Quantitative Assessment of the SR Ca^{2+} Leak-Load Relationship

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Abstract—Increased diastolic SR Ca^{2+} leak (J_{leak}) could depress contractility in heart failure, but there are conflicting reports regarding the J_{leak} magnitude even in normal, intact myocytes. We have developed a novel approach to measure SR Ca^{2+} leak in intact, isolated ventricular myocytes. After stimulation, myocytes were exposed to 0 Na$^+$, 0 Ca^{2+} solution ± 1 mmol/L tetracaine (to block resting leak). Total cell [Ca^{2+}] does not change under these conditions with Na$^+$-Ca^{2+} exchange inhibited. Resting [Ca^{2+}]$_i$ declined 25% after tetracaine addition (126$\pm$6 versus 94$\pm$6 nmol/L; $P<0.05$). At the same time, SR [Ca^{2+}] ([Ca^{2+}]$_{SRT}$) increased 20% (93$\pm$8 versus 108$\pm$6 μmol/L). From this Ca^{2+} shift, we calculate J_{leak} to be 12 μmol/L per second or 30% of the SR diastolic efflux. The remaining 70% is SR pump unidirectional reverse flux (backflux). The sum of these Ca^{2+} effluxes is counterbalanced by unidirectional forward Ca^{2+} pump flux. J_{leak} also increased nonlinearly with [Ca^{2+}]$_{SRT}$ with a steeper increase at higher load. We conclude that J_{leak} is 4 to 15 μmol/L cytosol per second at physiological [Ca^{2+}]$_{SRT}$. The data suggest that the leak is steeply [Ca^{2+}]$_{SRT}$-dependent, perhaps because of increased [Ca^{2+}]$_i$ sensitivity of the ryanodine receptor at higher [Ca^{2+}]$_{SRT}$. Key factors that determine [Ca^{2+}]$_{SRT}$ in intact ventricular myocytes include (1) the thermodynamically limited Ca^{2+} gradient that the SR can develop (which depends on forward flux and backflux through the SR Ca^{2+} ATPase) and (2) diastolic SR Ca^{2+} leak (ryanodine receptor mediated). (*Circ Res*. 2002;91:594-600.)

Key Words: Ca^{2+} cycling ■ membrane transport ■ sarcoplasmic reticulum ■ ryanodine receptors ■ excitation-contraction coupling

M uch is known about Ca^{2+}-induced Ca^{2+} release (CICR) and sarcoplasmic reticulum (SR) Ca^{2+} uptake in cardiac myocytes. SR Ca^{2+}-pump function is usually described as a unidirectional uptake mechanism (J_{pumpF}) and this can adequately describe Ca^{2+} transport during twitch relaxation and [Ca^{2+}]$_i$ decline.[1,2] Much less is known about how diastolic SR Ca^{2+} fluxes, especially SR Ca^{2+} leak flux (J_{leak}) and SR Ca^{2+} pump backflux (J_{pumpR}), alter excitation-contraction coupling (ECC). These important fluxes directly affect the SR Ca^{2+} content ([Ca^{2+}]$_{SRT}$), and free intra-SR [Ca^{2+}] ([Ca^{2+}]$_{SR}$) and can thus powerfully modulate ECC. Notably, the fractional SR Ca^{2+} release depends very steeply on [Ca^{2+}]$_{SRT}$, such that small changes in [Ca^{2+}]$_{SRT}$ can cause large changes in SR Ca^{2+} release during ECC.[3,4]

In the present study, we characterize diastolic SR Ca^{2+} fluxes. During diastole, [Ca^{2+}]$_i$ declines to low levels as Ca^{2+} is transported into the SR by J_{pumpF} and out of the cell (mainly via Na$^+$-Ca^{2+} exchange).[5] Thus, [Ca^{2+}]$_{SR}$ rises as [Ca^{2+}]$_i$ declines (Figure 1A). This rise increases both passive J_{leak} and Ca^{2+} pump–mediated J_{pumpR}. Two schools of thought describe diastolic SR Ca^{2+} flux as either (1) a strict forward pump versus leak balance ($J_{pumpF}=J_{leak}$ without appreciable J_{pumpR}, Figure 1B)[1,2,6] or (2) approaching a thermodynamic equilibrium where $J_{pumpF}=J_{pumpR}$ (with little J_{leak}, Figure 1C).[7–10] In both cases, the systolic rise in [Ca^{2+}]$_i$ stimulates net uptake of Ca^{2+} by the SR, which raises [Ca^{2+}]$_{SR}$. In the first scenario, this rise in [Ca^{2+}]$_{SR}$ causes J_{leak} to rise until it equals J_{pumpF} (which falls as [Ca^{2+}]$_i$ declines but is still 10% to 20% of V$_{max}$ at diastolic [Ca^{2+}]$_i$). In this case, J_{pumpR} is not considered and several ECC models or analyses have assumed a large SR Ca^{2+} leak (20 μmol/L cytosol per second).[1,2] Flux balance in this scenario requires this large resting J_{leak} and a large ATP consumption to simply retain constant diastolic SR Ca^{2+}.

