Potentiation of Fractional Sarcoplasmic Reticulum Calcium Release by Total and Free Intra-Sarcoplasmic Reticulum Calcium Concentration

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ABSTRACT Our aim was to measure the influence of sarcoplasmic reticulum (SR) calcium content ($[Ca]_{SRT}$) and free SR [Ca] ($[Ca]_{SR}$) on the fraction of SR calcium released during voltage clamp steps in isolated rabbit ventricular myocytes. $[Ca]_{SRT}$, as measured by caffeine application, was progressively increased by conditioning pulses. Sodium was absent in both the intracellular and in the extracellular solutions to block sodium/calcium exchange. Total cytosolic calcium flux during the transient was inferred from I_{Ca}, $[Ca]_{SRT}$, $[Ca]_i$, and cellular buffering characteristics. Fluxes via the calcium current (I_{Ca}), the SR calcium pump, and passive leak from the SR were evaluated to determine SR calcium release flux (J_{rel}). Excitation-contraction (EC) coupling was characterized with respect to both gain ($\int J_{rel}/\int I_{Ca}$) and fractional SR calcium release. Both parameters were virtually zero for a small, but measurable $[Ca]_{SRT}$. Gain and fractional SR calcium release increased steeply and nonlinearly with both $[Ca]_{SRT}$ and $[Ca]_{SR}$. We conclude that potentiation of EC coupling can be correlated with both $[Ca]_{SRT}$ and $[Ca]_{SR}$. While fractional SR calcium release was not linearly dependent upon $[Ca]_{SR}$, intra-SR calcium may play a crucial role in regulating the SR calcium release process.

INTRODUCTION

Calcium influx through L-type calcium channels causes graded calcium release from the sarcoplasmic reticulum (SR) resulting in a graded contraction of cardiac muscle (Beuckelmann and Wier, 1988; Näbauer et al., 1989; Bers, 1991). How the process of this excitation-contraction (EC) coupling is altered is the subject of intense research. However, SR calcium release is only rarely measured directly. It is most often assumed to be proportional to the more easily measured $[Ca]_i$ transient peak (Spencer and Berlin, 1995, 1997) or rate of rise (Yasui et al., 1994; Sham et al., 1995).

Several groups use an index of SR calcium release divided by the calcium trigger (I_{Ca}) to give a measure of "gain" (Wier et al., 1994; Santana et al., 1997). This concept has been useful when SR calcium load is constant, but if SR calcium load rises then a given I_{Ca} trigger is expected to increase the amount of SR calcium release, even if the EC coupling process is fundamentally unchanged. Thus it is not obvious how to interpret gain with changing SR calcium load, although the simplest expectation would be that gain would increase as a linear function of SR calcium content or $[Ca]_{SR}$.

As expected, many groups observe SR calcium release in proportion to total SR [Ca] ($[Ca]_{SRT}$) because there is more or less calcium available for release (Janczewski et al., 1995; Song et al., 1997). However, it has also been reported that SR calcium release increases disproportionately with the increase in $[Ca]_{SRT}$, indicating that there may be an effect of $[Ca]_{SRT}$ on the gating of the SR calcium release channel (Isenber and Han, 1994; Bassani et al., 1995; Spencer and Berlin, 1995, 1997; Santana et al., 1997; Dettbarn and Palade, 1997; Hüser et al., 1998).

The term fractional SR calcium release is used in studying the effect of $[Ca]_{SRT}$ upon SR calcium release. This requires measurement of SR calcium load and release. Bassani et al. (1995) completely blocked the SR calcium ATPase with thapsigargin while SR load was maintained. Then the SR calcium content was assessed by caffeine either before or after a single test twitch. The fraction of SR calcium content released during the test twitch (with no reuptake) was thus assessed. These investigators found a fractional release of ≈43% during a normal twitch, 4% at low SR calcium load, and 60% at maximal SR calcium load. Thus fractional release was a steeply nonlinear function of $[Ca]_{SRT}$.

The mechanism by which $[Ca]_{SRT}$ modulates release is unknown. One possibility is that free SR calcium ($[Ca]_{SR}$) may alter the properties of the SR calcium release channel (Sitsapesan and Williams, 1994; Donoso et al., 1995; Lukyanenko et al., 1996; Herrmann-Frank and Lehmann-Horn, 1996; Tripathy and Meissner, 1996). For instance, Lukyanenko et al. (1996) found that SR calcium release channel activity in planar lipid bilayers increased with $[Ca]_{SR}$ on the *trans* (lumenal) side of the membrane. This increased activity was due to increased mean open time and event frequency.

We hypothesize that SR calcium release increases as an increasingly steep function of $[Ca]_{SRT}$ at high $[Ca]_{SRT}$. In this case we test the findings of Bassani et al. (1995) with more detailed quantitative analysis and without blocking the SR calcium ATPase. We further hypothesize that the SR calcium buffering proteins are becoming saturated at high $[Ca]_{SRT}$ and that fractional SR calcium release is proportional to $[Ca]_{SR}$. To test this hypothesis, we have used a novel analytical method to quantitatively measure the gain

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($\int$ SR calcium release flux/$\int I_{Ca}$) and fractional SR calcium release in response to graded increases in both $[Ca]_{SRT}$ and $[Ca]_{SR}$. SR calcium release flux (J_{rel}) was obtained from total cytosolic calcium flux after all other fluxes into and out of the cytosol were accounted for. We found that the gain and fractional SR calcium release increased in a nonlinear manner with the relationship becoming much more steep at both higher $[Ca]_{SRT}$ and $[Ca]_{SR}$.

MATERIALS AND METHODS

The following is a brief explanation of the methods. All experimental methods are described in detail in the companion paper (Shannon et al., 2000).

The voltage clamp protocol was performed essentially as described by Ginsburg et al. (1998). Cells were sodium-depleted by incubating in a sodium- and calcium-free solution. After unloading the SR with a 10-s caffeine pulse (in normal Tyrode's solution containing sodium to allow sodium-calcium exchange to extrude calcium), the SR was progressively loaded with I_{Ca} in the absence of pipette or extracellular sodium in the whole cell patch mode. SR calcium content was measured by subsequent caffeine application (Ginsburg et al., 1998).

