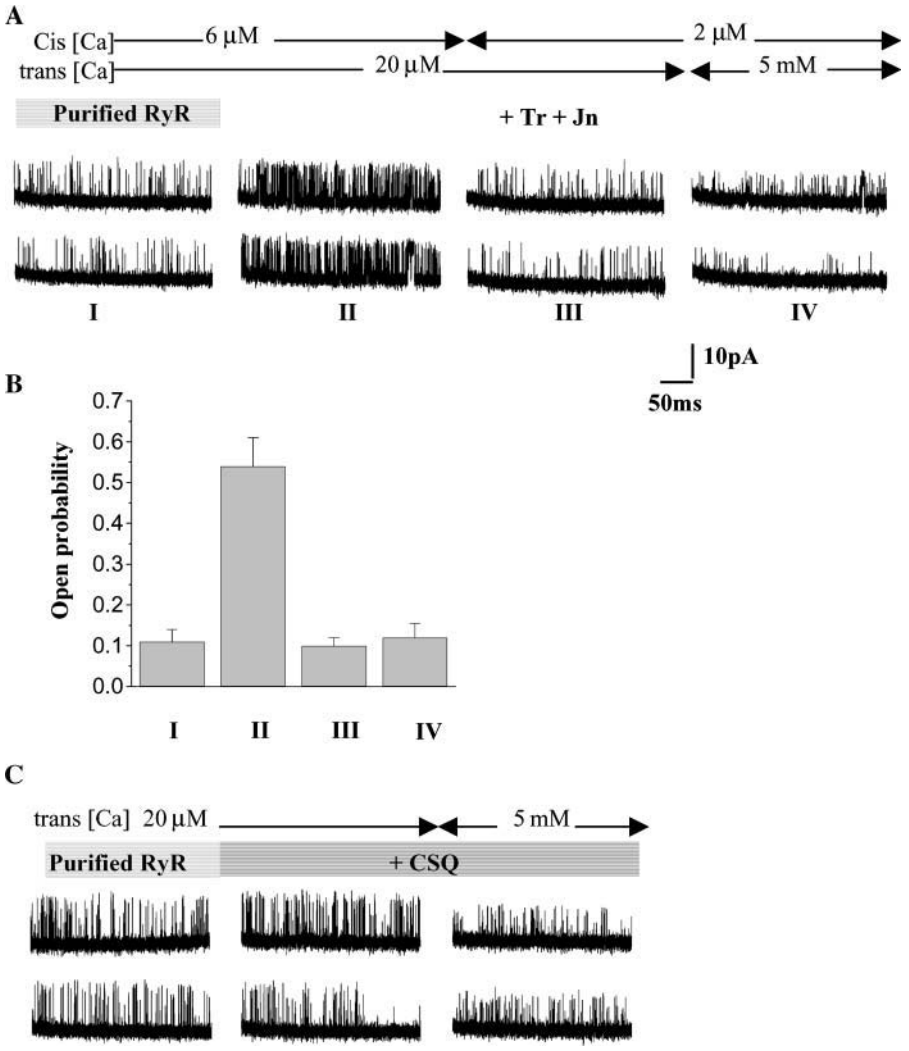

FIGURE 2 Effects of junctin and triadin 1 on activity of purified RyRs and their ability to respond to luminal Ca. (*A*) Representative traces of current recordings from a single purified RyR before (*I*) and after (*II*) addition of triadin 1 (Tr) and junctin (Jn) (5 μg/ml of each) to the *trans* chamber and after a subsequent increase in *trans* [Ca] from 20 μM to 5 mM (*IV*). Current recordings were obtained at +40 mV holding potential in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4. The *cis* [Ca] was 6 μM in control and after initial addition of triadin 1 and junctin; then *cis* [Ca] was decreased to 2 μM (*III*) to avoid excessive channel activity induced by these proteins. (*B*) Bar graph of pooled values of P_o for the same conditions. Data presented as mean ± SE ($n = 6$). (*C*) Representative traces illustrating the lack of effects of CSQ (5 μg/ml) added to the *trans* chamber on RyR channel activity ($n = 4$). Current recordings were obtained at +40 mV holding potential in symmetrical 350 mM $CsCh_3SO_3$, pH 7.4. Channel openings are upward.

effect on channel activity (not shown). Additional experiments in which CSQ alone (5 μg/ml) was added to the *trans* side yielded no significant change in P_o, nor did it recover luminal Ca sensitivity (Fig. 2 *C*). We interpret these results to suggest that neither triadin 1, junctin, nor CSQ alone mediate RyR responsiveness to luminal Ca, although triadin 1 and junctin have a dramatic, positive regulatory effect on RyR activity.