The second scenario (Figure 1C) is based on measurements showing very low SR Ca^{2+} leak rates in intact myocytes[7] and the appreciation that the SR Ca^{2+} pump is reversible (it can actually completely reverse and make ATP).[11,12] The pump can generate a [Ca^{2+}] gradient where [Ca^{2+}]$_{SR}$/[Ca^{2+}]$_i$ can approach a thermodynamic limit of 7000.[8,10] In this case (Figure 1C), most of the diastolic J_{pumpF} is balanced by J_{pumpR}, which rises with [Ca^{2+}]$_{SR}$ during [Ca^{2+}]$_i$ decline (with little J_{leak} required).[8–12] Note that J_{pumpR} cannot exceed J_{pumpF} in an intact cell, but as it approaches J_{pumpF}, it could create a steady-state balance where there is almost no net pump flux (J_{pump}), just enough to be counterbalanced by a very small J_{leak}. This would require very little ATP consumption to retain diastolic [Ca^{2+}]$_{SRT}$ but would make [Ca^{2+}]$_{SRT}$ quite sensitive to energetic state (eg, ΔG_{ATP}).
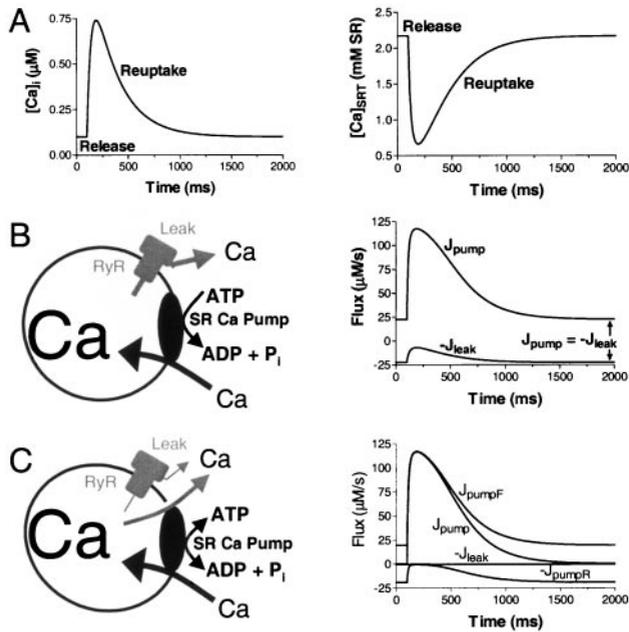
Figure 1. A, Theoretical curves describing $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SRT}$ during a typical contraction. As $Ca^{2+}$ is taken up, a $[Ca^{2+}]$ gradient builds across the SR membrane enhancing SR Ca^{2+} efflux, either by passive leak through spontaneous RyR openings (J_{leak}) or by a unidirectional reverse flux through the SR Ca^{2+} pump (J_{pumpR}). B, Theoretical profile of a pure pump-leak balance. With release, leak declines and the pump rate rises due to the fall of $[Ca^{2+}]_{SR}$ and the rise of $[Ca^{2+}]_i$, respectively. As SR uptake progresses, J_{leak} rises and J_{pump} (ie, J_{pumpF} with little or no J_{pumpR} present) falls until the two are equal at steady state. C, Flux profile of an alternative hypothesis where backflux accounts for a high percentage of SR Ca^{2+} efflux at steady state. In this case, $J_{pumpF}=J_{pumpR}+J_{leak}$, where J_{leak} is small.

The actual value of J_{leak} in intact ventricular myocytes is controversial and estimates have ranged from 0.3 μmol/L cytosol per second[7,9] to 5 to 30 μmol/L cytosol per second.[13] Models that do not consider J_{pumpR} require $J_{leak}\approx20$ μmol/L cytosol per second to compensate for diastolic J_{pumpF}.[1,2] Some of our data have more strongly favored values at the lower end of this J_{leak} range.[7–9] However, blocking J_{leak} with tetracaine can substantially raise SR Ca^{2+} content,[6,14,15] an effect that would be most consistent with relatively large J_{leak}. One factor that makes this an especially important issue is that if J_{leak} limits SR Ca^{2+} load, then it is energetically costly. It has also been suggested that during heart failure an increase in diastolic SR Ca^{2+} leak might be responsible (in part) for reducing SR Ca^{2+} content, thereby playing an important direct role in depressed cardiac contractility in heart failure.[16]

In the present study, we use a novel quantitative approach to directly address the roles of J_{pumpF}, J_{pumpR}, and J_{leak} in determining SR Ca^{2+} content in intact resting rabbit ventricular myocytes. Moreover, we examine the relative roles of J_{pumpR} and J_{leak} as a function of SR Ca^{2+} load itself. On block of J_{leak} by tetracaine, we measure both the fall in $[Ca^{2+}]_i$ and the complementary rise in $[Ca^{2+}]_{SRT}$ under conditions where transsarcolemmal Ca^{2+} fluxes are prevented. We find that J_{leak} depends very steeply on $[Ca^{2+}]_{SR}$, and this may explain some of the discrepancies in this field.
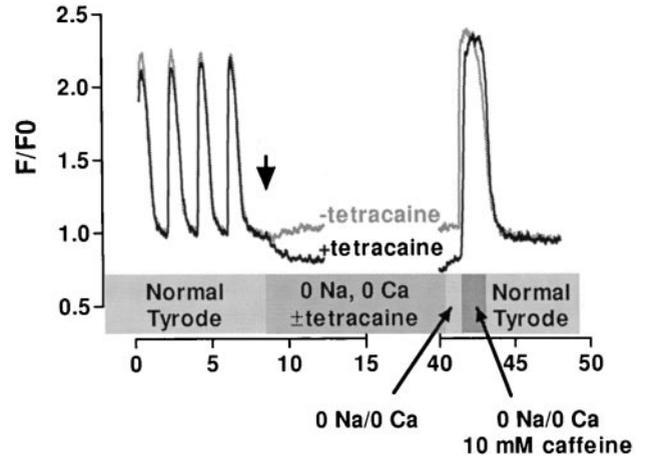
Figure 2. Protocol used to measure J_{leak}. The SR is loaded by pacing at 0.5 Hz, then solution is rapidly switched to 0 Na^+, 0 Ca^{2+} NT. After at least 30 seconds, $[Ca^{2+}]_{SRT}$ is measured with 10 mmol/L caffeine. Inclusion of 1 mmol/L tetracaine in the 0 Na^+, 0 Ca^{2+} NT causes steady-state $[Ca^{2+}]_i$ to drop and $[Ca^{2+}]_{SRT}$ to rise (ie, a shift of Ca^{2+} from cytosol to SR). Peaks of caffeine transients were the same indicating that total cellular $[Ca^{2+}]$ was the same in both cases.

Materials and Methods
All chemicals were from Sigma Chemical Co, except as indicated. 0 Na^+, 0 Ca^{2+} normal tyrode (NT) had Li^+ substituted for Na^+ and 10 mmol/L EGTA with no added Ca^{2+}. A more detailed description of the solutions and protocols can be found in the online data supplement (see http://www.circresaha.org).

Experimental Protocol
Experiments were conducted in accordance with the Guide for the Use of Experimental Animals at Loyola University Medical Center and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the NIH (publication No. 85-23, revised 1985). New Zealand White rabbit (Myrtle's Rabbitry, Inc, Thompson Station, Tenn) ventricular myocytes were isolated as described previously.[17] All experiments were performed at room temperature using the general protocol shown in Figure 2. Resting $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SRT}$ were measured using fluo-3 fluorescence in isolated myocytes in the presence and absence of J_{leak}. Cells were stimulated at least 20 times at 0.5 Hz or the indicated frequency in 2 mmol/L Ca^{2+} normal Tyrode to bring the cellular Ca^{2+} content to steady state. After the last pulse, the superfusate was rapidly switched to 0 Na^+, 0 Ca^{2+} NT $\pm$1 mmol/L tetracaine. Na^+-Ca^{2+} exchange, the primary Ca^{2+} influx and efflux mechanism at rest, was therefore blocked so that little or no Ca^{2+} entered or left the resting cell. In the control condition, $[Ca^{2+}]_i$ was monitored while 0 Na^+, 0 Ca^{2+} solution without tetracaine was perfused for a minimum of 30 seconds, then 10 mmol/L caffeine was added to cause SR Ca^{2+} release. The difference between the basal and peak total cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_T$) in the presence of caffeine is therefore $[Ca^{2+}]_{SRT}$.