Total cytosolic [Ca] ($[Ca]_T$) was calculated from $[Ca]_i$ using the calcium binding properties of known calcium buffers within the cell (Shannon et al., 2000). Because of the relatively rapid rise and fall of calcium during the transient, we did not assume steady-state binding kinetics, and instead calcium binding was calculated as being rate-dependent. Calcium binding to all of the calcium buffers was summed to determine the $[Ca]_T$ transient.

Calcium uptake rates and the SR calcium content were calculated from the change in $[Ca]_T$ over time. Only the late part of the $[Ca]_T$ transient was used to measure SR calcium uptake (i.e., the time after which SR release is assumed to be zero). SR calcium uptake data were fit to an equation that accounts for SR calcium pump forward flux and backflux and the SR calcium leak and buffering (Shannon et al., 2000). Fitted parameters that rose or fell to maximum or minimum constraints were occasionally used. These constraints were chosen to be physiological and were considered adequate for our purposes in this work.

Determination of SR calcium release flux

Fig. 2 shows the SR calcium release determination from changes in $[Ca]_T$ over time (Shannon et al., 2000). For the purposes of our experiments, the SL calcium pump was assumed to make only a negligible contribution to the calcium fluxes within the cell (Bassani et al., 1992). All of the other major nonrelease calcium fluxes into and out of the cytosol were accounted for by one of three methods:

1. *Inhibition:* Sodium/calcium exchange was inhibited by using 0 Na inside and outside the cell and mitochondrial influx was eliminated by using the mitochondrial uptake inhibitor RU360 inside the pipette.
2. *Measurement:* This included measurement of calcium influx via L-type calcium channels as current across the membrane. SR calcium content was also measured directly at the end of each loading sequence.
3. *Determination through nonlinear regression fits:* SR calcium pump flux and SR calcium leak were determined as described briefly above and in detail by Shannon et al. (2000). Constants for SR calcium pump activity were determined from fits of total cytosolic calcium flux late in the $[Ca]_i$ transient. An SR leak constant of 0.0047 ± 0.0004/s (Shannon et al., 2000) was used in the leak component of the equation. SR calcium buffering parameters were also determined. Using these parameters, fluxes were calculated for the early parts of the calcium transient by extrapolation.

Once all of the other major cellular calcium fluxes were determined by the methods above, these were subtracted from the total cytosolic calcium flux. The remainder was considered to be SR calcium release flux, the only major cellular calcium flux left:

$$J_{rel} = \frac{d[Ca]_T}{dt} - I_{Ca} - J_{pump} + J_{leak} \quad (1)$$

RESULTS

Rate dependence of calcium binding to indo-1

In order to infer an accurate $[Ca]_T$ transient in ventricular myocytes, we first corrected the measured $[Ca]_i$ for the kinetics of indo-1 binding to calcium (Shannon et al., 2000). Briefly, the ratio of indo-1 fluorescence at 405 and 485 nm when excited at 355 nm was directly related to the amount of indo-1 bound:

$$[Ca \cdot indo] = \frac{B_{max}}{1 + \dfrac{R_{max} - R}{\beta(R - R_{min})}} \quad (2)$$

where B_{max} is the total [indo-1] and [Ca · indo] is the calcium bound to indo-1. The derivative of this measurement over time was used in combination with the rate equation (Eq. 3 in Shannon et al., 2000) solved for $[Ca]_i$ over the course of the transient:

$$[Ca]_i = \frac{\dfrac{d[Ca \cdot indo]}{dt} + k_{off}[Ca \cdot indo]}{k_{on}(B_{max} - [Ca \cdot indo])} \quad (3)$$

where [Ca · indo] is the amount of bound indo. The result of this indo-1 binding kinetic correction is demonstrated for a typical $[Ca]_i$ transient in Fig. 1. Fig. 1 *A* shows the entire transient. The dashed line is the uncorrected transient derived from the Grynkiewicz equation (Grynkiewicz et al., 1985) which assumes indo-1 binding is instantaneous. The solid line is corrected for indo kinetics.

There is very little difference between the two transients in the declining phase. The active process during this phase of the transient is the SR calcium pump (note: sodium-calcium exchange is blocked here). The $[Ca]_i$ decline is slow compared to the indo-1 calcium binding rate, and therefore steady-state kinetics may be applied to this phase with little loss of accuracy in $[Ca]_i$ determination.

In contrast, there is a marked difference in the rising phase of the transient in Fig. 1 *B*. The kinetically corrected indo-1 $[Ca]_i$ transient rises faster by 10 ms to a slightly higher peak than the raw $[Ca]_i$ signal. The processes responsible for the rise in global $[Ca]_i$ are sarcolemmal calcium influx (i.e., I_{Ca}) and SR calcium release. However, calcium binding to cytosolic buffers and transport by the SR calcium pump will limit the rise in $[Ca]_i$. The $[Ca]_i$ so derived is then used as the driving function to calculate the time-dependent

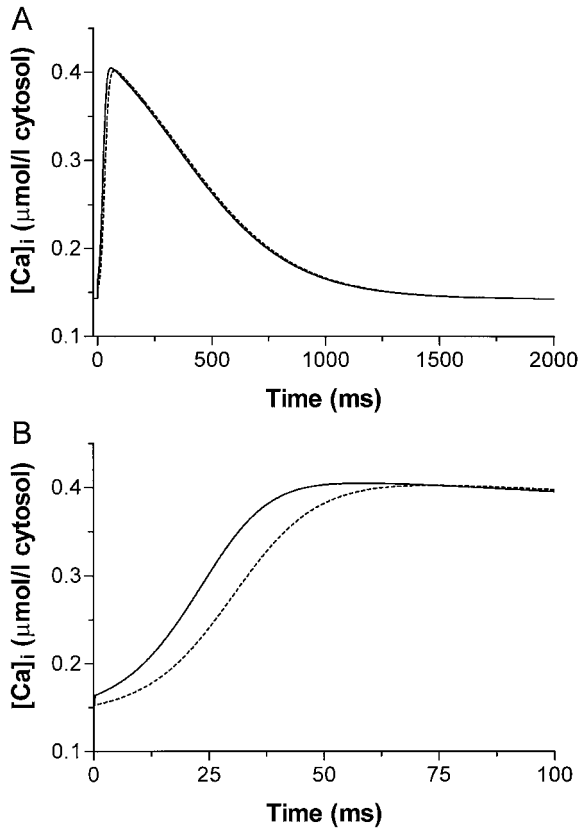

FIGURE 1 (*A*) A typical $[Ca]_i$ transient. Shown is the $[Ca]_i$ before (*dashed line*) and after (*solid line*) correction for the kinetics of calcium binding to indo. The lines appear almost superimposible on the time scale shown. (*B*) An expanded view of the first 100 ms of *A*. Notice that while the kinetic correction made very little difference in the appearance of the relative slow declining phase of *A*, the corrected transient reaches its peak ~10 ms earlier during the rapidly rising phase emphasized in *B*.

calcium binding to other cytosolic calcium buffers as described by Shannon et al. (2000).

