Section 1: Modification of the EGTA/phenol red method

This section gives details and justification for a modification of the EGTA/phenol red method as introduced previously with Fig. 3 in Pape et al. (2007). Fig. S1 shows results with fibers in which the pH indicator phenol red was the only dye present. The top trace shows the voltage measured in response to an 800-ms voltage-clamp pulse to $-30$ mV. Next (from top to bottom) are three absorbance change signals ($\Delta A$) measured at the same time as the voltage signal. As indicated, the signal with the smallest change is the raw (unprocessed) $\Delta A$ signal measured with the 690-nm filter, denoted as $\Delta A_{\text{raw}}$ (690). The signal with the largest amplitude is the corresponding signal measured at 570 nm, $\Delta A_{\text{raw}}$ (570). The phenol red–related absorbance change, denoted as $\Delta A_{\text{PR}}$ (570), was obtained by subtracting the estimated intrinsic absorbance change at 570 nm given by a scaled version of the $\Delta A_{\text{raw}}$ (690) signal using the procedure of Irving et al. (1987), summarized on page 322 of Pape et al. (2007).

Next in Fig. S1 A is the $\Delta p$H signal that was derived from the $\Delta A_{\text{PR}}$ (570) signal and measured resting pH as described previously (Irving et al., 1989; Pape et al., 1995; Pape and Carrier, 1998). The hashed line is the least-squares-best-fit line to the points during the last 300 ms of the pulse to $-30$ mV (range of points indicated by the cursor symbols). The line has a clear positive slope indicative of a change in direction of the $\Delta p$H signal. A similar positive slope was observed near the start of measurements ($\sim60$ min after permeabilization of the end-pool segments with saponin) in most of the other experiments examined though it was not always present. This reversal of the $\Delta p$H signal generally disappeared within 2 h of the start of experiments.

Results in Figs. 1 A and S3 A with the other Ca indicator used in this study, TMX, indicate that $[\text{Ca}^{2+}]$ in the

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**Figure S1.** Characterization of reversal of $\Delta p$H signal. (A) The top trace gives the voltage measured in the V1 end pool for an 800-ms voltage-clamp pulse to $-30$ mV, as indicated. The other traces in this panel are defined in the text. (B) Traces from another experiment in which SR $\text{Ca}^{2+}$ release occurring during two pulses instead of one. The format of the traces is the same as that in A. (C) Pairs of connected points plot $-d\Delta \text{pH}/dt$ versus the average value of $\Delta \text{pH}$ between pulses and after the last pulse, as illustrated in B. Each connected pair is from a different experiment. For A: fiber reference, 720992; time after saponin, 63.1 min; time after dye, 50.5 min; diameter, 130.7 µm; pH$_{o}$, 6.909; [phenol red], 0.94 mM; and holding current, $-25.8$ nA. For A: fiber reference, N01001; time after saponin, 60.1 min; time after dye, 48.2 min; diameter, 106.3 µm; pH$_{o}$, 6.962; [phenol red], 1.51 mM; and holding current, $-38.2$ nA. The fiber references for C were O31001 (open circle), N01001 (closed circle), 504011 (open square), O31011 (closed square), and N02011 (open triangle). Range of values for C: time after saponin, 60–85 min; time after dye, 46–70 min; diameter, 103–134 µm; pH$_{o}$, 6.55–7.12; [phenol red], 0.81–1.51 mM; and holding current, $-26$ to $-89$ nA.
SR approaches a constant level with no reversal of direction toward the end of pulses like that shown in Fig. 1. Therefore, the reversal of the $\Delta pH$ is not caused by the transport of Ca from the myoplasm back into the SR. Pape et al. (2007) suggested that the reversal could be caused by loss of protons from the myoplasm by some unknown process producing a $\Delta pH$ signal denoted as $\Delta pHi$. With somewhat limited justification given, Pape et al. (2007) assumed that the rate of this loss was proportional to the $\Delta pH$ signal so that

$$\frac{d\Delta pH_{i}}{dt} = k \cdot \Delta pH.$$  \hspace{1cm} (S1)

The signal labeled $\Delta pHi$ in Fig. 1 A was determined with this relationship by scaling the integral of the $\Delta pH$ signal so that its final slope during the last 300 ms of the pulse to $-30$ mV matched that of the $\Delta pH$ signal. (The $\Delta pHi$ signal was offset to allow comparison of its final slope with that of the $\Delta pH$ signal.) The corrected $\Delta pH$ signal, denoted as $\Delta pH_{cor}$ (not depicted), is given by

$$\Delta pH_{cor} = \Delta pH - \Delta pH_{i}.$$  \hspace{1cm} (S2)

As indicated in Fig. S1 A, the bottom pair of traces show the $\Delta [CaEGTA]$ and $\Delta [CaT]$ obtained from the $\Delta pH$ and $\Delta pH_{cor}$ signals, respectively, using Eqs. 1 and 2 in the article. Because of the correction, the slope of the $\Delta [CaT]$ signal is zero at the end of the pulse to $-30$ mV. Compared with the original EGTA/phenol red method (in which $\Delta [CaT]$ was equated with $\Delta [CaEGTA]$), it is clear that the modified approach taking into account the putative proton loss has very little effect on the early time course and magnitude of the $\Delta [CaT]$ signal. As seen later, however, the modified approach can significantly alter the relationship between $\Delta [CaT]$ and $\Delta [CaEGTA]$ with some types of multi-pulse protocols.

