Evidence for Ca\(^{2+}\) Activation and Inactivation Sites on the Luminal Side of the Cardiac Ryanodine Receptor Complex

Li Lien Ching, Alan J. Williams, Rebecca Sitsapesan

Abstract—We have used tryptic digestion to determine whether Ca\(^{2+}\) can regulate cardiac ryanodine receptor (RyR) channel gating from within the lumen of the sarcoplasmic reticulum (SR) or whether Ca\(^{2+}\) must first flow through the channel and act via cytosolically located binding sites. Cardiac RyRs were incorporated into bilayers, and trypsin was applied to the luminal side of the bilayer. We found that before exposure to luminal trypsin, the open probability of RyR was increased by raising the luminal [Ca\(^{2+}\)] from 10 \(\mu\)mol/L to 1 mmol/L, whereas after luminal trypsin exposure, increasing the luminal [Ca\(^{2+}\)] reduced the open probability. The modification in the response of RyRs to luminal Ca\(^{2+}\) was not observed with heat-inactivated trypsin, indicating that digestion of luminal sites on the RyR channel complex was responsible. Our results provide strong evidence for the presence of luminally located Ca\(^{2+}\) activation and inhibition sites and indicate that trypsin digestion leads to selective damage to luminal Ca\(^{2+}\) activation sites without affecting luminal Ca\(^{2+}\) inactivation sites. We suggest that changes in luminal [Ca\(^{2+}\)] will be able to regulate RyR channel gating from within the SR lumen, therefore providing a second Ca\(^{2+}\)-regulatory effect on RyR channel gating in addition to that of cytosolic Ca\(^{2+}\). This luminal Ca\(^{2+}\)-regulatory mechanism is likely to be an important contributing factor in the potentiation of SR Ca\(^{2+}\) release that is observed in cardiac cells in response to increases in intra-SR [Ca\(^{2+}\)]. (Circ Res. 2000;87:201-206.)

Key Words: ryanodine receptors ■ Ca\(^{2+}\) release ■ cardiac excitation-contraction coupling ■ sarcoplasmic reticulum

The involvement of the intra–sarcoplasmic reticulum (SR) [Ca\(^{2+}\)] as a regulator of excitation-contraction (EC) coupling in cardiac cells is currently a topic of considerable interest. Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels acts as a trigger for SR Ca\(^{2+}\) release and allows contraction of cardiac cells to occur in a graded manner.\(^{1,2}\) Increasing evidence now indicates that the loading of the SR directly alters the “gain” of EC coupling: a higher SR Ca\(^{2+}\) content leads to a greater SR Ca\(^{2+}\) release for a given Ca\(^{2+}\) trigger.\(^{3-5}\) Intuitively, this is expected, inasmuch as a larger SR/cytosolic Ca\(^{2+}\) gradient would lead to a greater Ca\(^{2+}\) current per cytosolic Ca\(^{2+}\) gradient.\(^{3-5}\) Shannon et al\(^{6}\) have found that the relationship between SR Ca\(^{2+}\) content and both the gain of EC coupling and fractional SR Ca\(^{2+}\) release becomes extremely steep at higher SR [Ca\(^{2+}\)]. These results indicate that the gating of RyR channels is altered by changes in SR Ca\(^{2+}\) content.

By reconstituting single RyR into bilayers, several investigators have already demonstrated that increasing the luminal [Ca\(^{2+}\)] leads to increases in open probability \((P_\text{o})\).\(^{3,7-10}\) The mechanism for this effect, however, is a matter of considerable debate at present because investigators are divided into 2 camps. It was originally suggested that changes in luminal [Ca\(^{2+}\)] cause changes in RyR gating by interacting with luminal binding sites on the channel.\(^7\) Subsequent work provided further evidence for this hypothesis.\(^3,8,11\) Other investigators, however, have suggested that luminal Ca\(^{2+}\) modulates P\(_t\), because it flows through the open channel and acts via cytosolic Ca\(^{2+}\) binding sites and does not interact with luminal sites.\(^9,10\) Unequivocal proof of the site of action of luminal Ca\(^{2+}\) has been confounded by the fact that RyR channels can be activated and inactivated by cytosolic Ca\(^{2+}\), making it very difficult to completely exclude the possibility that luminal Ca\(^{2+}\) has some access to cytosolic Ca\(^{2+}\) binding sites even at high positive trans-membrane potentials. Therefore, in the present study, we used a different approach to examine this question, namely, tryptic digestion of sites exposed on the luminal side of the bilayer. Elegant experiments in the squid giant axon set a precedent for using proteolysis as a means of separating activation and inactivation gating processes in ion channels.\(^12\) Our experiments provide compelling evidence to suggest that there are luminal Ca\(^{2+}\) binding sites located either on the cardiac RyR channels or on a closely associated protein that regulates RyR gating. Moreover, our results indicate for the first time the existence of luminal inhibition sites.
Materials and Methods