Cellular calcium fluxes

The sum of free and ligand bound calcium is the total cytosolic calcium ($[Ca]_T$), and how this changed with time ($d[Ca]_T/dt$) must be the sum of fluxes into and out of the cytosol (Eq. 1). I_{Ca} is directly measured, J_{pump} and J_{leak} are determined as described in Shannon et al. (2000). Thus SR calcium release flux (J_{rel}) can be directly calculated. Note that sodium-calcium exchange and mitochondrial calcium uptake are both blocked and SL calcium ATPase is considered negligible.

Fig. 2 shows the resulting calcium fluxes. SR calcium pump reversal (or "backflux") was a notable part of the SR calcium pump flux equation (Shannon et al., 2000). This resulted in two partial SR pump fluxes, which added up to the net SR calcium pump flux (J_{pump}) during the $[Ca]_i$ transient. The forward flux (J_{pumpf}) through the pump increased and backflux (J_{pumpr}) decreased almost immediately upon cellular stimulation due to the rapid increase in $[Ca]_i$ and decrease in $[Ca]_{SRT}$ and $[Ca]_{SR}$. As calcium is transported into the SR, $[Ca]_{SRT}$ and $[Ca]_{SR}$ increase, causing J_{pumpr} to rise at the same time that $[Ca]_i$ and J_{pumpf} are

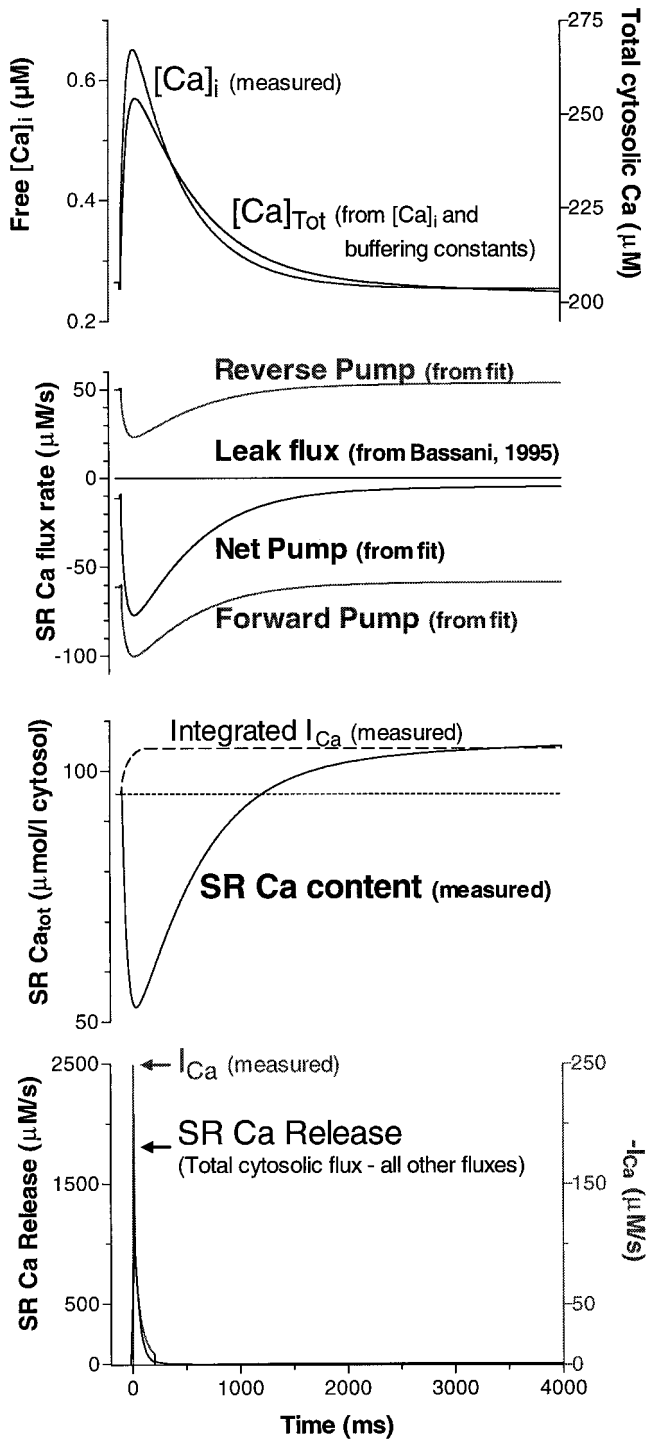

FIGURE 2 Cellular fluxes in a typical cardiomyocyte and the method by which each was determined.

decreasing. Eventually, the pump reaches a near steady state at resting $[Ca]_i$. At this point J_{pump} is equal to a small resting passive SR calcium leak flux, J_{leak} (Shannon et al., 2000), probably through occasional opening of the SR calcium release channels (Cheng et al., 1993; Bassani and Bers, 1995). Also note that the $[Ca]_{SRT}$ at the end of the pulse is the initial $[Ca]_{SRT}$ plus $\int I_{Ca}$ (or the amount of calcium that was brought into the cell).

J_{rel} parameters were closely examined. Peak J_{rel} was found to vary with $[Ca]_{SRT}$. These ranged from 52 to 1885 μmol/l cytosol/s at SR calcium loads of 80–115 μmol/l cytosol (17 pulse sets from five cells). In Fig. 2 the peak J_{rel} is 8 times the peak I_{Ca}. Time to peak release flux was 27.4 $\pm$ 4.0 ms and the time to 90% decline was 117 $\pm$ 20 ms. Neither of these changed with $[Ca]_{SRT}$ in pulse sets that had detectable release.