In the test condition, 0 Na^+, 0 Ca^{2+} NT was perfused with tetracaine added. Under this condition, J_{leak} is blocked and the measured changes in the steady-state condition were used to determine the magnitude of the three relevant SR fluxes: J_{pumpF}, J_{pumpR}, and J_{leak}.

Calculation of Cellular SR Fluxes
SR Ca^{2+} fluxes were calculated as described in the online methods. Briefly, at steady state, the leak rate is equal to the net influx through the SR Ca^{2+} pump:

$$(1) \qquad J_{leak}=J_{pump}=J_{pumpF}-J_{pumpR}$$
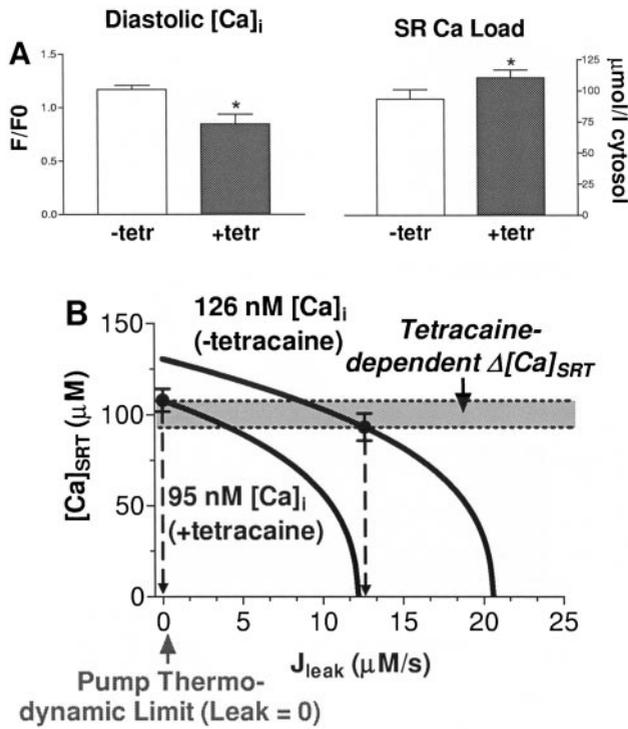
Figure 3. A, Tetracaine-dependent decrease in $[Ca^{2+}]_i$ and increase in $[Ca^{2+}]_{SRT}$ ($P<0.05$; t test). B, $[Ca^{2+}]_{SRT}$ vs J_{leak} relationship (Equations 3 and 4) before and after the shift of Ca^{2+} from cytosol to SR with 1 mmol/L tetracaine. Each curve represents the theoretical $[Ca^{2+}]_{SRT}$ vs J_{leak} relationship from the averaged data without tetracaine (upper curve) and with tetracaine. The arrows represent J_{leak} at those values (ie, 0 μmol/L cytosol per second with tetracaine and 12 μmol/L cytosol per second without tetracaine).

We calculated or inferred relevant SR Ca^{2+} pump parameters when the leak was 0 in the presence of tetracaine. At this point, $J_{pumpF}=J_{pumpR}$ (ie, $J_{pump}=0$). We then applied these parameters to the situation where leak was unblocked to calculate the net influx through the SR Ca^{2+} pump and therefore the net efflux through passive SR Ca^{2+} leak, which balances it.

Results

J_{leak} Determination Under Steady-State Conditions

Isolated rabbit myocytes were stimulated to steady state at 0.5 Hz, and $[Ca^{2+}]_i$ was monitored in 0 Na^+, 0 Ca^{2+} NT with and without tetracaine for at least 30 seconds, followed by caffeine application to measure $[Ca^{2+}]_{SRT}$. When we performed this protocol, resting $[Ca^{2+}]_i$ declined to 75% of control when tetracaine was added (126±6 versus 94±6 nmol/L, $P<0.05$; Figures 2 and 3A). Therefore, $[Ca^{2+}]_T$ declined. The peak of the subsequent caffeine transient, however, remained the same, indicating that the sum of the $[Ca^{2+}]_T$ and $[Ca^{2+}]_{SRT}$ was unchanged with and without tetracaine, and that there was no differential transport of Ca^{2+} into the mitochondria or out of the cell (eg, through the sarcolemmal Ca^{2+} pump). These results are in agreement with Bassani and Bers,[18] where no loss of SR $[Ca^{2+}]$ is observed after up to 5 minutes in 0 Na^+, 0 Ca^{2+} solution in rabbit or rat ventricular myocytes. Since the peak $[Ca^{2+}]_i$ in caffeine was the same but the baseline $[Ca^{2+}]_i$ was decreased, $[Ca^{2+}]_{SRT}$ was increased with tetracaine to 120% of control (93±8

versus 108±6 μmol/L, Figure 3A). This indicates that there is a shift of Ca^{2+} from the cytosol to the SR. The data clearly show that J_{leak} is large enough to have a measurable effect on the diastolic $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SRT}$ in the isolated intact myocyte, confirming results from other groups.[6,14,15]

This Ca^{2+} shift from the cytosol to the SR is the natural result of blocking the ryanodine receptor (RyR) (ie, J_{leak}), which causes the SR Ca^{2+} influx (J_{pumpF}) to become greater than the efflux (now only J_{pumpR}). Ca^{2+} is therefore transported from the cytosol to the SR causing $[Ca^{2+}]_i$, and therefore J_{pumpF}, to decline and at the same time, $[Ca^{2+}]_{SR}$, and therefore J_{pumpR}, to rise until the net SR Ca^{2+} flux (J_{SR}) is once again zero and steady state is achieved. The greater the magnitude of J_{leak} in the absence of tetracaine, the greater the shift of Ca^{2+} with tetracaine and the greater the differences in both $[Ca^{2+}]_i$ and $[Ca^{2+}]_{SRT}$.