Fig. S1 B shows results with two voltage pulses, an 800-ms voltage-clamp pulse to $-45$ mV followed by 600 ms at the resting potential of $-90$ mV and a 1,200-ms pulse to $-20$ mV. All of the signals have the same meaning as those at the same level in Fig. S1 A so that the same labels apply. As before, the intrinsic absorbance change is relatively small compared with the dye-related signal at 570 nm. As with the final level of the $\Delta pH$ signal during the pulse to $-30$ mV, the $\Delta pH$ signal during the period between two pulses has a positive slope (indicated by the hashed line fit to points from 10 ms after the pulse to $-45$ mV to 10 ms before the pulse to $-20$ mV). As with the final level of the signal, this positive slope occurs despite results shown later with TMX indicating that the concentration of Ca in the SR should be approximately constant during this period at $-90$ mV.

It is noted that the slope of the $\Delta pH$ signal before the end of the pulse to $-20$ mV is slightly less negative than the extrapolated hashed line. This change of slope is indicative of a small amount of voltage-dependent Ca$^{2+}$ release still occurring at the end of the pulse to $-20$ mV that terminated when the voltage returned to the holding potential. The corresponding results in Fig. S1 A showed no apparent change in slope. In general, changes of slopes were either undetectable or relatively small, in support of results discussed later indicating that the SR reached or approached nearly full depletion of Ca from the SR during such long depolarizations.

The lower and higher closed-circle symbols in Fig. S1 C plot the slopes of the lower and higher hashed lines,
Section 2: Determination of $\Delta A_{\text{ATMX}}$ at full depletion 

This section expands on an earlier method for assessing “remnant Ca”, the amount of Ca present in the SR at the end of a stimulation in which $[\text{Ca}^2+]_{\text{SR}}(t)$ or a related signal approaches a constant level, suggestive of “full depletion” of Ca from the SR (Part 6 of Appendix B of (Pape et al., 2007)). The main aim of this section is to estimate the value of $\Delta A_{\text{ATMX}}$ that would be measured if all of the Ca$^2+$ were depleted from the SR (denoted as $\Delta A_{\text{ATMX}}(\text{FD})$), a parameter used for determining the “remnant Ca”, the amount of Ca present in the SR at full depletion” of Ca from the SR (Part 6 of Appendix B of (Pape et al., 2007)).

An equation giving the mass balance for Ca in the SR is given by

$$V_{\text{SR}} \frac{d[\text{Ca}^2+]_{\text{SR}}}{dt} = -P \cdot A_{\text{SR}} \cdot [\text{Ca}^2+]_{\text{SR}} + V_{\text{SR}} \cdot U_T,$$  

(S3)

where $V_{\text{SR}}$ and $A_{\text{SR}}$ are the volume and surface area of the SR, respectively, $P$ is the permeability, and $U_T$ is the rate of uptake (pumping) of Ca$^2+$ into the SR expressed in terms of $d[\text{Ca}^2+]_{\text{SR}}/dt$. (This formulation uses the presumably more correct assumption that the driving force for SR Ca$^2+$ release is proportional to $[\text{Ca}^2+]_{\text{SR}}$ as opposed to $[\text{Ca}^2+]_{\text{SR}}$, as assumed previously in Pape et al., 2007.) Eq. S3 is equivalent to the relationship

$$\left( \frac{1}{V_{\text{SR}}/P \cdot A_{\text{SR}}} \right) \left( \frac{d[\text{Ca}^2+]_{\text{SR}}}{d[\text{Ca}^2+]_{\text{SR}}} \right) [\text{Ca}^2+]_{\text{SR}} = U_T,$$  

(S4)

where $U_T$ is the rate of uptake of the Ca$^2+$ into the SR expressed in terms of $-d[\text{Ca}^2+]_{\text{SR}}/dt$.

One approach for understanding Eq. S4 is to consider the denominator of the factor of $[\text{Ca}^2+]_{\text{SR}}$ as a time-dependent exponential time constant ($\tau$). If the permeability of the SR were constant, the value of $\tau$ would decrease with time (a behavior referred to here as an accelerating exponential decline) because the second factor in the denominator (the buffering power of the intrinsic buffers in the SR) decreases as SR Ca content decreases owing to the strong cooperative binding of Ca in the SR (Pape et al., 2007 and Table 1 in this article). As noted above, the permeability of the SR for Ca$^2+$ release was shown to decrease with decreasing SR Ca content so that the first term in the denominator should increase with time during the final time course thereby tending to offset the decreasing value of $\tau$ associated with the decreasing Ca buffering power. We refer to the “accelerating exponential decline” given by Eq. S4 as Model 1.