The second set of experiments involved addition of the three luminal proteins from the *trans* side of the bilayer (5 μg/ml each of triadin 1, junctin, and CSQ). *Cis* [Ca] was 6 μM and *trans* [Ca] was 20 μM for the control and the initial addition of triadin 1, junctin, and CSQ (Fig. 3 *A*, *I* and *II*). Then, *cis* [Ca] was reduced to 2 μM (Fig. 3 *A III*). Next, luminal [Ca] was increased to 5 mM (Fig. 3 *A IV*). Initial addition of all three proteins (i.e., triadin 1, junctin, and CSQ) produced no significant changes in P_o (Fig. 3 *B*). However, strikingly, the purified channel regained its ability to respond to elevated luminal Ca, with a significant increase in channel P_o from ~0.05 to ~0.22 (Fig. 3 *B*). These results suggested that a combination of CSQ and triadin 1 and/or junctin is required to endow the RyR with an ability to respond to changes in luminal Ca.

To assess the individual roles of each of the luminal auxiliary proteins in mediating RyR responsiveness to luminal Ca, we used a two-step protocol in which the initial addition of triadin 1 and/or junctin to the purified RyR was followed by subsequent addition of CSQ (5 μg/ml of each). *Cis* [Ca] remained 6 μM throughout this experiment and *trans* [Ca] was 20 μM for the control and initial addition of triadin 1 and/or junctin, as well as for the subsequent addition of CSQ (Fig. 4 *A*, *I–III*). Finally, luminal [Ca] was increased to 5 mM (Fig. 4 *A IV*). Initial addition of triadin 1 and junctin again caused a significant increase in P_o from 0.12 to 0.55, whereas the subsequent addition of CSQ gave a significant decrease in channel activity (P_o ~0.15). The increase of *trans* [Ca] to 5 mM once again resulted in a significant increase in channel P_o to ~0.45 (Fig. 4 *B*), indicating that the channels were responsive to luminal Ca. In two additional experiments triadin 1 and junctin were added separately and both found to have effects similar to those observed with the combination of these proteins. Thus triadin 1 and junctin might play interchangeable roles in mediating RyR responsiveness to luminal Ca, consistent with the similarities between the molecular structures of these proteins (Kobayashi and Jones, 1999; Jones et al., 1995; Kobayashi et al., 2000). Further studies are now on the way to provide a detailed characterization of the effects of triadin 1 and junctin on RyR. The restoration of RyR luminal Ca sensitivity is summarized in Fig. 5, which depicts the percentage of change compared to the control P_o for each combination of proteins used in these experiments.

An interesting aspect of our results is that CSQ inhibited the activity of RyR complexed with triadin and junctin, an effect that appeared to be reversed by increased luminal Ca (Fig. 4). To test if low luminal Ca is a necessary requirement for the inhibitory action of CSQ on RyR channel associated with triadin 1 and junctin, we carried out experiments in which CSQ was added after *trans* [Ca] had been raised to 5 mM. As shown in Fig. 6 *A*, under these conditions CSQ failed to produce any significant effects on channel activity. This result is consistent with the notion that luminal Ca acts by relieving the inhibitory influence of CSQ, possibly by dissociation of CSQ from the RyR channel.