We have previously shown[8] that resting SR Ca^{2+} fluxes can be described in the following manner:

$$(2) \qquad J_{SR}=J_{pumpF}-J_{pumpR}-J_{leak}$$

$$(3) \qquad J_{SR}=\frac{V_{max}([Ca^{2+}]_i/K_{mf})^H-V_{max}([Ca^{2+}]_{SR}/K_{mr})^H}{1+([Ca^{2+}]_i/K_{mf})^H+([Ca^{2+}]_{SR}/K_{mr})^H}$$
$$-k_{leak}([Ca^{2+}]_{SR}-[Ca^{2+}]_i)$$

$$(4) \qquad [Ca^{2+}]_{SRT}=[Ca^{2+}]_{SR}+\frac{B_{max-SR}[Ca^{2+}]_{SR}}{[Ca^{2+}]_{SR}+K_{d-SR}}$$

where B_{max-SR} and K_{d-SR} are the usual Michaelis parameters for intra-SR Ca^{2+} binding, K_{mf} and K_{mr} are the K_m values for forward and reverse unidirectional fluxes through the pump, respectively, k_{leak} is the rate constant for J_{leak}, V_{max} and H are the maximum Ca^{2+}-pump influx (and backflux) rate and the Hill coefficient, respectively. Values for the relevant parameters are in a table in the online data supplement.

The curves in Figure 3B show the dependence of $[Ca^{2+}]_{SRT}$ on J_{leak} (using Equations 3 and 4) at the two mean steady-state $[Ca^{2+}]_i$ values measured with and without tetracaine. The lower curve ($[Ca^{2+}]_i=95$ nmol/L) is with tetracaine where $J_{leak}=0$. Therefore the numerator in Equation 3 must be zero since $J_{SR}=0$ at steady state. In the following case:

$$(5) \qquad [Ca^{2+}]_{SR}/[Ca^{2+}]_i=K_{mr}/K_{mf}=7000$$

at this, the thermodynamically limiting $[Ca^{2+}]$ gradient.[10]

Thus, when $J_{leak}=0$ at steady state, $[Ca^{2+}]_{SRT}$ depends directly on $[Ca^{2+}]_i$ in a highly predictable manner (Equations 4 and 5) where J_{pumpF} must equal J_{pumpR}. As J_{leak} increases in the absence of tetracaine, the $[Ca^{2+}]_{SRT}$ declines and $[Ca^{2+}]_i$ rises. The upper curve is for $[Ca^{2+}]_i=126$ nmol/L and the measured $[Ca^{2+}]_{SRT}$ is 86% of the value at $J_{leak}=0$. From this, we infer that $J_{leak}=12$ μmol/L per second in the absence of tetracaine.

Using this analysis, we determine J_{leak} to be 12 μmol/L per second or 30% of the total diastolic efflux from the SR. The remaining 70% is accounted for by J_{pumpR}.

J_{leak} as a Function of $[Ca^{2+}]_{SRT}$

The data above account for leak under only one loading condition (pacing at 0.5 Hz). However, Ca^{2+} spark frequency increases with SR Ca^{2+} load and since the gain of ECC and fractional release both increase as steep, nonlinear functions of $[Ca^{2+}]_{SRT}$, it might be expected that diastolic release (ie, J_{leak}) will vary substantially with
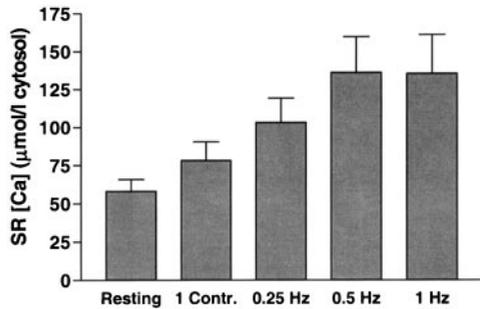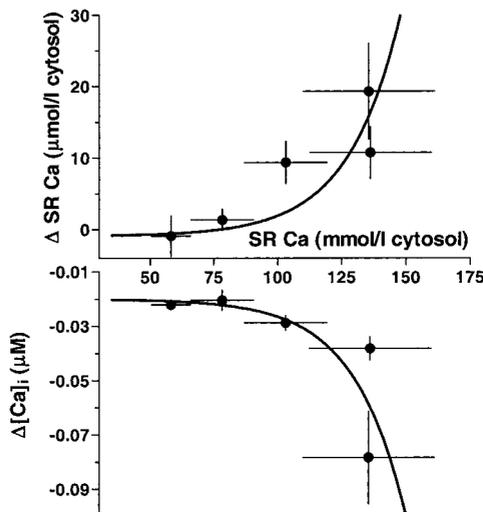
Figure 4. $[Ca^{2+}]_{SRT}$ without tetracaine at each loading protocol. The lowest SR Ca²⁺ load was achieved in myocytes allowed to load at rest. The next was achieved by a single contraction. Higher loads were achieved at higher pacing frequencies.

total SR $[Ca^{2+}]$. We therefore tested the hypothesis that there is a higher relative J_{leak} at higher $[Ca^{2+}]_{SRT}$.

The same general protocol described above was used (Figure 2). $[Ca^{2+}]_{SRT}$ was altered by varying the protocol before the switch to 0 Na⁺, 0 Ca²⁺ solution ±1 mmol/L tetracaine. The SR was emptied of Ca²⁺ in all cases by addition of caffeine in 0 Ca²⁺ NT (allowing Na⁺-Ca²⁺ exchange to extrude the SR Ca²⁺). When the SR was allowed to load passively in NT for 30 seconds to 5 minutes, $[Ca^{2+}]_{SRT}$ (without tetracaine) stayed relatively low (Figure 4). Stimulation once during reloading gave a slightly higher $[Ca^{2+}]_{SRT}$ and pacing at increasing frequencies gave progressively higher loads above this point.