Model 2 corresponds to case that the final time course of $[\text{Ca}^2+]_{\text{SR}}(t)$ follows a simple exponential decline (constant $\tau$). Assuming that the declining permeability exactly offsets the rate of decline of the second factor in the denominator of Eq. S4, Eq. S4 becomes

$$\frac{d[\text{Ca}^2+]_{\text{SR}}}{dt} + \frac{1}{\tau} \left[\text{Ca}^2+\right]_{\text{SR}} = U_T.$$  

(S5)

Also assuming that $U_T$ is constant (as is nearly the case; see below), the solution of Eq. S5 is given by

$$[\text{Ca}^2+]_{\text{SR}} = (\left[\text{Ca}^2+\right]_{\text{SR}}(t_{0})) - U_T \tau \exp (-t-t_{0})/\tau + U_T \tau,$$  

(S6)

so that $[\text{Ca}^2+]_{\text{SR}}$ at infinite time is given by

$$[\text{Ca}^2+]_{\text{SR}}(\infty) = U_T \tau.$$  

(S7)

During the final part of the SR Ca$^2+$ release signal, the $\Delta A_{\text{ATMX}}$ signal should be essentially linearly related to $[\text{Ca}^2+]_{\text{SR}}(t)$ by the relationship

$$\Delta A_{\text{ATMX}}(t) - \Delta A_{\text{ATMX}}(\text{FD}) \propto [\text{Ca}^2+]_{\text{SR}}(t),$$

so that Eq. S6 becomes

$$\Delta A_{\text{ATMX}}(t) = \Delta A_{\text{ATMX}}(\text{FD}) + \epsilon \cdot \exp (-t-t_{0})/\tau + U_T \tau,$$  

(S8)

where $\epsilon = \Delta A_{\text{ATMX}}(t_{0}) - \Delta A_{\text{ATMX}}(\text{FD})$ and $U_T \tau$ is the constant uptake rate given in terms of $d\Delta A_{\text{ATMX}}/dt$. With the extrapolated final value of $\Delta A_{\text{ATMX}}$ at infinite time defined as $\Delta A_{\text{ATMX}}(\infty)$, Eq. S8 gives that

$$\Delta A_{\text{ATMX}}(\text{FD}) = \Delta A_{\text{ATMX}}(\infty) - U_T \tau.$$  

(S9)

Fig. S2 shows results that illustrate the application and justification of Eq. S9 for estimating $\Delta A_{\text{ATMX}}(\text{FD})$. It
shows the same voltage pulse (to $-20$ mV) and $\Delta A_{\text{TMX}}$ signals during the last voltage-clamp pulse in the series of pulses seen in Fig. S3 A. (As seen in Fig. S3 A, about half of decrease in the $\Delta A_{\text{TMX}}$ signal occurred before this final pulse shown here in Fig. S2). The red trace in Fig. S2 is a least-squares best fit of an exponential plus a constant function to the $\Delta A_{\text{TMX}}$ points between and including the points marked by the red cursors (the + symbols; the last red cursor is obscured with the $\Delta A_{\text{TMX}}$ trace but is evident with the voltage trace). The constant part of the fitted function had a value of $-0.006115$, which is the value of $\Delta A_{\text{TMX}}(\infty)$ indicated in the figure (recall that $\Delta A_{\text{TMX}}$ is the change with respect to the $A_{\text{TMX}}$ signal at rest, i.e., before the multi-pulse stimulation as seen with Fig. S3). The best-fit value of $\tau$ for the exponential function was 219.7 ms. The value of the uptake parameter $U$ is given by the slope of the least-squares-best-fit line (shown in green) fit to the points after the stimulation (those points between and including the green cursors). As seen the reuptake is well described by a linear function, thereby justifying the assumption of constant $U$ in the derivation of Eq. S9. The value of $U$ was $1.779 \times 10^{-7}$ ms$^{-1}$ so that the $U \tau = 0.000039 (1.779 \times 10^{-7} \times 219.7)$ and $\Delta A_{\text{TMX}}(\text{FD}) = -0.006154 (-0.006115-0.000039)$. It is noted that the added amount of $\Delta A_{\text{TMX}}$ caused by remnant Ca in the SR when steady state is reached ($\Delta A_{\text{TMX}}(\text{FD}) - \Delta A_{\text{TMX}}(\infty)$ or $U \tau$) is only 0.64% (100 $\times$ -0.000039 $\div$ -0.006115) of the magnitude of the $\Delta A_{\text{TMX}}$ signal. The corresponding percentages in the other two experiments in this study were also small, 0.55 and 2.23% (fiber numbers 724011 and 423021, respectively; see Table 1).