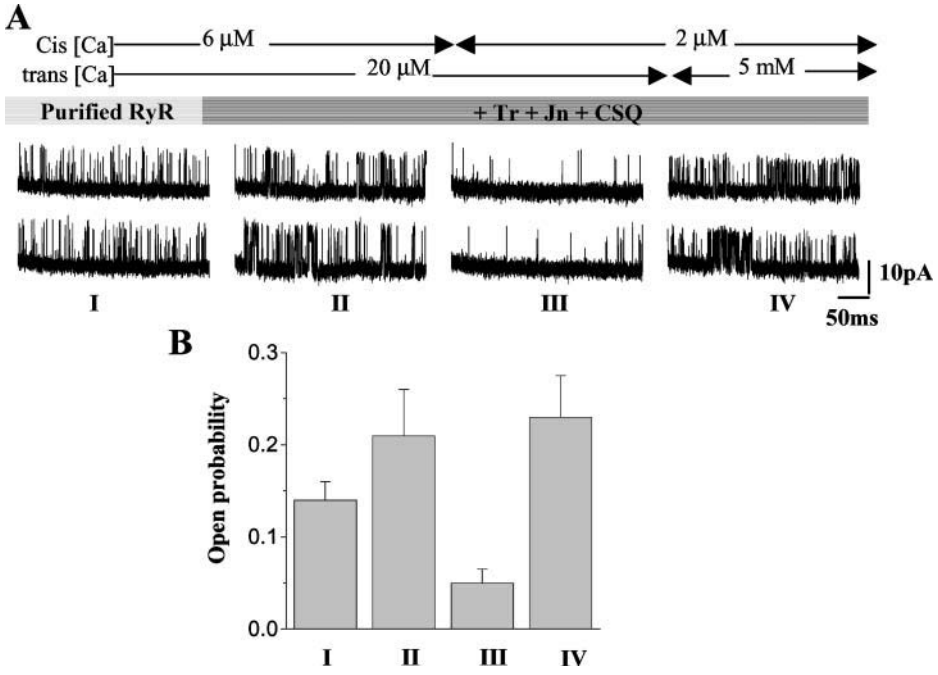

FIGURE 3 Effects of combined addition of junctin, triadin 1, and calsequestrin on activity of purified RyRs and their ability to respond to luminal Ca. (*A*) Representative traces of current recordings from a single purified RyR before (*I*) and after (*II*) simultaneous addition of triadin 1, junction, and calsequestrin (5 μg/ml of each) to the *trans* chamber and after subsequent increase in *trans* [Ca] from 20 μM to 5 mM (*IV*). The *cis* [Ca] was 6 μM in control and after initial addition of triadin 1 and junctin; then *cis* [Ca] was decreased to 2 μM (*III*). Channel openings are upward. (*B*) Bar graph of pooled values of P_o for the same conditions. Data presented as mean ± SE ($n = 7$).