There is a distinct advantage to using this protocol to determine the load dependence of J_{leak}. Obviously, we expect J_{leak} to rise at least to some extent as $[Ca^{2+}]_{SRT}$ goes up. This is simply because the amount of Ca²⁺ available for leak is increased (ie, the SR Ca²⁺ gradient, and therefore the driving force, is increased). However, the leak may also increase because $[Ca^{2+}]_{SR}$ (and therefore $[Ca^{2+}]_{SRT}$) affects the leak


Figure 5. Changes in $[Ca^{2+}]_{SRT}$ and $[Ca^{2+}]_i$ with tetracaine addition. When tetracaine is included in the protocol, $[Ca^{2+}]_i$ decreases and $[Ca^{2+}]_{SRT}$ increases. The magnitude of these changes increases as $[Ca^{2+}]_{SRT}$ (without tetracaine) increases. This suggests that J_{leak} increases as $[Ca^{2+}]_{SRT}$ without tetracaine increases. Lines through data are modified exponentials.
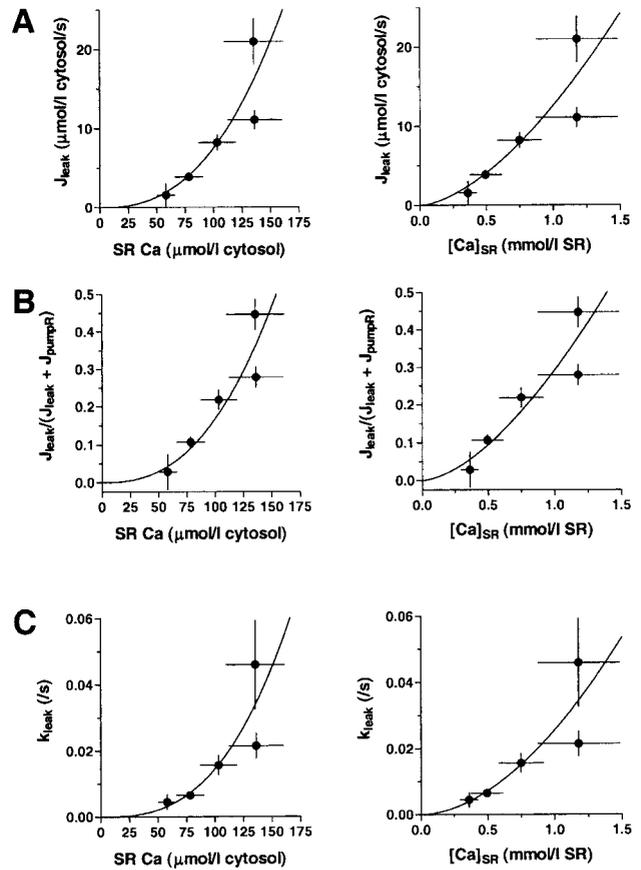
Figure 6. J_{leak} increases as $[Ca^{2+}]_{SRT}$ and free SR $[Ca^{2+}]$ ($[Ca^{2+}]_{SR}$) increase. Lines through the data are modified exponentials. A, J_{leak} is calculated from data in Figure 5 as described in the online data supplement. B, J_{leak} is expressed as a fraction of total SR Ca²⁺ efflux ($J_{pumpR}+J_{leak}$). This makes the determination less dependent on our value for V_{max}. C, The leak rate constant, k_{leak}, varies with both $[Ca^{2+}]_{SRT}$ and $[Ca^{2+}]_{SR}$ indicating that the leak rate constant depends on $[Ca^{2+}]_{SR}$ (ie, that $[Ca^{2+}]_{SR}$ has an effect on the release process itself).

process itself. Increased $[Ca^{2+}]_{SR}$ may increase the P_o of the RyR as shown in bilayers.[19,20] The key is that any increase in J_{leak} strictly due to an increase in the SR Ca²⁺ gradient will be linearly related to $[Ca^{2+}]_{SR}$. However, this relationship may be highly nonlinear if there is an effect on RyR gating. This technique, therefore, discriminates between J_{leak} changes due to an increase in SR Ca²⁺ gradient and those due to altered leak gating (ie, an effect on the leak rate constant, k_{leak}, Equation 3).

We measured J_{leak} as in Figure 2 at different SR Ca²⁺ loads. As $[Ca^{2+}]_{SRT}$ (without tetracaine) rose, the application of tetracaine caused larger decreases in resting $[Ca^{2+}]_i$ and, at the same time, larger increases in $[Ca^{2+}]_{SRT}$ (Figure 5). Therefore, more Ca²⁺ shifted from the cytosol to the SR when tetracaine was included in the protocol (Figure 5). Since this shift rises with J_{leak}, the data show that J_{leak} does, indeed, rise with $[Ca^{2+}]_{SRT}$, even without further analysis. When the measurements are analyzed quantitatively (Figure 6), it can be seen that J_{leak} rises in a highly nonlinear manner with a particularly steep increase at higher loads (still within the physiological range, Figure 6A). J_{leak}, therefore, is between 4 and 15 µmol/L
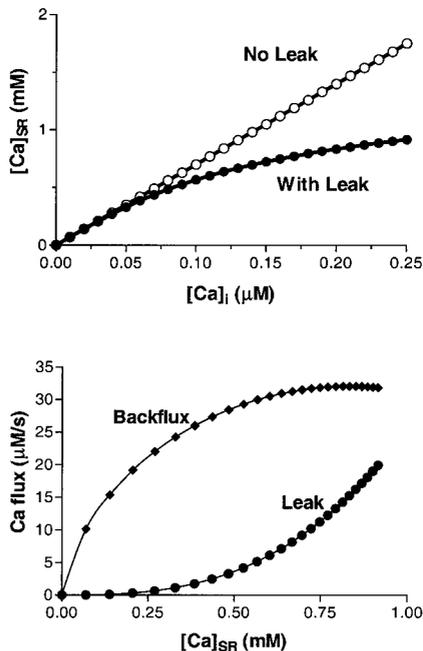
Figure 7. A, $[Ca^{2+}]_{SR}$ in relation to $[Ca^{2+}]_i$ both with and without tetracaine. $[Ca^{2+}]_{SR}$ with tetracaine varies linearly with $[Ca^{2+}]_i$ (slope=7000, SR thermodynamic limit). $[Ca^{2+}]_{SR}$ without tetracaine is close to this line at low $[Ca^{2+}]_i$ but falls away from this thermodynamic limit at higher values as J_{leak} increases (ie, as k_{leak} increases with $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_i$). B, J_{pumpR} (backflux) and J_{leak} (leak) depend on $[Ca^{2+}]_{SR}$. Curves are based on Equation 3 and k_{leak} as in Figure 6C.

cytosol per second, depending on the $[Ca^{2+}]_{SRT}$ (and $[Ca^{2+}]_{SR}$), within physiological limits. Figure 6B represents the leak as a fraction of the total SR Ca^{2+} efflux (ie, in a manner that is independent of the V_{max}, which we chose for the SR Ca^{2+} pump). Note that even when $[Ca^{2+}]_{SRT}$ is high, J_{leak} is still only approaching the level of J_{pumpR}.