Single-Channel Experiments

SR membrane vesicles were prepared from sheep hearts and fused with planar phosphatidylethanolamine lipid bilayers as previously described. Vesicles were incorporated in a fixed orientation such that the cis chamber corresponded to the cytosolic side of the channel and the trans chamber corresponded to the SR lumen. The trans chamber was held at ground, and the cis chamber was held at potentials relative to ground. After vesicle fusion, the cis chamber was perfused to give a symmetrical solution containing 250 mmol/L CsOH and 140 mmol/L PIPES, pH 7.2. The pH and free 

Materials

All chemicals were AnalaR or the best available grade from Sigma Chemical Co or BDH.

Results

Effects of Luminal Ca\(^{2+}\) on Channels Activated by Cytosolic Ca\(^{2+}\) Plus ATP

We find that the gating of RyR channels activated solely by cytosolic Ca\(^{2+}\) is not affected by changes in luminal [Ca\(^{2+}\)]. Therefore, we have examined the effects of luminal Ca\(^{2+}\) on native sheep cardiac RyR channels activated by cytosolic Ca\(^{2+}\) plus ATP. The effects of luminal Ca\(^{2+}\) under these conditions can be observed in Figure 1. In the presence of symmetrical 10 μmol/L Ca\(^{2+}\) and in the absence of other channel activators, P\(_o\) was ≈0. The addition of 1 mmol/L ATP to the cytosolic channel side increased P\(_o\) to 0.157±0.100 at +40 mV and 0.110±0.03 (mean±SEM, n=8) at −40 mV. When channels were thus activated synergistically by cytosolic Ca\(^{2+}\) and ATP, increasing the luminal [Ca\(^{2+}\)] was then able to cause further increases in P\(_o\) at both +40 and −40 mV. After the addition of 1 mmol/L luminal Ca\(^{2+}\), P\(_o\) was 0.369±0.132 at +40 mV and 0.254±0.100 at −40 mV (mean±SEM, n=8). Figure 1 illustrates the typical response of the channels to increases in luminal [Ca\(^{2+}\)] from 10 μmol/L to 1 mmol/L. The results demonstrate that the effects of luminal Ca\(^{2+}\) on sheep cardiac RyR channels activated by the combination of micromolar cytosolic Ca\(^{2+}\) and ATP are similar to the effects observed in sheep skeletal and canine cardiac channels. It is important to note that under these experimental conditions, luminal Ca\(^{2+}\) was no less effective at activating channels at +40 mV than at −40 mV. These results correspond with our previous observations with skeletal RyRs and indicate that luminal Ca\(^{2+}\) does not have to flow through the channel and bind to cytosolic Ca\(^{2+}\) binding sites to regulate P\(_o\).
Effects of luminal Ca\(^{2+}\) and Ryanodine Receptors

Figure 3. Effects of lowering the luminal [Ca\(^{2+}\)] after trypsin incubation at +40 mV (left) and −40 mV (right) in a typical experiment in which 3 RyR channels have been incorporated into the bilayer. Arrows indicate zero current level. After trypsin incubation, increasing luminal [Ca\(^{2+}\)] from 10 μmol/L to 1 mmol/L reduces \(P_o\), and the top traces illustrate the low \(P_o\) at both +40 and −40 mV under these conditions. The inhibitory effect of luminal Ca\(^{2+}\) was removed by lowering luminal [Ca\(^{2+}\)] to ∼90 nmol/L with 10 mmol/L EGTA (bottom traces).