Fig. 3 takes a closer look at the calcium fluxes near the beginning of the transient. It is immediately apparent that I_{Ca} peaks before all other fluxes. Most importantly, it peaks before the peak of J_{rel}. This is consistent with the idea that I_{Ca} stimulates release. Sodium/calcium exchange, which has been proposed to stimulate release (Wasserstrom and Vites, 1996; Litwin et al., 1998), is absent. There was no cyclic AMP in the pipette and the experiments were at room temperature. These are conditions that make other putative triggers of J_{rel} unlikely to be factors here (Howlett and Ferrier, 1997; Santana et al., 1998).

Note that $[Ca]_T$ peaks after $[Ca]_i$. This is consistent with the fact that cytosolic calcium buffers have finite calcium binding kinetics and are not close to equilibrium early in the transient (Shannon et al., 2000). Therefore, at the $[Ca]_i$ transient peak, cytosolic calcium buffers are still "catching up" with I_{Ca} and J_{rel}. For this reason, calcium binding to all of the major cytosolic calcium buffers is calculated as being time-dependent. The $[Ca]_i$ therefore begins to decline only when net cytosolic calcium flux (J_{pump}, J_{rel}, and I_{Ca}) equals the flux of calcium onto cytosolic calcium buffers.

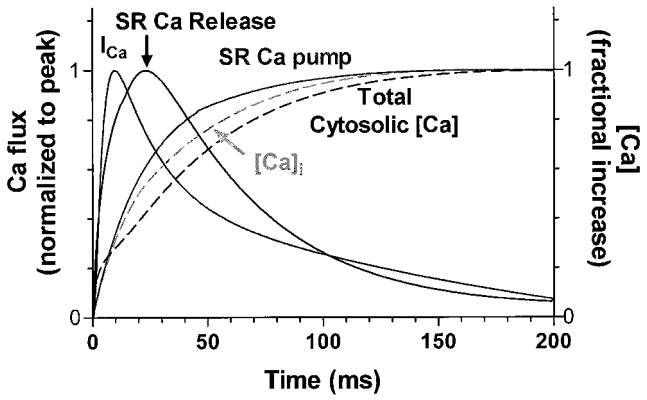

FIGURE 3 A closer look at the rise and fall of the I_{Ca} and calcium release flux rates under low $[Ca]_{SRT}$. I_{Ca} peaks before SR calcium release flux consistent with I_{Ca} being the major stimulus of release. Total cytosolic [Ca] peaks after $[Ca]_i$ due to the effect of time-dependent calcium binding to buffers within the cytosol. Note the early peak $[Ca]_i$ corresponding to I_{Ca} influx.

I_{Ca} inactivation and the rate of SR calcium release

Differences in I_{Ca} inactivation rate have been proposed to reflect differences in $[Ca]_i$ at the L-type calcium channel mouth (Hadley and Hume, 1987; Sipido et al., 1995; Adachi-Akahane et al., 1996; Sham, 1997; Puglisi et al., 1999). We have used the method of Puglisi et al. to determine the time-to-peak J_{rel} in the junctional space. I_{Ca} from the first and last pulses in a set were normalized to their peaks (Fig. 4 *A*). The first pulse was the current with calcium-depleted SR and no release. The last pulse included inactivation due to SR calcium release from the loaded SR. The difference current (I_{diff}) was taken to reflect local $[Ca]_i$ due to J_{rel}. The derivative (dI_{diff}/dt) was determined as an indicator of rate of local $[Ca]_i$ change in the cleft and thus J_{rel}. The relationship between dI_{diff}/dt and J_{rel} is a complicated one, but the time course of each process should be similar.

Fig. 4 *A* shows that peak J_{rel} sensed by calcium channels (maximum dI_{diff}/dt) occurs within 11 ms of depolarization. In Fig. 4 *B* mean data from this type of analysis are compared with the time-to-peak SR calcium release determined by overall calcium flux analysis (as in Figs. 2 and 3). The peak of J_{rel} was much earlier when sensed locally by I_{Ca} (10.2 $\pm$ 0.6 ms) than when determined by bulk calcium flux analysis (27.4 $\pm$ 4.0 ms).

Load-dependence of SR calcium release

We used two measures of EC coupling efficacy in our cells. Gain is the calcium released normalized to the amount of I_{Ca} trigger ($\int J_{rel}/\int I_{Ca}$). It is, therefore, a measure of $[Ca]_T$ transient amplification due to EC coupling (Stern, 1992). Qualitatively, our results were the same when peak I_{Ca} was used in our analysis instead of $\int I_{Ca}$ (but, of course, in this case the gain value would be higher due to a smaller denominator). At low SR calcium loads there appeared to be no J_{rel} despite a calcium load that could be readily measured during caffeine application. Thus there appeared to be a threshold $[Ca]_{SRT}$ required for significant release. No J_{rel} was detected in some cases up to a $[Ca]_{SRT}$ of 45 μmol/l.

Not surprisingly, we found that the gain increased with increased $[Ca]_{SRT}$. Gains approached 10 at "normal" SR calcium contents of $\approx$100 μmol/l cytosol and were as high as 50 at extremely high $[Ca]_{SRT}$. This increase was related nonlinearly with $[Ca]_{SRT}$ (Fig. 5, *top*). It was gradual at lower $[Ca]_{SRT}$ but increased dramatically at higher $[Ca]_{SRT}$ with a Hill slope $>$10. If $\int J_{rel}$ increased with $[Ca]_{SRT}$ only because there was more calcium available for release, we would predict that the gain would be directly proportional to $[Ca]_{SRT}$ (Fig. 5, *top*, *dashed line*). Because the gain was a