The highly nonlinear nature of the relationship between J_{leak} and $[Ca^{2+}]_{SR}$ (Figures 6A and 6B) indicates that J_{leak} is increasing not just because the SR free $[Ca^{2+}]$ gradient increases but also because the P_o of the RyR increases as well. This is further demonstrated by the observed rise in k_{leak} as a function of $[Ca^{2+}]_{SRT}$ and $[Ca^{2+}]_{SR}$ (Figure 6C), as this parameter is entirely gradient independent and would otherwise be constant (Equation 3).

Discussion

Experimental Technique

We have described a new quantitative technique to assess J_{leak} in intact isolated myocytes. Even without the detailed analysis, the technique has the advantage that all of the results are paired and J_{leak} is directly related to the amount of Ca^{2+} shift from cytosol to SR.

We also determined that the sarcolemmal Ca^{2+} pump and mitochondrial Ca^{2+} uptake do not remove significant amounts of Ca^{2+} from the cytosol during the holding period in 0 Na$^+$, 0 Ca^{2+} NT (where Na$^+$-Ca^{2+} exchange is inhibited, as previously shown[18]). This interpretation is explained by 2 factors: (1) These mechanisms are known to be extremely slow relative to Na$^+$-Ca^{2+} exchange, the major efflux path-

way,[2] and (2) The peaks of the caffeine transients with and without tetracaine are the same.

This last point deserves further explanation. The difference in $[Ca^{2+}]_{SRT}$ with and without tetracaine results from a decrease in baseline $[Ca^{2+}]_i$, not a difference in peak $[Ca^{2+}]_i$ with caffeine. The caffeine peaks are the same because, regardless of whether tetracaine is present in the 0 Na$^+$, 0 Ca^{2+} NT solution, the total cellular Ca^{2+} has not changed during the holding period. Any net transport into a compartment other than the cytosol or the SR (eg, extracellular) should change the caffeine transient peak. This was not observed.

Magnitude of the SR Ca^{2+} Leak

The measured J_{leak} is RyR-dependent. The only source of diastolic Ca^{2+} flux from the SR (other than backflux) that has been directly observed in cardiac myocytes is from the RyR, typically in the form of Ca^{2+} sparks. These elementary units of SR Ca^{2+} release are observed by confocal microscopy using a Ca^{2+}-sensitive fluorescent dye. It is possible that flux through the RyR at rest can explain nearly all of the J_{leak}. A resting J_{leak} of ≈ 10 μmol/L cytosol per second requires about 50 Ca^{2+} sparks per second in the cell (or ≈ 2 sparks/pL per second). This is a typical resting Ca^{2+} spark frequency in ventricular myocytes[21] and depending on the spark current (3 to 20 pA[22,23]) could explain the entire resting J_{leak} of Ca^{2+} from the SR. In addition, direct measurement of SR Ca^{2+} leak rate in permeabilized myocytes in the presence of ruthenium red (to block RyR) and thapsigargin (to block the SR Ca^{2+} pump) was insignificant compared with other fluxes.[24] This indicates that any non-RyR–mediated leak is insignificant. Also consistent with this hypothesis, $[Ca^{2+}]_{SR}$ within SR vesicles approaches the thermodynamic limit of the SR Ca^{2+} pump when RyRs are blocked by ruthenium red.[10]

The Controversy

Determining J_{leak} and its SR Ca^{2+} load dependence in intact cardiac myocytes was a major goal of this study. Results from previous attempts to measure J_{leak} directly in isolated myocytes are mixed. In one example, Bassani and Bers[7] determined J_{leak} by measuring the rate of SR Ca^{2+} loss with the SR Ca^{2+} pump completely blocked. $[Ca^{2+}]_{SRT}$ decreased with a τ of 385 seconds, giving an initial J_{leak} of 0.3 μmol/L cytosol per second at $[Ca^{2+}]_{SRT} \approx 100$ μmol/L cytosol (in both rat and rabbit). This is a very low value. Because $J_{pumpF} \approx 25$ μmol/L cytosol per second at resting $[Ca^{2+}]_i$ must equal $J_{leak} + J_{pumpR}$ at steady state, this implies that J_{pumpR} is nearly equal J_{pumpF} at rest.

Such a low J_{leak} would hardly affect diastolic $[Ca^{2+}]_{SRT}$. Ginsburg et al[9] tested this hypothesis by measuring maximal steady-state $[Ca^{2+}]_{SRT}$ in rabbit cardiac myocytes under voltage clamp with SR pump stimulation or inhibition. If $[Ca^{2+}]_{SRT}$ is limited by J_{leak}, it should be sensitive to these maneuvers. Slowing the pump with thapsigargin or accelerating it with isoproterenol resulted in nearly the same maximum $[Ca^{2+}]_{SRT}$ as control, implying that the pump operates close to its thermodynamic limit against a small leak.

However, not all results agree with this interpretation. For instance, two groups[6,14,15] found that tetracaine block of J_{leak}

in rat ventricular myocytes nearly doubled $[Ca^{2+}]_{SRT}$, an unexpected effect if the leak were very small. These data conflict with the conclusions above[9] and imply that altering J_{leak} in cardiac ventricular cells may affect ECC through alteration of $[Ca^{2+}]_{SRT}$.

We used a variation on the tetracaine technique above with Na^+-Ca^{2+} exchange blocked (Figure 2). Thus, the cell effectively becomes a closed system preventing significant sarcolemmal Ca^{2+} flux. This has at least 2 effects: (1) it makes more quantitative measurements possible (a critical improvement) and (2) it prevents SR Ca^{2+} loading or loss that can occur through Na^+-Ca^{2+} exchange. The data clearly show that J_{leak} has measurable effects on $[Ca^{2+}]_{SRT}$ within ventricular myocytes. The magnitude of J_{leak} after pacing at 0.5 Hz was 12 μmol/L per second or $\approx$30% of the total efflux rate. The rest is J_{pumpR}, emphasizing the significance of this flux as well.

Load Dependence of the Leak: The Resolution?