Figure 4. Time-dependent effects of trypsin incubation on the \(P_o\) of a typical cardiac RyR channel. The histogram shows average \(P_o\) calculated in 30-second segments at a holding potential of −40 mV. The channels were activated by 10 μmol/L cytosolic Ca\(^{2+}\) and 1 mmol/L ATP in the presence of 1 mmol/L luminal Ca\(^{2+}\). Arrow indicates the time of luminal trypsin addition. The initial apparent increase in \(P_o\) was followed by a decline in \(P_o\) over the 6-minute incubation period.

Effects of Luminal Trypsin on Conductance

Figure 5 illustrates the changes in RyR current amplitude at +40 and −40 mV that occur with changes in luminal [Ca\(^{2+}\)] and luminal trypsin addition. Addition of 1 mmol/L luminal Ca\(^{2+}\) caused a reduction in current amplitude at both +40 mV and −40 mV (Figure 5b), as previously observed\(^7,8\) and expected from a knowledge of the conductance and relative permeabilities of divalent and monovalent cations in the cardiac RyR.\(^9,10\) This was followed by a further reduction in current amplitude by trypsin (Figure 5c). For example, at the addition of trypsin (400 μg/mL) to the luminal chamber. After 3 and 6 minutes of incubation, \(P_o\) declined to 0.108±0.059 and 0.031±0.019 (mean±SEM, n=4), respectively. A similar decrease in \(P_o\) with time was observed at +40 mV.

ATP and 10 μmol/L cytosolic Ca\(^{2+}\) (top trace) and subsequently activated further by increasing luminal [Ca\(^{2+}\)] from 10 μmol/L to 1 mmol/L (bottom trace). Trypsin (400 μg/mL) was added to the luminal side of the bilayer, in the continued presence of 1 mmol/L luminal Ca\(^{2+}\). After 6 minutes of incubation, the reaction was stopped by the removal of trypsin and 1 mmol/L luminal Ca\(^{2+}\) by perfusion with solution containing 250 mmol/L Cs\(^+\) and 10 μmol/L Ca\(^{2+}\). The gating of the channels after perfusion is shown in the right panel (top trace) of Figure 2. The right panel (lower trace) also demonstrates that readmission of 1 mmol/L luminal Ca\(^{2+}\) now results in a marked lowering of \(P_o\). For example, at −40 mV, \(P_o\) was 0.092±0.102 (mean±SEM, n=4) after perfusion and was reduced to 0.016±0.011 (mean±SEM, n=4) after readmission of 1 mmol/L luminal Ca\(^{2+}\). The reduction in \(P_o\) caused by 1 mmol/L luminal Ca\(^{2+}\) in trypsin-treated channels was observed at both +40 mV and −40 mV. Reversal of the Ca\(^{2+}\)-induced inhibition was also observed at both holding potentials after lowering the free luminal [Ca\(^{2+}\)] to 90 nmol/L and is demonstrated in a representative experiment in Figure 3. After incubation with trypsin, \(P_o\) was 0.053±0.038 and 0.016±0.011 (mean±SEM, n=4) at +40 mV and −40 mV, respectively, in the presence of 1 mmol/L luminal Ca\(^{2+}\) and was 0.110±0.096 and 0.103±0.104 (mean±SEM, n=4) at +40 mV and −40 mV, respectively, after lowering the luminal free [Ca\(^{2+}\)] to 90 nmol/L.