As noted above, Model 1—corresponding to the case of constant permeability during the final part of the $\Delta A_{\text{TMX}}$ signal—predicts an “accelerating exponential decline”. In contrast to this prediction, the decline in the $\Delta A_{\text{TMX}}$ signal was more rapid earlier on (corresponding to a smaller value of $\tau$ earlier on) as seen by the more rapid earlier decline in the $\Delta A_{\text{TMX}}$ signal compared with the extrapolated fitted exponential function. This behavior—denoted as “slowing exponential decline”—was also seen in one of the two other experiments (fiber 423021). In the other experiment (fiber 724011), the final time course was approximately exponential during a longer period of the final decline (the $\Delta A_{\text{TMX}}$ signal for this latter experiment is shown in Fig. 1 A). A third model (Model 3) giving a “slowing exponential decline” of the final time course of the $\Delta A_{\text{TMX}}$ signal was also assessed. In this model, the rate of change of the $\Delta A_{\text{TMX}}$ signal ($-\frac{d\Delta A_{\text{TMX}}}{dt}$) was considered to be proportional to the square of $\Delta A_{\text{TMX}}$ instead of proportional to $\Delta A_{\text{TMX}}(\text{FD})$ as with Model 2. Least-squares best fits of an analytical solution of Model 3 (not given) provided very poor matches to the data (not depicted). (The value of $\Delta A_{\text{TMX}}(\infty)$ with Model 3 as opposed to $U \tau$ for Model 2 suggesting a possible upper limit of $2U \tau$ caused by remnant Ca.) Likewise, an analytical solution of Model 1 assuming a Hill coefficient for cooperative binding of 3 (not given) also provided a very poor match to the data (not depicted). The poor matches to the data of Models 1 and 3 and the relatively reasonable match of Model 2 indicate that the latter model provides a very good estimate of remnant Ca and

Figure S3. Phenol red and TMX absorbance signals with multiple steps and cooperative Ca$^{2+}$–calsequestrin-binding reaction. This figure has the same format as Fig. 2. As in Fig. 2 C, the red curve in C is the least-squares best-fitted model of cooperative binding to the data points using Eqs. 14 and 15. In this case, the fitted values of $[\text{CaB}]_{\text{nor}}$, the Hill coefficient ($n$) and $K_{\text{CB}}$ were 55.1 mM, 2.31, and 0.93 mM, respectively. The experiment in this figure is the same one used for Figs. 3–5 of Pape et al. (2007). Fiber reference, 211021; time after saponin treatment, 80 min; time after adding dye, 54 min; sarcotrial length, 3.9 µm; fiber diameter, 84 µm; holding current, $-26$ nA; 16.5°C.
its related value here, $\Delta \text{ATMX}(\approx) - \Delta \text{ATMX(FD)}$. The $[\text{Ca}^{2+}]_{\text{SR}}(t)$ signals in this article were obtained with estimates of $\Delta \text{ATMX(FD)}$ using Model 2, as described with Fig. S2.

As noted, above, the better match with Model 2 compared with Model 1 supports earlier results indicating that the permeability for SR $\text{Ca}^{2+}$ release decreases with decreasing $[\text{Ca}^{2+}]_{\text{SR}}$ when the Ca content of the SR decreases from $\sim$1/10th its physiological value to near zero.

Section 3: Correction of ratio $V_{\text{myo}}/V_{\text{SR}}$ and parameters used from Lamboley and Pape (2011)

Because TMX has such a low affinity for Ca, there should be essentially no CaTMX in the myoplasm so that essentially all of the CaTMX signal in the resting state should arise from the SR. However, the Ca-free form of TMX distributes in both the myoplasm and SR. To determine the distribution of Ca-free TMX at rest, Lamboley and Pape (2011) separated the myoplasmic and SR components by rapidly permeabilizing the surface membrane of frog cut fibers with the detergent saponin thereby washing out the myoplasmic component and leaving the SR component. This article uses values of $K_{\text{app}}$ and $[\text{Ca}^{2+}]_{\text{SR,SR}}$ from Lamboley and Pape (2011) as defined with Eq. 5 and associated text in the article. The upper and lower limits for values of $K_{\text{app}}$ described in this section require correction because of the updated value below for the ratio $V_{\text{myo}}/V_{\text{SR}}$. As described in Methods (in the paragraph with Eq. 5), values of $[\text{Ca}^{2+}]_{\text{SR,SR}}$ for cases 1–3 were given by the value of $K_{\text{app}}$ multiplied by the average ratio of $[\text{Ca}^{2+}]_{\text{SR,SR}}/K_{\text{app}}$ of 0.256 determined in Lamboley and Pape (2011), a value that does not depend on the $V_{\text{myo}}/V_{\text{SR}}$ ratio and other factors that can affect $K_{\text{app}}$.

As noted in Methods, the value of the ratio $V_{\text{myo}}/V_{\text{SR}}$ in frog skeletal muscle used in Pape et al. (2007) and Lamboley and Pape (2011) is almost certainly lower than the actual value. The following corrected estimate for this ratio of 8.0 is 1.49-fold greater than the ratio of 5.385 given in A5 of Lamboley and Pape (2011). The value of 8.0 is obtained by dividing 0.88, the fraction of fiber volume outside the SR and mitochondria, by 0.11, the fraction of fiber volume occupied by SR (the origin of both of these estimated values, 0.88 and 0.11, are given at the top of page 629 in Baylor et al., 1983). The ratio of 8.0 assumes that the fraction of volume occupied by water is the same in the SR and myoplasmic compartments.