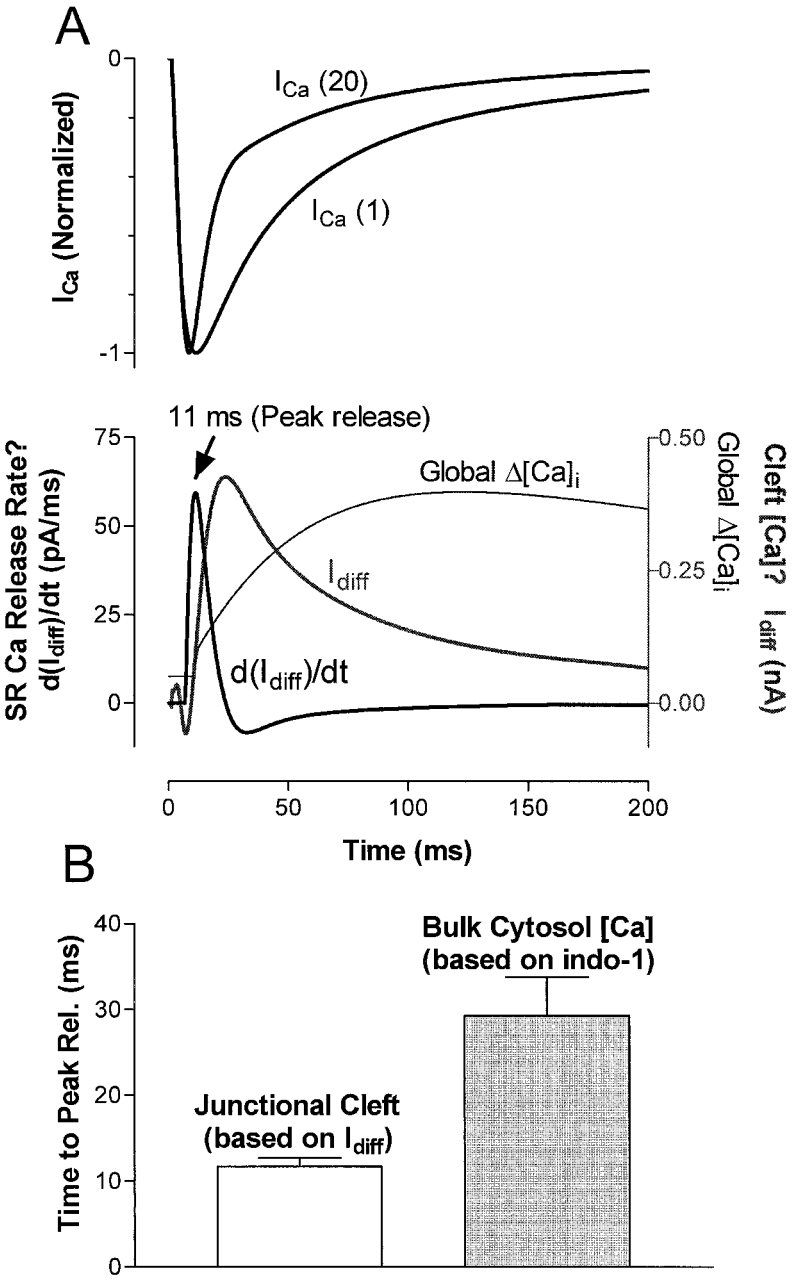

FIGURE 4 Time to peak release rate from time to I_{Ca} inactivation. (*A*) The process by which the time to peak release within the junctional space was estimated. I_{Ca} in the myocyte with unloaded and loaded SR were normalized to peak current and the difference current (I_{diff}) was determined. The dI_{diff}/dt is an indication of the rate of SR calcium release-dependent inactivation of I_{Ca} and therefore SR calcium release within the junctional space. (*B*) The time to peak release within the junctional space is much shorter than the time to peak release as sensed within the bulk cytosol.

nonlinear function of $[Ca]_{SRT}$, we conclude that $\int J_{rel}$ is not simply proportional to $[Ca]_{SRT}$ and that the process of EC coupling must be affected by $[Ca]_{SRT}$ in some way. The $[Ca]_{SRT}$ appeared to reach a maximal level of ~100–120 μmol/l cytosol and at this level the curve was very steep.

In further support of this conclusion, we also measured fractional SR calcium release as a function of $[Ca]_{SRT}$ ($\int J_{rel}/[Ca]_{SRT}$, Fig. 5, *bottom*). Fractional release varied with $[Ca]_{SRT}$ (for a given I_{Ca}) getting as high as 150% at high $[Ca]_{SRT}$. If the $\int J_{rel}$ were simply due to the increase in available calcium for release with no effect upon the release process itself, we would expect a constant fractional release at all SR calcium loads (Fig. 5, *bottom*, *dashed line*). This is clearly not the case. As with the EC coupling gain, fractional release increased nonlinearly with $[Ca]_{SRT}$ and with a much steeper increase at higher $[Ca]_{SRT}$. It was certainly not constant.

The fractional SR calcium release was frequently >100% of the $[Ca]_{SRT}$ at very high $[Ca]_{SRT}$. On the surface, this would appear to be surprising. However, a close look at Figs. 2 and 3 reveals that SR calcium uptake begins almost immediately with release and continues at a high rate throughout the release process. The amount of calcium released can exceed $[Ca]_{SRT}$ because calcium that has been taken up by the SR calcium pump can be re-released. However, we also observe that SR calcium content was

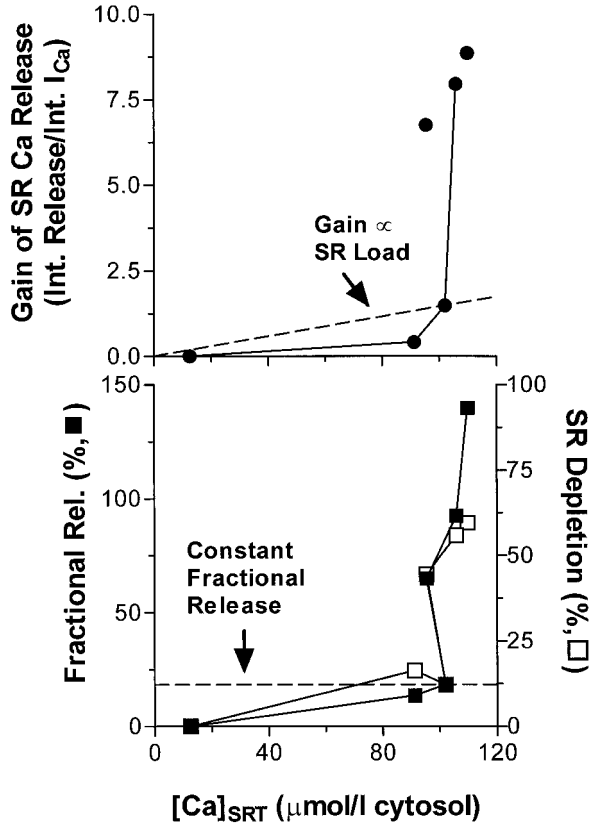

FIGURE 5 The gain of EC coupling and the fractional SR calcium release (■) as a function of $[Ca]_{SRT}$. Both parameters increased as a nonlinear function of $[Ca]_{SRT}$ (*solid lines*), contrary to the expected linear relationship if the release mechanism was unchanged with $[Ca]_{SRT}$ (*dashed lines*). The relationship to SR calcium depletion (minimum $[Ca]_{SRT}$ during the transient/initial $[Ca]_{SRT}$, □) is similar.