The addition of trypsin to the luminal side of the bilayer caused a time-dependent effect on channel gating at both +40 mV and −40 mV and is shown in Figure 4. \(P_o\) appeared to increase immediately after luminal trypsin addition but was followed within 1 minute by a gradual decline in \(P_o\). For example, at −40 mV, average \(P_o\) was 0.303±0.216 (mean±SEM, n=4) in the first 30 seconds immediately after
-40 mV, current amplitude was reduced from 18.68±0.37 to 17.61±0.51 pA (mean±SEM, n=4) after increasing luminal [Ca\(^2+\)] from 10 μmol/L to 1 mmol/L and was further reduced to 14.01±0.67 pA at -40 mV (mean±SEM, n=4) after trypsin incubation. Removal of trypsin and returning the free luminal [Ca\(^2+\)] to 10 μmol/L by perfusion of the luminal chamber restored current amplitude to control levels (Figure 5d). Readdition of 1 mmol/L luminal Ca\(^2+\) again lowered current amplitude to the same level that was observed before trypsin incubation (results not shown). Thus, although the effects of trypsin incubation on RyR gating were irreversible, the effects on single-channel conductance were reversible, indicating that the conductance-induced changes were not caused by tryptic digestion of the channel.

Effects of Heat-Inactivated Trypsin on RyR Function

To examine whether the change in RyR response to luminal Ca\(^2+\) observed after treatment with trypsin was the result of its proteolytic activity, control experiments were performed with the use of heat-inactivated trypsin. Trypsin was boiled for 10 minutes, and the loss of enzymatic activity was qualitatively tested with SDS-PAGE by comparing its ability to cleave BSA with that of the active enzyme. We found that heat-inactivated trypsin produced a similar reversible reduction in Cs\(^+\) current amplitude at both +40 and -40 mV, as was observed with active trypsin. For example, at -40 mV, current amplitude was 18.87±0.61 pA before and 15.58±0.70 pA (mean±SD, n=3) after luminal addition of 1 mmol/L Ca\(^2+\) and 400 μg/mL inactivated trypsin. The effects of heat-inactivated trypsin on luminal Ca\(^2+\) modulation of RyR gating is shown in a representative experiment in Figure 6. There was no significant difference in the effects of heat-inactivated trypsin at +40 or -40 mV, but for clarity, only the effects at -40 mV are shown. The figure demonstrates that the channels respond normally to changes in luminal [Ca\(^2+\)] before incubation with heat-inactivated trypsin; increasing the luminal [Ca\(^2+\)] from 10 μmol/L to 1 mmol/L produced an increase in P_o (top and middle traces). P_o was 0.113±0.019 at +40 mV and 0.069±0.063 at -40 mV (mean±SD, n=3) in the presence of 10 μmol/L of luminal Ca\(^2+\). After increasing luminal Ca\(^2+\) to 1 mmol/L, the P_o decreased by 0.448±0.217 at +40 mV and 0.261±0.150 at -40 mV (mean±SD, n=3). Subsequent incubation of the channels with 400 μg/mL heat-inactivated trypsin for 6 minutes did not cause any time-dependent reduction in P_o, as was observed with active trypsin (see Figure 4). For example, the P_o at +40 mV after perfusing away the heat-inactivated trypsin from the luminal channel side and reestablishing the luminal [Ca\(^2+\)] at 1 mmol/L was 0.447±0.030 (mean±SD, n=3). The results with the heat-inactivated trypsin indicate that the decrease in P_o caused by incubation with active trypsin is the result of tryptic digestion of luminal sites.

Discussion

Cardiac RyRs localized in junctional SR membranes are thought to complex with a number of other proteins, including junctin, triadin, and calsequestrin.17 Some of these proteins have a luminal location or have luminally exposed domains and may still be associated with RyRs after reconstruction into bilayers. This may occur with native RyRs (as in the present study) and even with “purified” RyRs, inasmuch as it has been shown that certain proteins are still bound to RyRs even after purification with CHAPS solubilization.17 Therefore, it is possible that the effects of luminal Ca\(^2+\) observed in our experiments do not result from the direct effect of luminal Ca\(^2+\) on the channel itself but are mediated by an associated protein to which Ca\(^2+\) binds.

We find that the P_o of sheep cardiac RyR channels activated by 1 mmol/L ATP in the presence of 10 μmol/L cytosolic and luminal Ca\(^2+\) (top trace). Luminal Ca\(^2+\) (1 mmol/L) further increased P_o (middle trace). After 6-minute luminal incubation with heat-inactivated trypsin (400 μg/mL) in the presence of 1 mmol/L luminal Ca\(^2+\), heat-inactivated trypsin was removed by perfusion. Readdition of 1 mmol/L luminal Ca\(^2+\) did not cause the channel to become inhibited as was observed with active trypsin. P_o was similar to that observed before incubation (bottom trace).
previously described,13 provide no further clues as to the precise location of the luminal Ca\(^{2+}\) binding sites on the RyR channel complex (see online supplementary information, available at http://www.circresaha.org).