Most of the results in Lamboley and Pape (2011)—including measurements of $f_{\text{TMX}}$ (defined with Eq. 5 in the article)—do not depend on the ratio of $V_{\text{myo}}/V_{\text{SR}}$ including columns 2–9 in Table 1 and all of the results in Table 2. The main effect of the change of the ratio was on possible values of $K_{\text{app}}$ and, thereby, $[\text{Ca}^{2+}]_{\text{SR}}$. The average value for the ratio of the concentrations of TMX in the SR to that in the myoplasm ($[\text{TMX}]_{\text{SR}}/[\text{TMX}]_{\text{myo}}$) was 2.8 in Lamboley and Pape (2011; their Section 3.4 and Column 10 of their Table 1), which is updated to 4.17 with the new ratio of $V_{\text{myo}}/V_{\text{SR}}$ (4.17 = 1.49 × 2.8). The value for $[\text{TMX}]_{\text{SR}}/[\text{TMX}]_{\text{myo}}$ being >1 was considered to be caused by binding of Ca-free TMX to sites in the SR. The upper limit for $K_{\text{app}}$ was obtained by assuming that all of the excess Ca-free TMX in the SR is bound to sites and is unable to complex Ca and that none of the CaTMX form binds to such sites (Section 3.11 of Lamboley and Pape, 2011). The first estimate of the upper limit was then corrected for the dependence of $K_{\text{LTMX}}$ on the concentration of total TMX as described in Section 3.14 in Lamboley and Pape (2011). Following the same procedure with the new value of $V_{\text{SR}}/V_{\text{myo}}$, the old upper limit for $K_{\text{app}}$ of 6.6 mM becomes 9.1 mM, which has an associated value for $[\text{Ca}^{2+}]_{\text{SR,SR}}$ of 2.35 mM ($2.35 = 0.256 \times 9.1$). This upper limit corresponds to case 3 in the article.

The lower limit for $[\text{Ca}^{2+}]_{\text{SR,SR}}$ corresponded to the unlikely condition that none of the excess TMX in the SR was bound to sites in the SR. Following the same procedure described in section 3.14 of Lamboley and Pape (2011) to take into account the dependence of $K_{\text{LTMX}}$ on TMX concentration with the updated value for $V_{\text{SR}}/V_{\text{myo}}$, the corrected lower limit of $[\text{Ca}^{2+}]_{\text{SR,SR}}$ determined with TMX is 0.26 mM (down from the previous lower limit of 0.43 mM).

The general form of the equation for determining the time course of $[\text{Ca}^{2+}]_{\text{SR}}$ from the $\Delta \text{ATMX}$ signal in response to a voltage stimulation is given by Eq. 23 in Lamboley and Pape (2011) and reproduced here with a small modification:

$$[\text{Ca}^{2+}]_{\text{SR}}(t) = [\text{Ca}^{2+}]_{\text{SR,SR}}(t) \cdot \frac{1 - \Delta \text{ATMX}(t)}{\Delta \text{ATMX(FD)}}, \quad \frac{[\text{Ca}^{2+}]_{\text{SR,SR}}(t)}{1 + \frac{\Delta \text{ATMX}(t)}{\Delta \text{ATMX(FD)}}}$$

As defined in Section 2, $\Delta \text{ATMX(FD)}$ is the value of the $\Delta \text{ATMX}$ signal that would be reached if $[\text{Ca}^{2+}]_{\text{SR}}$ were able to reach zero, i.e., full depletion. The small modification is that $\Delta \text{ATMX(FD)}$ here replaces $\Delta \text{ATMX}(\approx)$ used in Lamboley and Pape (2011) because they did not take into account the small difference between $\Delta \text{ATMX(FD)}$ and $\Delta \text{ATMX}(\approx)$ caused by remnant Ca as described with Section 2. In contrast to the form of this equation given by Eq. 7 in the article, $K_{\text{app}}$ can change with time in this general form, from a starting value of $K_{\text{app,0}}$ (when $t = 0$). This equation can be considered to have 3 parts or factors; the first and second are $[\text{Ca}^{2+}]_{\text{SR,SR}}$ and the ratio $K_{\text{app,0}}(t)/K_{\text{app,0}}$, respectively, and the third is the remainder of the equation. Because $\Delta \text{ATMX}$ and $\Delta \text{ATMX(FD)}$ are measured and the ratio $[\text{Ca}^{2+}]_{\text{SR,SR}}/K_{\text{app,0}}$ was measured and its value does not depend on factors influencing $K_{\text{app,0}}$ (see above), the third part of Eq. S10 is reliably determined from measured values. Another important
feature to note with the third part is that the value of 0.256 determined in Lamboley and Pape (2011) for the ratio \([Ca^{2+}]_{SR,R}/K_{app,R}\) is relatively small so that the denominator of the third part changes significantly less than the numerator so that the whole third part varies approximately linearly with \(\Delta A_{TMX}\). Because of the dependence of \(K_{d,TMX}\) on total TMX concentration noted above, \(K_{app}\) can vary with the concentration of total TMX not bound to sites in the SR (denoted as \([TMX_T]_{free}\)). For the case that both CaTMX and Ca-free TMX bind in the same proportion, \([TMX_T]_{free}\) and, thereby, \(K_{app}\) would not vary during a stimulation so that the second part of Eq. S10 would have a constant value of 1. On the other hand, \([TMX_T]_{free}\) could change if only the Ca-free form of TMX binds to sites in the SR, as conversion of CaTMX to TMX in response to SR Ca\(^{2+}\) release would increase unbound Ca-free TMX resulting in increased binding. This latter possibility was assessed in Section 3.14 of Lamboley and Pape (2011) where it was shown to have an almost negligible effect on the normalized time course of the derived \([Ca^{2+}]_{SR}(t)\) signal (see their Fig. 9). Moreover, the effect of taking into account the change in \(K_{app}\) results in a more linear relationship between the normalized \([Ca^{2+}]_{SR}\) signal and the underlying \(\Delta A_{TMX}\) signal. The same conclusions are reached taking into account the updated value for the ratio \(V_{myo}/V_{SR}\). In this case, the difference between the calculated \([Ca^{2+}]_{SR}\) signals with and without taking into account the time dependent change in \(K_{app}\) was somewhat greater but still almost negligible (not depicted). For this reason, Eq. 7 (in which \(K_{app}\) is assumed constant) is used to derive \([Ca^{2+}]_{SR}(t)\) from the \(\Delta A_{TMX}(t)\) signal in the article instead of Eq. S10.