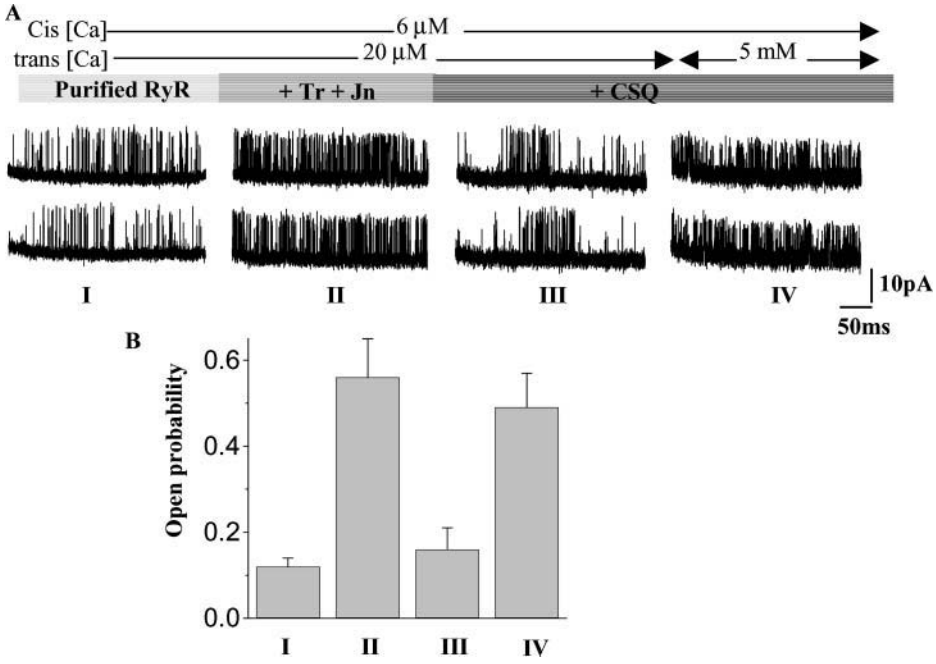

FIGURE 4 Effects of sequential addition of junctin and triadin 1 and calsequestrin on activity of purified RyRs and their ability to respond to luminal Ca. (*A*) Representative traces of current recordings from a single purified RyR before (*I*) and after (*II*) addition of triadin 1 and junctin (5 μg/ml of each) to the *trans* chamber, after subsequent addition of calsequestrin (*III*), and after an increase of *trans* [Ca] from 20 μM to 5 mM (*IV*). Current recordings were obtained at +40 mV holding potential in symmetrical 350 mM $CsCH_3SO_3$, pH 7.4. The *cis* [Ca] was 6 μM throughout the experiment. Channel openings are upward. (*B*) Bar graph of pooled values of P_o for the same conditions. Data presented as mean ± SE ($n = 3$).

High [Ca] and increased ionic strength have been reported to dissociate CSQ from RyR (Zhang et al., 1997; Beard et al., 2002). To assess the impact of elevated Ca on RyR/triadin/junctin-CSQ interactions under the conditions of our experiments, we tested the reversibility of the effects of luminal Ca on purified RyRs reassociated with triadin 1, junctin, and CSQ and on native RyR channels in SR vesicles.

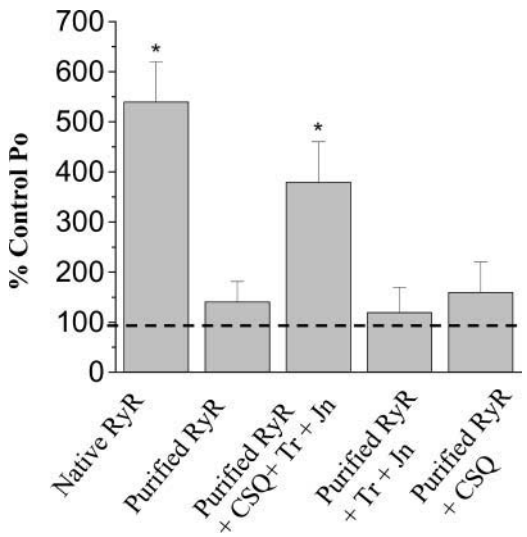

FIGURE 5 The role of calsequestrin, junctin, and triadin 1 in conferring the luminal Ca sensitivity of RyR. Relative changes in P_o upon increasing *trans* [Ca] from 20 μM to 5 mM for native RyR and for purified RyRs in the presence of different combinations of luminal auxiliary proteins triadin 1 (Tr), junctin (Jn), and calsequestrin (CSQ) in the *trans* chamber. Data presented as mean ± SE ($n = 3 - 14$) from experiments documented in Figs. 1–4. * $P < 0.05$.

Luminal Ca was reduced by chelating the excess Ca with EGTA. Only in two out of six native RyR channels that responded to increases in luminal Ca were the effects of luminal Ca found to be reversible; in the four other channels reverting to low *trans* [Ca] did not restore the initial low activity (Fig. 6 *B*). These results suggest that 5 mM luminal Ca results in the loss of CSQ from most but not all of the native RyR channels in the bilayer. Interestingly, in channels that maintained their high activity on returning to low luminal Ca, addition of CSQ (5 μg/ml) to the *trans* chamber reduced P_o to original low levels (Fig. 5 *B*). In purified RyRs reassociated with triadin 1, junctin, and CSQ, the effects of increased luminal Ca were reversible in all experiments performed ($n = 3$) (Fig. 6 *C*). These results could be ascribed to CSQ molecules present in the *trans* compartment reassociating with the RyR/triadin/junctin complex.

Taken together, the results of our experiments point toward CSQ as a luminal Ca sensor that negatively affects RyR channel activity at low luminal [Ca] when RyR is complexed with triadin 1 and/or junctin. This inhibitory effect is overcome at increased luminal [Ca] as a result of Ca binding to, and dissociation of CSQ from, the RyR.