As shown previously for skeletal\(^1\) and cardiac\(^2\) RyRs activated synergistically by cytosolic ATP and Ca\(^{2+}\), we find that the stimulatory effects of luminal Ca\(^{2+}\) are not significantly different at +40 mV or at −40 mV. The different holding potentials produce a marked difference in the amount of Ca\(^{2+}\) flux through the channel\(^9,18\); therefore, as we suggested in our earlier study,\(^8\) our control results with luminal Ca\(^{2+}\) argue against the possibility of luminal Ca\(^{2+}\) acting by binding to cytosolic sites.

Trypsin incubation altered RyR function in at least 2 respects. First, trypsin reduced conductance; second, the response of the channel to luminal Ca\(^{2+}\) was altered. The 2 effects appear to be the result of different interactions of trypsin with the channel because the conductance change was reversible after perfusing away the trypsin, whereas the gating change was irreversible. Further distinction between the 2 effects was obtained by the use of heat-inactivated trypsin. Heat-inactivated trypsin also caused a similar reversible reduction in conductance in but did not cause the trypsin-induced change in RyR gating. These data lead us to conclude that the conductance effect is the result of a protein-protein interaction between RyR and trypsin, whereas the change in the response of RyR to luminal Ca\(^{2+}\) involves cleavage of exposed luminal sites. Therefore, the results strongly suggest that the increase in \(P_o\) observed in response to an increase in luminal [Ca\(^{2+}\)] is due to the binding of Ca\(^{2+}\) to luminal sites on the RyR protein complex and not the result of Ca\(^{2+}\) flowing through the channel to act on cytosolic sites. Furthermore, the fact that trypsin completely abolishes any stimulatory effect of luminal Ca\(^{2+}\) and does not leave a residual stimulatory effect argues against any contribution to a luminal Ca\(^{2+}\)-induced increase in \(P_o\) by Ca\(^{2+}\) flowing through the channel and accessing cytosolic binding sites.

The unexpected reduction in \(P_o\) caused by increasing luminal [Ca\(^{2+}\)] after incubation with trypsin indicates that there are also luminal Ca\(^{2+}\) binding sites on the RyR protein complex that mediate the inhibition of RyR activity. The simplest explanation for our results is that there are luminal Ca\(^{2+}\) activation and Ca\(^{2+}\) inhibition sites but that trypsin cleavage of the RyR protein complex damages the activation site(s) while allowing the inhibitory site(s) to remain functional. Changing the holding potential and, therefore, the Ca\(^{2+}\) flux through the channel does not affect the inhibitory action of luminal Ca\(^{2+}\), indicating that the sites mediating the inhibition do not have a cytosolic location. This is the first time that an inhibitory effect of luminal Ca\(^{2+}\) has been observed in cardiac RyRs, although high luminal [Ca\(^{2+}\)] has been reported to inhibit purified rabbit skeletal RyRs.\(^9,10\) It is possible that skeletal inhibitory luminal Ca\(^{2+}\) binding sites exhibit a relatively higher affinity for Ca\(^{2+}\) than do the cardiac inhibitory sites or that the purification procedure has led to alterations to luminal activation sites. The fact that 1 mmol/L luminal Ca\(^{2+}\) causes activation of the cardiac RyR before trypsinization but channel inactivation after trypsinization indicates either that the luminal activation sites have a higher affinity for Ca\(^{2+}\) or that they transduce a greater change in \(P_o\) than do the inhibition sites.