never fully depleted during the course of SR calcium release (see Fig. 2). We therefore looked at fractional SR calcium release in another way. That is, we evaluated the maximum "SR calcium depletion" occurring during a twitch:

$$\text{SR calcium depletion} = \frac{(\text{initial SR calcium} - \text{minimum SR calcium})(100)}{\text{initial SR calcium}}$$

For instance, in Fig. 2 the initial SR calcium content is 96 μmol/l cytosol and the minimum SR calcium content during the transient is 53 μmol/l cytosol. The SR calcium depletion therefore is 45%.

Similar to the fractional release, SR calcium depletion varied nonlinearly with SR calcium content with a steep slope at higher $[Ca]_{SRT}$ (Fig. 5, *bottom*, *right axis*). Therefore, when initial $[Ca]_{SRT}$ is high, more release takes place leading to a higher $[Ca]_i$ and at the same time, a lower SR calcium content (and $[Ca]_{SR}$). Both factors tend to increase the net SR calcium uptake rate leading to an amplification of the $[Ca]_{SRT}$ effect on fractional release due to calcium uptake and re-release.

The steep nonlinear relationship of gain and fractional release versus $[Ca]_{SRT}$ at high $[Ca]_{SRT}$ led us to hypothesize that the major factor controlling the EC coupling load dependence may be $[Ca]_{SR}$. As $[Ca]_{SRT}$ increases, the SR calcium buffering proteins may saturate and $[Ca]_{SR}$ may increase at a steep rate, causing increased release. We therefore used the SR calcium buffering fit parameters to convert $[Ca]_{SRT}$ to $[Ca]_{SR}$ (Shannon et al., 2000). The relationship between EC coupling and $[Ca]_{SR}$ was examined (Fig. 6). Once again, the gain and fractional release increased in a nonlinear manner that was more steeply dependent upon $[Ca]_{SR}$ at higher levels. The relationship between EC coupling and bound SR calcium ($[Ca \cdot L]$) was also similar (not shown).

In order to more fully define the shape of the relationships in Figs. 5 and 6, we determined $(\Delta[Ca]_T - \int I_{Ca})/\int I_{Ca}$ (a measure of gain) and $(\Delta[Ca]_T - \int I_{Ca})/[Ca]_{SRT}$ (a measure of fractional release) as a function of $[Ca]_{SRT}$ in for each pulse in 6 sets of 20 pulses (Fig. 7). We could not do precisely the same analysis as in Figs. 5 and 6 for all 20 pulses because we did not directly measure SR calcium load at each pulse (only the points in Figs. 5 and 6). Instead, we have used the shape of the relationship between $[Ca]_{SRT}$ and pulse number in Fig. 4 *A* of our companion paper to extrap-

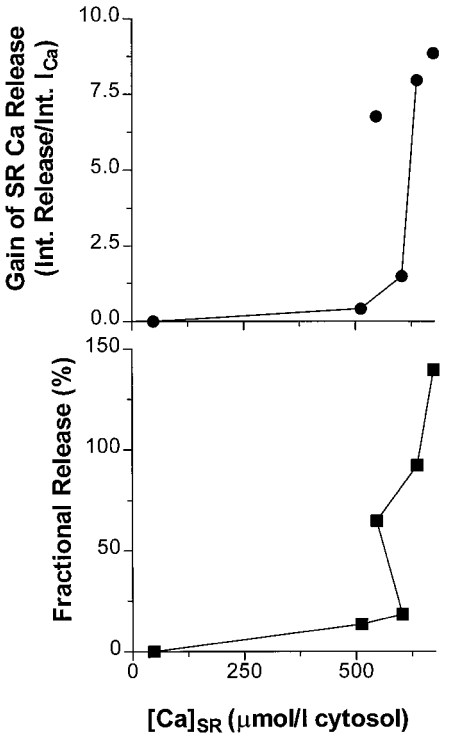

FIGURE 6 The gain of EC coupling and the fractional SR calcium release as a function of $[Ca]_{SR}$. There was no direct correlation between the two parameters and $[Ca]_{SR}$. Nevertheless, an effect of $[Ca]_{SR}$ upon SR calcium release cannot be ruled out.

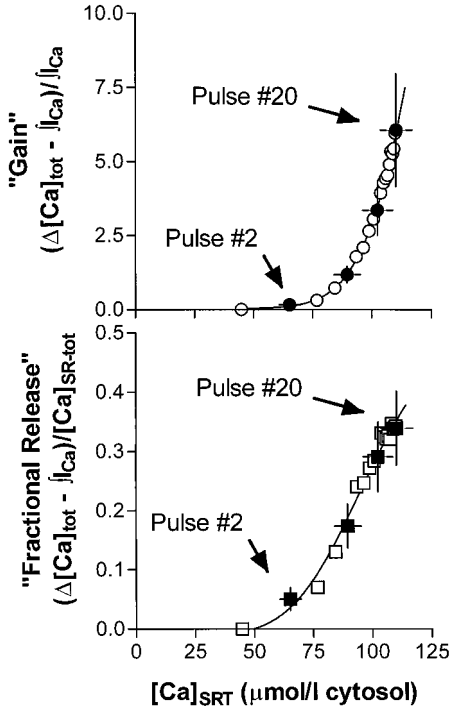

FIGURE 7 $(\Delta[Ca]_T - \int I_{Ca})/\int I_{Ca}$ (a measure of gain) and $(\Delta[Ca]_T - \int I_{Ca})/[Ca]_{SRT}$ (a measure of fractional release) as a function of $[Ca]_{SRT}$. Error bars are only shown at pulses 2, 5, 10, and 20 (*filled circles*). The parameters are determined at loads intermediate to those shown in Figs. 5 and 6. Both accelerate as a smooth function which is arbitrarily described here as a sigmoid curve. *Top:* Hill slope = 8.8, EC_{50} = 130 μM; *bottom:* Hill slope = 5.6, EC_{50} = 100 μM.

olate SR calcium content to every pulse between 1 and 19, normalizing to the $[Ca]_{SRT}$ measured at pulse 20 for all six pulse sets (Shannon et al., 2000).