DISCUSSION

Modulation of RyRs by Ca inside the SR has emerged as an important control mechanism for CICR and Ca cycling in the heart (see Györke et al., 2002, for review). However, the molecular basis of RyR regulation by luminal Ca remains to be determined. In the present study, we have investigated the molecular identity of the cardiac luminal Ca sensor by reconstituting purified RyR together with the luminally

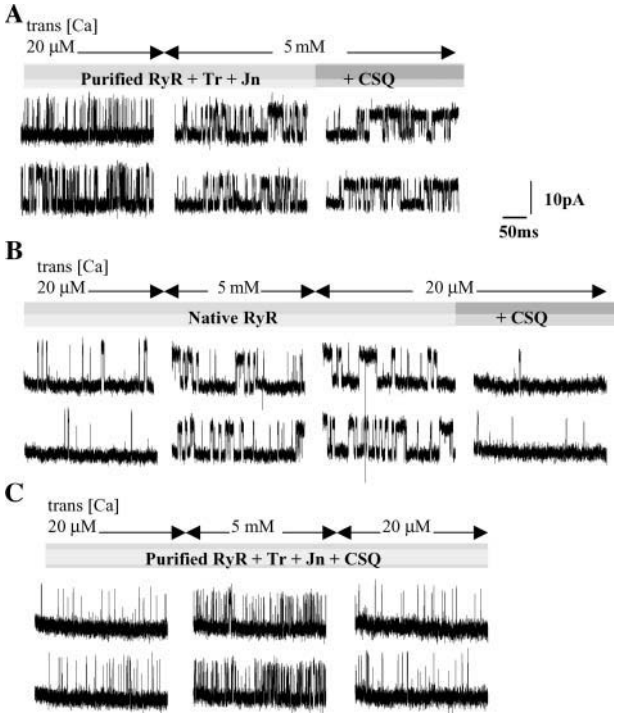

FIGURE 6 Modulation by calsequestrin of native RyR channels and purified RyRs reassociated with triadin 1 and junctin. (*A*) Representative single-channel traces illustrating the absence of effects on P_o of CSQ in purified RyRs reassociated with triadin 1 and junctin under conditions of elevated luminal Ca (from 20 μM to 5 mM). The P_o values were 0.43 $\pm$ 0.08, 0.38 $\pm$ 0.06, and 0.46 $\pm$ 0.07 ($n = 3$) for low *trans* [Ca] (20 μM), high *trans* [Ca] (5 mM), and high *trans* Ca + CSQ (5 μg/ml), respectively. (*B*) Representative single-channel traces illustrating the irreversibility of the effects of 5 mM luminal Ca and restoration of initial low activity by CSQ added to the *trans* chamber in native RyRs. The P_o values were 0.06 $\pm$ 0.02, 0.36 $\pm$ 0.07, 0.31 $\pm$ 0.06, and 0.03 $\pm$ 0.01 ($n = 4$) for low *trans* [Ca] (20 μM), high *trans* [Ca] (5 mM), reverting to low *trans* [Ca] (20 μM), and 5 μg/ml *trans* CSQ, respectively. (*C*) Representative single-channel traces illustrating the reversibility of the effects of 5 mM luminal Ca on purified RyRs reassociated with triadin 1, junction, and calsequestrin. The P_o values were 0.09 $\pm$ 0.03, 0.34 $\pm$ 0.07, and 0.05 $\pm$ 0.02 ($n = 3$) for low *trans* [Ca] (20 μM), high *trans* [Ca] (5 mM), and reverting to low *trans* [Ca] (20 μM), respectively. In all experiments the *cis* [Ca] was 6 μM. Low luminal Ca was restored by adding 5 mM EGTA to the *trans* chamber. Triadin 1, junctin, and calsequestrin were added to the *trans* chamber at 5 μg/ml each. All current recordings were obtained at +40mV holding potential in symmetrical 350 mM $CsCH_3S0_3$, pH 7.4.

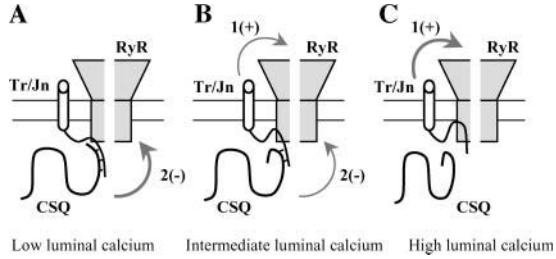

FIGURE 7 The hypothesized functional interactions between calsequestrin, junctin, triadin 1, and RyR in the cardiac SR membrane. Based on our results, we conclude that CSQ serves as luminal Ca sensor for RyR. Triadin 1 (Tr) and junctin (Jn) are required to physically link RyR and CSQ. At the same time they stabilize RyR in an increased activity mode (1(+)). CSQ acts by removing the potentiatory influence of triadin 1 and junctin on the RyR channel through Ca-dependent interaction with triadin 1 and junctin (2(−)). At low luminal Ca concentrations, CSQ inhibits the RyR channel complex via strong interactions with triadin 1 and junctin (*A*). On elevating luminal Ca this inhibition is gradually relieved as Ca binding sites on CSQ become increasingly occupied with Ca and CSQ-triadin/junctin interactions weaken, allowing stimulation of channel activity (*B*); the channel becomes maximally active when CSQ dissociates from the RyR complex at >5 mM Ca (*C*).