In summary, the only significant effect arising from uncertainty in \(K_{app}\) is in the value of \([Ca^{2+}]_{SR,R}\), which is the first factor in Eqs. 7 and S10. As a result, TMX is expected to provide a very reliable estimate of the time course of \([Ca^{2+}]_{SR}(t)\) during voltage-activated SR Ca\(^{2+}\) release though there is considerable uncertainty in its magnitude.

Section 4: Matching the final values and slopes of the measured \([Ca^{2+}]_{SR}\) and \([Ca^{2+}]_{SR,model}\) signals

As indicated above, the \(\Delta pH\) signal was adjusted for putative loss of protons from the myoplasm so that its final slope was zero, which gives the condition of no net Ca\(^{2+}\) flux from the SR (\(d[Ca^{2+}]_T/dt = 0\)). The main justification for this was that the \(\Delta A_{TMX}\) signal approaches a constant level at the end indicating that full depletion of the SR is approached. However, there is generally a small negative slope in the \(\Delta A_{TMX}\) at the end of the final pulses indicative of a continued net release of Ca\(^{2+}\) from the SR (see Fig. S2 and arrows pointing to \(\Delta A_{TMX}\) signals in Figs. 1 A and S3 A). To account for this, the final part of the measured and modeled \([Ca^{2+}]_{SR}\) signals were constrained to provide a match of their final slopes and val-

![Figure S4. Results with model of linear binding component of intrinsic buffers.](image)

A. The top trace shows the same voltage signal shown in Figs. 3 A and 4 A. Each pair of traces shows a \([Ca^{2+}]_{SR}\) (black trace) and a \([Ca^{2+}]_{SR,model}\) (red trace) signal. The \([Ca^{2+}]_{SR,model}\) traces were determined with the assumed fraction of resting total SR Ca content displaying linear binding \((f_{LB})\) shown on the right from the same stimulation shown in Figs. 3 and 4. The assumed values for \([Ca^{2+}]_{SR,R}\) and \(K_{app}\) (for TMX) for all of the results were 0.6656 and 2.6 mM, respectively, i.e., the case 1 values and the same values used for Figs. 3 and 4. The best-fit parameters from Eq. 15 obtained with the fits were, for \(f_{LB} = 0, 0.1, 0.2, 0.3, 0.4, \) and 0.5, respectively, were: for \(K_{CB}\), 0.94, 0.82, 0.71, 0.63, 0.58, and 0.56 mM; for \(n\), 2.28, 2.54, 2.96, 3.60, 4.61, and 6.08; for \([Ca^{2+}]_{max}\), 18.92, 19.04, 19.10, 19.10, 19.11, and 19.16 mM. From the values of \([Ca^{2+}]_{SR,R}\), \(n\), and \(K_{CB}\), the fraction of total cooperative Ca-binding sites occupied by Ca (denoted as \(f_{CB,R}\)) were 0.313, 0.371, 0.452, 0.549, 0.654, and 0.741; and the concentration of Ca bound to cooperative binding sites at rest (denoted as \([Ca^{2+}]_{R}\)) and given by \(f_{CB,R}\) times \([Ca^{2+}]_{max}\) were 17.7, 15.9, 14.0, 12.1, 8.6, and 8.3 mM (values given with the same order of increasing \(f_{LB}\) values from 0–0.5 as for the other parameters). See text for additional details.
values at the end of the last pulse as described previously in Pape et al. (2007) and reviewed in this section.