The transition from low to high gain and fractional release is a smooth but very steep function of load at high $[Ca]_{SRT}$ for both gain and fractional release. These relationships are arbitrarily described in terms of a sigmoid curve that has a high Hill slope, consistent with the data in Fig. 5. We conclude that any mechanism or model of ECC must be able to explain this SR calcium load-dependent alteration in gain and fractional release.

DISCUSSION

Analytical technique

The problem of measuring SR calcium release has been approached in at least four ways: 1) measure the derivative of the $[Ca]_i$ transient rising phase (Cleeman and Morad, 1991; Konishi and Berlin, 1993; Berlin and Konishi, 1993). 2) Measure the experimental derivative of the calcium binding rate to high concentrations of EGTA and Oregon-green-BAPTA 5N (Sham et al., 1998). 3) Measuring the $[Ca]_{SRT}$ after a twitch when reuptake has been completely blocked by thapsigargin (Bassani et al., 1995). 4) Obtain SR calcium release flux from $[Ca]_T/dt$ after accounting for all other calcium fluxes into and out of the cytosol (Sipido and Wier, 1991; Wier et al., 1994). The last method is similar to those used previously in skeletal muscle (Baylor et al., 1983; Melzer et al., 1984, 1987), and is more indirect, but allows continued measurement from both the same cell and other cells in the chamber after an initial release measurement.

We have chosen to use technique 4 with significant modification to quantify release in this paper. Our characterization includes all of the known major cytosolic calcium buffers and backflux through the SR calcium pump (Shannon et al., 2000).

Some assumptions made here merit consideration. To accurately evaluate SR pump (and leak) flux it is ideal to have conditions where these fluxes are isolated and not overlapping with J_{rel}. We have done this by characterizing SR calcium pump and leak properties late in the time course of the calcium transient (Shannon et al., 2000), starting at 300–500 ms where J_{rel} is assumed to be zero. The resulting time to 90% decline of J_{rel} was 117 $\pm$ 20 ms. To some extent this is circular, but SR calcium pump and leak properties were also fit when there was no J_{rel} (first pulse after emptying SR). We therefore assume that little error due to continued release is present.

We also assume that cellular fluxes we did not account for will not have a major impact on the results (e.g., sodium-calcium exchange and mitochondrial calcium uniport were blocked). The sarcolemmal calcium pump flux can almost certainly be neglected based upon the slow calcium disappearance from the cytosol in the absence of sodium-calcium exchange, mitochondrial uptake, and SR calcium uptake in rabbit and rat ventricular myocytes (Bassani et al., 1993, 1994; $\tau > 20$ s).

Indo, like many commonly used fluorescent dyes, has a high affinity for calcium (in vivo $K_d \approx 800$ nM). This makes it more likely to influence the calcium transient by buffering and the slow off rate tends to make it slower to respond to $[Ca]_i$. Using lower affinity calcium indicators like furaptra (Konishi and Berlin, 1993; Berlin and Konishi, 1993) can minimize these problems, but they add other problems. These include magnesium sensitivity, low signal-to-noise ratio, and greater motion artifacts. The problems with indo-1 can also be minimized by using low concentrations (Evans and Cannell, 1997) and by kinetic correction as we have done here (see also Berlin and Konishi, 1993). This speeds the rising phase of $[Ca]_i$ (Fig. 1), but should still be considered to be a lower limit. These dyes still underestimate the peak J_{rel} because of spatial calcium gradients within the cell, and these are global $[Ca]_i$ measurements. Initial calcium release causes a faster and larger $[Ca]_i$ spike near the ryanodine receptors of the SR membrane.

Consider, for example, the fact that J_{rel} sensed by calcium channels (Fig. 4) occurs much faster than peak SR calcium release determined by the indo fluorescence transient and

calcium flux analysis (Figs. 2 and 3). The results are consistent with predictions based upon mathematical modeling (Langer and Peskoff, 1996).

There are at least two possible reasons for this:

1. *Spatial Nonuniformity:* The appearance of the peak in the release rate as sensed in the bulk cytosol is limited by the time it takes for calcium to diffuse within the cytosol. Experimental evidence for nonuniformity of $[Ca]_i$ during release exists (Cheng et al., 1993; Cannell et al., 1994; Gómez et al., 1996; Wier et al., 1997) and theoretical estimates for the time to near uniformity in muscle are near our time-to-peak SR calcium release as measured in the bulk cytosol (Cannell and Allen, 1984).
2. *Saltatory Conduction:* Another possible delay may come in the form of saltatory conduction of calcium-induced calcium release from release channel to release channel along the SR (Spencer and Berlin, 1995). Such a mechanism presupposes the existence of SR that does not come into contact with the sarcolemma (extended junctional SR and corbular SR). Nonjunctional sarcoplasmic reticulum could make up as much as 25% of the cardiac SR (Jorgensen et al., 1993). For these to contribute to the global calcium signal, calcium released from the junctional SR would have to diffuse to the site of nonjunctional SR, causing further release. Thus the peak release might be delayed. It is not known whether nonjunctional SR participates in calcium release during normal contraction.

Both the time-to-local-peak I_{diff}/dt and the time-to-peak global J_{rel} are important. The time-to-peak within the junctional space is an indication of how quickly high subsarcolemmal calcium can be achieved within the cell, affecting SL membrane processes such as L-type calcium current and sodium-calcium exchange. However, the release rate sensed by indo-1 within the bulk cytosol will be closer to that sensed by the contractile fibers.

This consideration emphasizes that indo-1 is sensing a damped and more global version of J_{rel} than a local sensor in the junctional cleft. Thus, the true maximum J_{rel} is probably higher than indicated by Fig. 2 and occurs earlier in time. However, integrated J_{rel} should still be accurate. The amount of SR calcium release flux can be used for quantitative evaluation of the SR calcium load-dependence of calcium release.