located auxiliary proteins triadin 1, junctin, and CSQ. Our results indicate that the luminal Ca sensitivity of RyR is conferred by a complex of CSQ, triadin 1 and/or junctin. The hypothesized functional interactions between these proteins in the SR membrane are illustrated in Fig. 7. Based on our results, we conclude that CSQ serves as the actual luminal Ca sensor for RyR. Triadin 1 and/or junctin are required to physically link RyR and CSQ. At the same time, they stabilize the RyR in an increased activity mode and by this fashion, prime the RyR channel for Ca-dependent regulation by CSQ. When CSQ binds to triadin 1 and/or junctin, it inhibits the activity of the RyR channel complex at low luminal Ca concentrations. On raising luminal Ca, this inhibition is gradually relieved as the Ca binding sites on CSQ become increasingly occupied with Ca, leading to weakened interactions between CSQ and triadin 1 (junctin) and increased channel open probability; the channel becomes maximally active when CSQ dissociates completely from the RyR complex at >5 mM Ca. Which of these two possible forms of control by CSQ (i.e., changes in tightness of binding interactions or dynamic dissociation/reassociation from/to the RyR complex) operates inside cardiac myocytes remains to be determined.

Our proposed scheme for functional relationships between RyR, CSQ, triadin 1, and junctin is supported by current views of binding interactions between these proteins within the RyR complex (Zhang et al., 1997; Shin et al., 2000; Kobayashi et al., 2000). Biochemical studies have shown that triadin 1 and junctin directly bind to RyR and CSQ, thus anchoring CSQ to the junctional membrane in proximity to the RyR channel (Zhang et al., 1997). It has been reported that the interaction of triadin 1 and junctin with CSQ occurs at highly charged homologous luminal domains within the structure of these proteins (Shin et al., 2000; Kobayashi et al., 2000). These domains are characterized by the presence of alternating positively and negatively charged amino acids referred to as the KEKE motif (Zhang et al., 1997; Jones et al., 1995; Kobayashi et al., 2000). The same luminal domains appear to be involved in interactions of junctin and triadin 1 with RyR (Zhang et al., 1997). The triadin-binding domain of skeletal muscle CSQ was localized to an Asp-rich region at the carboxyl-terminus of CSQ, a region which is also implicated in Ca binding (Shin et al., 2000). Binding of

Ca to cardiac (Scott et al., 1988) and skeletal (Shin et al., 2000) CSQ is expected to weaken the electrostatic interactions between CSQ and triadin (junctin) by screening the negatively charged interaction sites, thereby accounting for the relief in inhibition of the Ca release channel activity by CSQ at increased luminal Ca. At still higher concentrations, Ca results in dissociation of CSQ from triadin 1 and junctin (Zhang et al., 1997; Shin et al., 2000). On the other hand, junctin and triadin 1 remain tightly bound to the RyR, independent of intraluminal Ca concentration (Zhang et al., 1997).

Previous lipid bilayer studies on native and purified RyRs have demonstrated that luminal Ca can affect RyR by at least two different mechanisms. In native cardiac RyRs, it has been found that luminal Ca acts at Ca sensing sites localized at the luminal side of the RyR complex (Györke and Györke, 1998; Ching et al., 2000). Experiments on purified cardiac and skeletal RyRs have convincingly shown that in some cases, luminal Ca, after passing the pore, can induce RyR openings by acting at the cytosolic activation sites instead of influencing channel activity at luminal Ca sensing sites (Xu and Meissner, 1998). Apparently, purification can result in loss of some regulatory proteins that are involved in luminal Ca sensing. At the same time, purification may lead to alterations of the cytosolic Ca regulatory sites, making the sites more accessible for activation for Ca passing through the channel. In this study, the observed failure of purified RyRs to respond to changes in [Ca] at the luminal side of the protein is consistent with the notion that purification causes disruption of the luminal Ca regulatory sites. As to why the channels did not exhibit changes in activity that could be attributed to the ''feed-through'' activation from the *cis* side reported in other purified RyR studies is not clear. One possibility is that the changes to RyR activation sites depend on the degree of purification of RyR. It is conceivable that harsher dissociation procedures make the channel more prone to feed-through Ca activation since native RyR channels in SR vesicles lack this mechanism (Lukyanenko et al., 1996; Györke and Györke, 1998; Ching et al., 2000). The difference could also be due to the fact that all our measurements were performed in the presence of millimolar [Mg] that may antagonize the effects of Ca passing through the pore at the activation sites.