The mechanism underlying the reduced current amplitude observed in the presence of trypsin or heat-inactivated trypsin is not known. It is possible that both molecules interact with RyRs near the luminal mouth of the pore and block the flow of permeant ions, but equally, trypsin (and heat-inactivated trypsin) may bind elsewhere on the RyR protein complex, resulting in a conformational change of RyR that leads to the reduction in conductance. Further experiments are required to understand the conductance changes observed with these molecules. The importance of these results lies in the fact that the irreversible change in RyR gating is not associated with any measurable changes in conductance either when Ca\(^{2+}\) is the permeant ion or when appreciable Ca\(^{2+}\) current is evident (see Figure 5), indicating that the trypsin cleavage of luminal Ca\(^{2+}\) sites do not lead to any major changes in the pore structure of the channel. Therefore, these results provide strong evidence that the Ca\(^{2+}\) binding sites involved in the effects of luminal Ca\(^{2+}\) on channel gating are distinct from any cation binding sites within the channel pore.

Where are the luminal Ca\(^{2+}\) binding sites? The precise location is not yet known, but they must be situated either on RyR or on a protein that remains associated with RyRs after incorporation into the bilayer. Trypsin preferentially cleaves amino acid residues with positively charged side chains.\(^19\) RyRs have a number of lysine and arginine residues in the C-terminal domain that are predicted to have a luminal location and that could be the putative sites for cleavage in the present experiments,\(^20–23\) but so also do the luminal domains of junctin and triadin.\(^17,24\) In a number of cases, these residues are located close to negatively charged residues, which could form Ca\(^{2+}\) binding regions. Trypsin does not cleave calsequestrin under our conditions of trypsin incubation (in the presence of 1 mmol/L Ca\(^{2+}\)),\(^25\) suggesting that the observed changes to luminal Ca\(^{2+}\) modulation of \(P_o\) are not caused by damage to calsequestrin.

Our observation that luminal Ca\(^{2+}\) can regulate cardiac RyR gating from the luminal side of the bilayer and does not require that Ca\(^{2+}\) should first flow through the channel before binding to cytosolic sites is crucial for a full understanding of the mechanisms determining the dependence of SR Ca\(^{2+}\) release on SR Ca\(^{2+}\) content. Unlike the situation in the bilayer, it would be expected that in the restricted space of the cleft region in cardiac cells, the Ca\(^{2+}\) flowing through RyRs would feed back to regulate RyR gating by binding to Ca\(^{2+}\) activation and inhibition sites. The luminal regulatory sites proposed in the present study would provide an additional distinct mechanism by which the SR [Ca\(^{2+}\)] could modulate the release mechanism, thus allowing a far greater degree of potentiation in the gain of EC coupling. Therefore, our results may help to explain the nonlinear relationship between SR Ca\(^{2+}\) content and SR Ca\(^{2+}\) release and the hugely steep increases in Ca\(^{2+}\) release and in the gain of EC coupling at high SR Ca\(^{2+}\) levels, described by Shannon et al.\(^6\) Importantly, the SR [Ca\(^{2+}\)] at which the authors observed steep changes in Ca\(^{2+}\) release corresponds closely to the levels of luminal Ca\(^{2+}\) that have been shown to activate isolated cardiac RyR channels reconstituted into bilayers.\(^3,7,11,26\) Be-
cause changes in SR load can occur under pathological conditions such as heart failure and ischemia, the above discussion would suggest that small changes in SR Ca\(^{2+}\) content may lead to large changes in contractility. Therefore, it is crucial to understand the mechanisms responsible for regulating the gating of the cardiac RyR channel by luminal [Ca\(^{2+}\)].

In summary, we demonstrate that the cardiac RyR response to luminal Ca\(^{2+}\) can be altered by physical protein modifications at the luminal side of the bilayer, indicating that luminal Ca\(^{2+}\) can regulate channel gating by binding to luminal sites on the RyR channel complex. Our results reveal for the first time the presence of luminal Ca\(^{2+}\) activation and inactivation sites, although the exact location of the sites is yet to be elucidated. Luminal Ca\(^{2+}\) binding sites would play an important role in regulating SR Ca\(^{2+}\) release and contractility in cardiac muscle by providing an increased potential for increasing the gain of EC coupling.

Acknowledgment
This study was supported by The British Heart Foundation

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_Circ Res._ 2000;87:201-206
doi: 10.1161/01.RES.87.3.201

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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