The $[Ca^{2+}]_{SRT}$ dependence of J_{leak} also complicates accurate leak measurement. This was emphasized by recent work using permeabilized myocytes where resting $[Ca^{2+}]_i$ was clamped.[13,25] Ca^{2+} spark frequency increased as $[Ca^{2+}]_{SRT}$ (and $[Ca^{2+}]_{SR}$) rose at constant $[Ca^{2+}]_i$ (making it clear that $[Ca^{2+}]_{SR}$ alters diastolic RyR gating in situ). Quantitatively, this measurement is complicated by uncertainty about the flux though a single spark (3 to 20 pA)[22,23] and because Ca^{2+} spark frequency is often much higher in permeabilized myocytes.[25]

In this study, we measured the $[Ca^{2+}]_{SRT}$ dependence of J_{leak} in intact, unpermeabilized myocytes, thus avoiding the dialysis of potentially important proteins and other substances from the cytosol. We hypothesized that the leak would rise in a nonlinear fashion with increasing steepness at higher $[Ca^{2+}]_{SR}$. The basis for this hypothesis is related to our ECC study[3] of the relationship between fractional release versus $[Ca^{2+}]_{SRT}$ and $[Ca^{2+}]_{SR}$. We found that both ECC and fractional SR Ca^{2+} release increased sharply as a function of either $[Ca^{2+}]_{SRT}$ or $[Ca^{2+}]_{SR}$ especially at high $[Ca^{2+}]_{SRT}$. Because the increased fractional release is most likely because of enhanced $[Ca^{2+}]_i$ sensitivity of the RyR at high $[Ca^{2+}]_{SR}$,[19,20] we expected a similar relationship between SR $[Ca^{2+}]$ and resting J_{leak}.

Our measurement of J_{leak} as a function of $[Ca^{2+}]_{SRT}$ was consistent with the ECC data. J_{leak} rose in a highly nonlinear manner with a particularly steep increase at higher loads (Figure 6). The magnitude was between 4 and 15 μmol/L cytosol per second, depending on $[Ca^{2+}]_{SRT}$, within the physiological range.

This relationship presents a possible resolution to the apparent dichotomous results of J_{leak} large enough to limit $[Ca^{2+}]_{SRT}$[6,13] and those of a low leak, which has little effect on $[Ca^{2+}]_{SRT}$.[7,9] High J_{leak} may occur at higher $[Ca^{2+}]_{SRT}$ and the $[Ca^{2+}]_{SRT}$ will be more sensitive to J_{leak} at these loads. Note that higher $[Ca^{2+}]_{SRT}$ does not mean overloaded (eg, causing Ca^{2+} waves or spontaneous contractions), and the leak may have a significant effect on normal diastolic $[Ca^{2+}]_{SRT}$ and be manifest as increased spark frequency.

Physiological and Pathophysiological Significance

The mere fact that SR Ca^{2+} load increases when J_{leak} is blocked provides valuable information about how diastolic fluxes control $[Ca^{2+}]_{SRT}$ and therefore ECC. If the SR Ca^{2+} load is determined to a significant extent by J_{leak} and, at the same time, J_{leak} is determined by $[Ca^{2+}]_{SRT}$, then $[Ca^{2+}]_{SRT}$ is, in effect, inherently self-limiting. Figure 7A demonstrates this by showing the relationship between $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_i \pm J_{leak}$. At higher $[Ca^{2+}]_i$, the higher J_{leak} prevents the large increases in $[Ca^{2+}]_{SR}$, which can be seen with J_{leak} blocked. Figure 7B shows how both J_{leak} and J_{pumpR} depend on $[Ca^{2+}]_{SR}$ in Figure 7A. Consider the relationship as a simple cellular feedback loop. As $[Ca^{2+}]_{SRT}$ increases, J_{leak} increases. As J_{leak} increases, $[Ca^{2+}]_{SRT}$ decreases. What this means functionally is that one may approach the thermodynamic $[Ca^{2+}]_{SR}/[Ca^{2+}]_i$ limit when $[Ca^{2+}]_{SRT}$ is low to moderate. However, at high $[Ca^{2+}]_{SRT}$, there is an inherent maximal $[Ca^{2+}]_{SRT}$ below the thermodynamic limit (note apparent maximal $[Ca^{2+}]_{SRT}$ in Figures 5 and 6). Anything that shifts this relationship could change the SR Ca^{2+} load for a given diastolic $[Ca^{2+}]_i$.

Marx et al[16] suggested that a high persistent diastolic SR Ca^{2+} leak may exist in heart failure myocytes. A high J_{leak} combined with increased Na^+-Ca^{2+} exchange and reduced J_{pump}[26] could limit $[Ca^{2+}]_{SRT}$. The greater ATP consumption resulting from a high rate of pump-leak futile cycling could further exacerbate this effect. Reducing $[Ca^{2+}]_{SRT}$ could have severe effects on ECC and contraction in the failing heart.[3,4] Our data therefore suggest that if J_{leak} is increased in heart failure, it may reduce $[Ca^{2+}]_{SRT}$ and SR Ca^{2+} release during systole.[3,4]

Because J_{pumpR} also makes up >50% of the total SR Ca^{2+} efflux (Figure 6), it is important to emphasize that J_{pumpR} might also change under different conditions. In wild-type versus phospholamban (PLB) knockout mice, we found that PLB, in addition to decreasing the rate of SR Ca^{2+} uptake (ie, J_{pumpF}), also increased J_{pumpR}. This resulted in a decreased steady-state $[Ca^{2+}]_{SRT}$ and $[Ca^{2+}]_{SR}$.[24] Therefore, PLB phosphorylation may cause an increase in the efficiency of the SR Ca^{2+} pump and thus an increase in the thermodynamic limit of the pump, causing an increase in $[Ca^{2+}]_{SRT}$.

In summary, our studies show that the two major SR diastolic effluxes, J_{pumpR} and J_{leak}, both play a role in determining $[Ca^{2+}]_{SRT}$ (and $[Ca^{2+}]_{SR}$). Both J_{pumpR} and/or J_{leak} are potentially involved in a variety of cardiac disturbances including heart failure.[16,24,27] Moreover, decreasing either or both of these fluxes may enhance ECC,[24,27,28] probably by changing $[Ca^{2+}]_{SR}$, which has a powerful effect on ECC.[3,4] The proteins that mediate these fluxes may be good potential targets for pharmacological manipulation of the inotropic state, possibly for treatment of cardiac diseases.

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Circulation Research

Quantitative Assessment of the SR Ca^{2+} Leak-Load Relationship

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The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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Data were collected with PClamp (Axon Instruments, Foster City, CA). Further mathematical data manipulation was performed using Microsoft Excel (Microsoft Corporation, Seattle, WA). Smoothing of the data was performed with TableCurve (SPSS Software Products, San Rafael, CA).