The approach to achieve these constraints was to adjust the final slope of the $\Delta p$H signal with the value of $k$ above in Eq. S1 and to adjust $[\text{Ca}]_{\text{T,SR,R}}$ via an adjustment of the assumed value of the final level of the $\Delta p$H signal ($\Delta p$H$_{\text{min}}$). One reason for adjusting values associated with the $\Delta p$H signal as opposed to the $\Delta A_{\text{T MX}}$ signal is that there was already some uncertainty in the value of $\Delta p$H$_{\text{min}}$ and in the final time course of the $\Delta A_{\text{R}}$ signal owing to the corrections by Eqs. S1 and S2 for the putative loss of protons. Another is that the signal from TMX should be more reliable as full depletion is approached because the $\Delta A_{\text{T MX}}$ is approximately linearly related to $[\text{Ca}^{2+}]_{\text{SR}}$ and relative changes in $[\text{Ca}^{2+}]_{\text{SR}}$ compared with $[\text{Ca}]_{\text{T,SR}}$ should be greater as full depletion is approached owing to the cooperative nature of the Ca-binding properties of the intrinsic buffers (e.g., Figs. 1 C, 2 B, and S3 C).

The iterative procedure of imposing the constraints of matched slopes and values of the measured and modeled $[\text{Ca}]_{\text{T,SR}}$ signals at the end of the final pulse are described in detail in seven “User Steps” in Part 4 of Appendix B in Pape et al. (2007). The only actual difference is that $[\text{Ca}^{2+}]_{\text{SR,R}}$ is fixed and $K_{\text{IB}}$ is an adjustable parameter in this study whereas the reverse was the case in Pape et al. (2007). As a result, $K_{\text{SB}}$ (in Pape et al., 2007) replaces $[\text{Ca}^{2+}]_{\text{SR,R}}$ as the adjustable parameter in User Step 5 on page 356 of Pape et al. (2007). Another difference in User Step 5 is that $[\text{Ca}^{2+}]_{\text{SR}}(t)$ was calculated with Eq. 7 in this study whereas it was calculated with an equivalent method involving several steps in Pape et al. (2007).

As seen with the pair of $\Delta p$H signals at the bottom of Fig. 2 A, the effect of imposing the constraints of matched slopes and values was quite minor for the first model of Ca binding above, i.e., with no linear binding. Although not explicitly shown, it became more important as the assumed degree of linear binding (the value of $f_{\text{LB}}$) increased in the second model of Ca binding to intrinsic buffers. A main reason that this adjustment only produced small changes is that more than 99.7% of the total Ca in the SR is estimated to have been released by the end of the last voltage pulse for the experiments in this study. The value of 99.7% is from the amount of remnant Ca given in columns 10 of Table 2 of Pape et al. (2007).

Section 5: Possibility that a portion of the intrinsic Ca$^{2+}$ buffers display linear binding

Shortly after their initial discovery of calsequestrin (MacLennan and Wong, 1971), MacLennan and colleagues reported two other intrinsic Ca-binding proteins in the SR of skeletal muscle (MacLennan et al., 1973; Ostwald and MacLennan, 1974)). One they termed the high molecular weight protein, later designated as calreticulin. The other was a set of three “low molecular weight acidic proteins”, almost all in the 33–38-kD range. Based on the data with 100 mM KCl present in Figs. 4 and 8 of Ostwald and MacLennan (1974), replotted on linear scales, Ca binding to calreticulin and to the 33–38 kD proteins are well described by linear binding (the amount of Ca bound to the proteins being proportional to $[\text{Ca}^{2+}]$) for $[\text{Ca}^{2+}]$ values up to 1 mM. The only data point above this range was for a value of $[\text{Ca}^{2+}]$ of 5 mM for the 33–38 kD proteins indicating a modest deviation from linearity. Given these results, it seems reasonable to assume a model of linear Ca binding for these proteins in the physiological range of $[\text{Ca}^{2+}]_{\text{SR}}$ values (i.e., < 2.35 mM; see upper limit for $[\text{Ca}^{2+}]_{\text{SR,R}}$ given above).

Two other Ca$^{2+}$-sequestering proteins were later identified, a histidine-rich 165-kD protein and a 150-kD protein termed sarcalumenin, both of which are present in small amounts and distributed throughout the SR (Beard et al., 2004). The 160-kD protein (Orr and Shoshan-Barmatz, 1996) and sarcalumenin (Leberer et al., 1990) appear to have Ca-binding properties similar to those of calsequestrin. The model given by Eqs. 11 and 12 in the article includes two components of the intrinsic buffers in the SR, a linear binding component (tentatively associated with calreticulin, the “low molecular weight acidic proteins” and possibly a linear Ca-binding component of calsequestrin) and a component displaying cooperative binding (likely caused mostly by calsequestrin with minor contributions from sarcalumenin and the histidine-rich 165-kD protein).