Variation in EC coupling with SR calcium load

Some investigators have seen no $[Ca]_{SRT}$ effect upon the release process (Janczewski et al., 1995) while others have observed release potentiation at high $[Ca]_{SRT}$ (Isenber and Han, 1994; Spencer and Berlin, 1995, 1997; Bassani et al., 1995; Santana et al., 1997; Dettbarn and Palade, 1997; Hüser et al., 1998). $[Ca]_{SRT}$ increased both gain and fractional release nonlinearly in our experiments (Figs. 5 and 7). It is possible that reports that did not demonstrate a $[Ca]_{SRT}$ effect were performed over a range of $[Ca]_{SRT}$ where fractional release does not change very much (e.g., relatively low $[Ca]_{SRT}$). Most investigations that include high loads find a $[Ca]_{SRT}$ effect at higher and increasing $[Ca]_{SRT}$. Their results fit well with the right part of our relationship where the curve becomes increasingly steep with increasing $[Ca]_{SRT}$.

In some cases, such as that in Figs. 5 and 6, our fractional release exceeded 100%. As can be seen in Figs. 2 and 3, SR calcium uptake begins immediately upon SR calcium release. Therefore, uptake takes place while the SR calcium release channels are open. In fact, all calcium exceeding the actual extent of SR depletion can be accounted for by reuptake and re-release of calcium through these open channels. If the relationship between $[Ca]_{SRT}$ and SR calcium depletion (i.e., minimum SR [Ca]/initial SR [Ca], Fig. 2) is examined we see that the relationship is very consistent with previously reported measurements (Bassani et al., 1995) where the fractional release is 40–60% when reuptake was blocked at normal to maximal $[Ca]_{SRT}$ (Fig. 5). This, combined with the fact that no release was detected at loads below this level, leads us to speculate that SR depletion to $\approx$50% may contribute somehow to a "shut off" signal for SR calcium release.

The mechanism(s) by which high $[Ca]_{SRT}$ increases release is a matter of debate. There are two general categories: 1) extra-SR effects via local cytosolic $[Ca]_i$, and 2) intra-SR effects attributed to lumenal calcium (free or bound). These need not be mutually exclusive.

Extra SR $[Ca]_i$ near the release site would be expected to be higher at a higher $[Ca]_{SRT}$ for any given RyR opening. This is a simple consequence of higher $[Ca]_{SRT}$ corresponding to higher $[Ca]_{SR}$, and therefore higher calcium flux for a given channel opening. However, this effect by itself would only increase SR calcium release in proportion to increased $[Ca]_{SR}$. This higher $[Ca]_i$ would also be more likely to activate neighboring RyR and increase overall J_{rel}. Evidence supporting this mechanism comes from bilayer studies where increased RyR gating with high lumenal side [Ca] depends on calcium flux to the cytosolic side (Tripathy and Meissner, 1996; Xu and Meissner, 1998). Spencer and Berlin (1995, 1997) also showed in cellular experiments that strong buffering of cytosolic $[Ca]_i$ (high BAPTA or indo) or using Sr release from the SR could prevent the increased fractional SR release at higher $[Ca]_{SRT}$.

There is also strong evidence for a more direct effect of lumenal SR calcium to increase RyR open probability. Several studies in bilayers have shown that lumenal [Ca] in the mM range increases P_o for a given activating cytosolic [Ca] or caffeine (Sitsapesan and Williams, 1994; Donoso et al., 1995; Lukyanenko et al., 1996; Herrmann-Frank and Lehmann-Horn, 1996; Györke and Györke, 1998). It is possible that this lumenal calcium effect is in part mediated by other proteins associated with the RyR in vivo such as

calsequestrin, triadin, or junctin (Jones et al., 1995; Zhang et al., 1997). Since calcium alters the interaction of these SR proteins, this is a plausible mechanism and would explain why different results were found with more highly purified RyR2 (Xu and Meissner, 1998) than native preparations (Györke and Györke, 1998). Our experiments with intact cells cannot readily distinguish between intra- and extra-SR effects, but we speculate that both might contribute to the remarkably steep relationship between $[Ca]_{SRT}$ and fractional SR calcium release.

We hypothesized that $[Ca]_{SR}$ would be more simply and directly related to fractional SR calcium release than is $[Ca]_{SRT}$. However, the qualitative relationship between fractional SR calcium release and $[Ca]_{SR}$ was much the same as for $[Ca]_{SRT}$. This does not of course rule out $[Ca]_{SR}$ as a factor in the effect. However, if it is a mediator, the steepness of the J_{rel} relationship at high $[Ca]_{SR}$ indicates a high degree of cooperativity in the effect. For instance, a K_m of $\sim$600 μM $[Ca]_{SR}$ and a Hill coefficient $>$10 describes the results in Fig. 6. The mechanism would be more complex than a simple 1:1 binding relationship with a moderate affinity and with a proportional response on RyR open probability and calcium flux.

The question of how $[Ca]_{SRT}$ affects SR calcium release is an important one. Pathologically, a reduction in J_{pump} would lead to decreased $[Ca]_{SRT}$ and $\int J_{rel}$ during cardiac hypertrophy or failure (Arai et al., 1994; Pieske et al., 1998). The nonlinearity in fractional SR calcium release means that even a moderate reduction in $[Ca]_{SRT}$ could disproportionately depress SR calcium release and contractility.

However, SR calcium overload may lead to spontaneous SR calcium release because the ryanodine receptors become more sensitive to $[Ca]_i$ and release calcium at a near-resting $[Ca]_i$ stimulus. For instance, calcium spark frequency is known to increase with load (Satoh et al., 1997) and spontaneous SR calcium release in rabbit ventricular myocytes takes place at SR calcium loads of $\geq$110 μmol/l cytosol (Pogwizd et al., 1998).

Pharmacologically, many drugs such as sodium pump inhibitors, catecholamines, and PDE inhibitors act via sodium-calcium exchange or the SR calcium pump to increase $[Ca]_{SRT}$ and indirectly increase SR calcium release. Determination of the mechanism by which $[Ca]_{SRT}$ increases release may aid rational design of more specific inotropic agents that directly increase EC coupling.

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