Solutions

Normal tyrode (NT) solution was of a standard composition (in mM): 140 mM NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 glucose, and 5 HEPES, pH 7.4 with NaOH. 0 Na, 0 Ca NT had the same compostion with no added Ca, 10 EGTA, and 140 LiCl substituted for NaCl (pH 7.4 with LiOH).

Ca calibration

Cells were loaded with 10 μM fluo-3 for 40 minutes at room temperature followed by 30 minutes of de-esterification to obtain adequate signal for accurate measurement of resting fluorescence. Because of its high fluorescence efficiency, fluo-3 signals were less noisy than signals from other dyes such as indo-1. Fluo-3 therefore allowed better detection of small changes in [Ca]$_i$ at rest. Before each leak measurement cells were stimulated electrically at 0.5 Hz for at least 20 pulses. The diastolic fluorescence (F0) between beats at this point was collected and [Ca]$_i$ was assumed to be 0.12 μM ([Ca]$_d$). Fluorescence (F) during the subsequent protocol was calibrated by using a pseudoratio:

$$[Ca]_i = \frac{F/F0(K_{d(Ca)})}{((K_{d(Ca)}/[Ca]_d) - F/F0 + 1)} \tag{1}$$

where $K_{d(Ca)}$ is the affinity of the dye for Ca, 1.1 μM. By mesuring diastolic flourescence between beats in this manner before each run, we were able to account for drifts in fluorescence due to factors such as photobleaching of the dye over the course of the experiment. Fluo-3 was found to be quenched 5% by 1 mM tetracaine when added to a cuvette with stirring (data not shown). Fluorescence in the presence of tetracaine was therefore corrected for this artifact.

[Ca]$_i$ was converted to total cytosolic [Ca] ([Ca]$_T$) as follows:

$$[Ca]_T = \frac{B_{max}}{1 + \frac{K_d}{[Ca]_i}} + [Ca]_i \tag{2}$$

where B_{max} and K_d have thier usual meanings and are 272 μmol/l cytosol and 0.673 μM, respectively.[1] In addition a Bmax of 150 μmol/l cytosol and a K_d of 1.1 μM was used for binding to fluo-3.

Calculation of Cellular SR Fluxes

In 0 Na, 0 Ca NT, Ca flux across the SL is essentially 0. J_{pump} is the sum of J_{pumpF} and J_{pumpR}:

$$J_{pump} = J_{pumpF} - J_{pumpR} \tag{3}$$

J_{pump} is always in the forward direction and the SR Ca pump *never completely reverses under normal physiological circumstances*. In other words, it is never used to actually cause a net production of ATP.

J_{leak} along with J_{pumpR}, account for Ca efflux from the SR. As J_{pumpR} increases, J_{pump} slows until eventually it becomes equal and opposite to the passive leak of Ca back across the SR membrane. At this point the system is in steady-state:

$$J_{SR} = J_{pump} - J_{leak} \tag{4}$$

where J_{SR}, the net flux of Ca across the SR, is 0.

Further analysis makes this measurement more quantitative. The system behaves according to the following equation:

$$V = \frac{V_{max}\left(\frac{[Ca]_i}{K_{mf}}\right)^H - V_{max}\left(\frac{[Ca]_{SR}}{K_{mr}}\right)^H}{1 + \left(\frac{[Ca]_i}{K_{mf}}\right)^H + \left(\frac{[Ca]_{SR}}{K_{mr}}\right)^H} + k_{leak}([Ca]_{SR} - [Ca]_i) \tag{5}$$

where K_{mf} and K_{mr} are the K_m values for forward and reverse unidirectional fluxes through the pump, respectively, k_{leak} is the rate constant for J_{leak}, and V_{max} and H are the maximum influx (and efflux) rate and Hill coefficient, respectively.

This equation accounts for SR Ca pump activity (first term) as well as leak (second term). Given this relationship, the +tetracaine case can be examined where J_{leak} (second term) is 0 leaving only SR Ca pump activity to be determined. Known variables are $[Ca]_i$ and $[Ca]_{SRT}$. Under these conditions, the SR Ca gradient, $[Ca]_{SR}/[Ca]_i$, is 7000:1.[2] Therefore:

$$[Ca]_{SR} = [Ca]_i(7000) \tag{6}$$

Also note for later use that the Haldane equation can be applied so that:

$$K_{mr} = K_{mf}(7000) \tag{7}$$

Since:

$$[Ca \cdot L]_{SR} = [Ca]_{SRT} - [Ca]_{SR} \tag{8}$$

where $[Ca \cdot L]$ is SR Ca bound and since:

$$[Ca \cdot L]_{SR} = \frac{B_{max-SR}([Ca]_{SR})}{[Ca]_{SR} + K_{d-SR}} \tag{9}$$

B_{max-SR} can be calculated given $[Ca]_{SR}$ (Equation 6) and $[Ca]_{SRT}$ and a K_{d-SR} of 638 μM.[2]

Now the case without tetracaine can be examined. All terms of the equation, J_{pump} and J_{leak}, are non-zero. But all of the pump parameters which were determined for the case with tetracaine are taken to be the same as above. Therefore, the only unknown parameter left, k_{leak}, can be calculated and J_{leak} at steady-state determined:

$$k_{leak}([Ca]_{SR} - [Ca]_i) = \frac{V_{max}\left(\frac{[Ca]_i}{K_{mf}}\right) - V_{max}\frac{[Ca]_{SR}}{K_{mr}}}{1 + \frac{[Ca]_i}{K_{mf}} + \frac{[Ca]_{SR}}{K_{mr}}} \tag{10}$$

On the whole, this analysis builds upon our previous work.[3–5] The parameters which are taken from these reports provide quantitative value to our assessment (Table 1). Two of the values which we have used are taken from experiments in isolated membrane vesicles, the SR Ca gradient with no leak (7000:1, Equations 6 and 7), and the K_{d-SR} (638 μM, Equation 9, a value very close to the in vitro K_d of calsequestrin).[2] We have taken the opportunity to apply these values to the physiological environment of the intact isolated myocyte.

Parameter	Value
V_{max}	137 μmol/l cytosol/s
K_{mf}	0.260 μM
K_{mr}	1.8 mmol/l SR
H	0.75
B_{max-SR}	calculated
K_{d-SR}	0.630 mmol/l SR
SR volume	3% of cell volume
cytosolic volume	65% of cell volume

Table 1: Parameters used for passive SR Ca leak calculation at steady-state. Values are from Shannon and Bers, 1997 and Shannon, et al. 2000[2,3]

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