Fig. S4 shows the application of this model based on the same approach described with Fig. 2 A except that $[\text{CaB}]_{\text{LB}}$ from Eq. 12 is used in Eq. 11 instead of $[\text{CaB}]_{\text{IB}}$. The top trace in Fig. S4 A shows the same voltage signal shown in Figs. 2 A and S3 A. The next pair of traces (from top to bottom) show the same $[\text{Ca}]_{\text{SR}}$ (black trace) and $[\text{Ca}]_{\text{SR,mod}}$ (red trace) signals shown in Fig. 2 A. The next five pairs of traces show the same corresponding pairs of traces with values of $f_{\text{LB}}$ varying from 0.1 to 0.5, as indicated. As with Figs. 1 and 2, values of $[\text{Ca}^{2+}]_{\text{SR,R}}$ and $K_{\text{app}}$ for case 1 were 0.6656 mM and 2.6 mM, respectively, and the cooperative binding constants, $[\text{CaB}]_{\text{max}}$, $K_{\text{IB}}$, and $n$, were adjusted to give the least-squares best fit of the $[\text{Ca}]_{\text{SR,mod}}$ signal to the $[\text{Ca}]_{\text{SR}}$ signal. In each case, the same two constraints as above were imposed, namely that the slope and final value of the $[\text{Ca}]_{\text{SR}}$ signal matched that of the $[\text{Ca}]_{\text{SR,mod}}$ signal at the end of the pulse to $-20$ mV. As before, these constraints were achieved by adjusting the rate constant $k$ for the proton removal correction while concurrently adjusting $\Delta p$H$_{\text{min}}$. It is noted that the constraint that the final slopes matched became more important as $f_{\text{LB}}$ increased. It is seen in Fig. S4 A that the $[\text{Ca}]_{\text{SR,mod}}$ signals appear to provide reasonably good fits to their associated $[\text{Ca}]_{\text{SR}}$ signals for values of $f_{\text{LB}}$ up to 0.4 but that a significant deviation occurred toward
the end, best seen with the traces with $f_{LB}$ set to 0.5. The sums of squares for the 6 values of $f_{LB}$ (0, 0.1, 0.2, 0.3, 0.4, and 0.5) were 83.8, 82.1, 93.0, 126.9, 228.5, and 499.7, respectively, so that there was a slight improvement in the fit with $f_{LB} = 0.1$ compared with that with no linear binding, i.e., $f_{LB} = 0$. The best-fit values for the Hill coefficient $n$ for the six cases (2.28, 2.54, 2.96, 3.60, 4.61, and 6.08) increased with increasing $f_{LB}$ as expected.

The six signals under the voltage trace in Fig. S4 B are the $[\text{CaT}]_{\text{SR}}(t)$ signals shown in A minus the component associated with linear binding. These signals, therefore, give mainly the cooperative-binding (nonlinear) part of the $[\text{CaT}]_{\text{SR}}$ signals associated with the assumed values of $f_{LB}$ shown to the right of the traces. For several of the traces, the final part of the signal shows a negative undershoot as indicated by the arrow under the trace with $f_{LB}$ set to 0.5 (the hashed-line segments indicate zero calcium levels). The reason for the negative undershoot is seen by the pair of traces at the bottom of Fig. S4 B showing $[\text{CaT}]_{\text{SR}}(t)$ (black trace) and the linear part of the $[\text{CaT}]_{\text{SR}}(t)$ signal (red trace). The negative undershoot occurs because the slower decaying linear component is greater than the measurement-derived $[\text{CaT}]_{\text{SR}}(t)$ signal toward the end of the pulse (see part indicated by the arrow; also see parts indicated by arrows in Figs. 1 C and S3 C). Because negative calcium levels are not physically possible, it seems reasonable to rule out the models where the negative components are clearly evident, those with $f_{LB}$ values from 0.3 to 0.5. Because the $f_{LB}$ value of 0.1 actually gave a somewhat better fit than that with $f_{LB} = 0$ (see end of preceding paragraph), the results actually suggest the presence of a linear binding component at the level of $\sim$10% of the resting SR Ca load. Given the sum-of-squares values at the end of the preceding paragraph and the visual evidence in Fig. S4 A, it seems reasonable to place an upper limit of 0.2 on the value of $f_{LB}$ for this experiment.

Data from the experiment in Fig. 1 were analyzed in the same way as those shown here in Fig. S4. In this case, the sum of squares for $f_{LB} = 0, 0.1, 0.2, 0.3, 0.4$ were 299, 251, 231, and 335 mM$^2$. In this case, the best fit occurred with $f_{LB} = 0.2$. The possibility of $f_{LB} = 0.3$ is ruled out for the same reasons as above for the results with $f_{LB} = 0.3$ in Fig. S4, namely, a negative undershoot in the cooperative binding component. The best-fit values for the Hill coefficient, $n$, for the same 4 values of $f_{LB}$ were 3.95, 4.91, 6.23, and 8.75. The value of 6.23 for $n$ for the case with $f_{LB} = 0.2$ is about double the largest values of $n$ for calsequestrin observed in vitro studies (see Discussion), which tends to make the value of $f_{LB} = 0.2$ less likely.

In summary, the results from the two experiments here suggest that the linear Ca$^{2+}$ buffer component accounts for 20% or less of the total Ca bound at rest.

References