Several previous studies have addressed the effects of CSQ on RyR activity using RyRs isolated from skeletal muscle. Although in some of these studies adding skeletal muscle CSQ to the luminal side of the channel was found to activate RyR (Kawasaki and Kasai, 1994; Ohkura et al., 1998; Szegedi et al., 1999), in other studies CSQ reduced RyR activity (Beard et al., 2002). In the latter report, skeletal muscle CSQ had no significant effects on the purified skeletal RyR, but reduced the activity of the native RyR in SR membranes depleted of CSQ. Similarly, in a preliminary study, cardiac CSQ was reported to inhibit the activity of cardiac RyRs stripped of their CSQ by treatment with 10 mM luminal Ca (Wang et al., 2001). Our findings are in line with these results. Although the reasons behind the discrepancies between different studies are not known, they could involve differences in experimental conditions. For example, our measurements with purified cardiac RyRs, as well as those by Beard et al. (2002) with CSQ-extracted skeletal muscle membranes, were performed in the presence of physiological concentrations of ATP, whereas the other studies were carried out with cytosolic Ca as the sole RyR activating ligand. The effects of luminal Ca seem to require the presence of allosteric modulators of RyR, such as ATP, caffeine, or sulmazole (Györke and Györke, 1998; Ching et al., 2000). Thus, the conditions of some studies might not have been optimal for defining the role of CSQ as a luminal Ca sensor. Variation in the degree of purification of RyR could also contribute to the differences in results. Since inhibition of RyR by CSQ occurs only in the presence of triadin and/or junctin (Beard et al., 2002; this study), it is possible that the RyR preparations from some of the studies lacked these proteins. In a previous work, addition of triadin to the cytosolic side of the skeletal RyR inhibited channel activity (Ohkura et al., 1998). Although in our experiments cytosolic triadin did not affect the cardiac RyR channel open probability, potentiation of the channel specifically on addition of luminal triadin suggests that this effect was due to interaction of the protein with the putative intraluminal triadin-binding domain of the RyR (Zhang et al., 1997).

In conclusion, our results determine, for the first time, the identities and modes of interactions of molecules involved in luminal Ca regulation of single cardiac RyR channels. The levels of luminal Ca are sensed by CSQ; then, this information is transmitted to RyR via the intermediate proteins, triadin 1 and/or junctin (Fig. 7). The regulation consists of a luminal Ca-dependent relief of inhibition that Ca-free CSQ exerts on the activity of the RyR channel complexed with triadin 1 and junctin. This dynamic, Ca-dependent modulation of RyR by CSQ has important ramifications for SR Ca handling in cardiac myocytes. Before the onset of Ca release when the $[Ca]_{SR}$ is high, a large fraction of RyRs would be in a CSQ-uninhibited mode. During Ca release when $[Ca]_{SR}$ declines, the increased level of Ca-free CSQ would result in inhibition of the RyR channels, thereby providing a molecular mechanism for the luminal Ca-dependent termination of Ca release in cardiac muscle (Terentyev et al., 2002, 2003). At the same time, the excessively large fraction of CSQ-uninhibited RyR channels at elevated basal $[Ca]_{SR}$ could contribute to the increased diastolic SR Ca leak (Lukyanenko et al., 2001; Shannon et al., 2002) and increased frequency of spontaneous Ca sparks and Ca waves (Cheng et al., 1996; Lukyanenko et al., 1996, 2001) under the conditions of Ca overload. Future studies will have to define the specific structural domains involved in the described intermolecular interactions and to verify the roles of these interactions in controlling SR Ca release inside living cells.

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