Calcium buffering properties of sarcoplasmic reticulum and calcium-induced Ca\(^{2+}\) release during the quasi-steady level of release in twitch fibers from frog skeletal muscle

Karine Fénelon, Cédric R.H. Lamboley, Nicole Carrier, and Paul C. Pape

Département de physiologie et biophysique, Université de Sherbrooke Faculté de Médecine et des Sciences
de la Santé, Sherbrooke,
Québec J1H5N4, Canada

Experiments were performed to characterize the properties of the intrinsic Ca\(^{2+}\) buffers in the sarcoplasmic reticulum (SR) of cut fibers from frog twitch muscle. The concentrations of total and free calcium ions within the SR ([Ca\(_{T}\)\(_{SR}\)] and [Ca\(^{2+}\)\(_{SR}\)]) were measured, respectively, with the EGTA/phenol red method and tetramethylmurexide (a low affinity Ca\(^{2+}\) indicator). Results indicate SR Ca\(^{2+}\) buffering was consistent with a single cooperative-binding component or a combination of a cooperative-binding component and a linear binding component accounting for 20\% or less of the bound Ca\(^{2+}\). Under the assumption of a single cooperative-binding component, the most likely resting values of [Ca\(^{2+}\)\(_{SR}\)] and [Ca\(_{T}\)\(_{SR}\)] are 0.67 and 17.1 mM, respectively, and the dissociation constant, Hill coefficient, and concentration of the Ca-binding sites are 0.78 mM, 3.0, and 44 mM, respectively. This information can be used to calculate a variable proportional to the Ca\(^{2+}\) permeability of the SR, namely d[Ca\(_{T}\)\(_{SR}\)]/dt ÷ [Ca\(^{2+}\)\(_{SR}\)] (denoted release permeability), in experiments in which only [Ca\(_{T}\)\(_{SR}\)] or [Ca\(^{2+}\)\(_{SR}\)] is measured. In response to a voltage-clamp step to –20 mV at 15°C, the release permeability reaches an early peak followed by a rapid decline to a quasi-steady level that lasts ~50 ms, followed by a slower decline during which the release permeability decreases by at least threefold. During the quasi-steady level of release, the release amplitude is 3.3-fold greater than expected from voltage activation alone, a result consistent with the recruitment by Ca-induced Ca\(^{2+}\) release of 2.3 SR Ca\(^{2+}\) release channels neighboring each channel activated by its associated voltage sensor. Release permeability at ~60 mV increases as [Ca\(_{T}\)\(_{SR}\)] decreases from its resting physiological level to ~0.1 of this level. This result argues against a release termination mechanism proposed in mammalian muscle fibers in which a luminal sensor of [Ca\(^{2+}\)\(_{SR}\)] inhibits release when [Ca\(_{T}\)\(_{SR}\)] declines to a low level.

INTRODUCTION

Contraction in skeletal muscle is initiated when Ca\(^{2+}\) stored in the SR is released into the myoplasm in response to membrane depolarization. Studies of the mechanisms that control SR Ca\(^{2+}\) release are aided by methods that permit estimating the Ca permeability of the SR membrane or, equivalently, the extent of activation of the SR Ca\(^{2+}\) release channels. This quantity is proportional to the rate of Ca\(^{2+}\) release divided by the driving force for release. Because the free calcium concentration in the myoplasm ([Ca\(^{2+}\)\(_{myo}\)]) is generally small relative to that in the SR ([Ca\(^{2+}\)\(_{SR}\)]), and because the SR membrane potential is expected to be near zero (Somlyo et al., 1977; Labarca and Miller, 1981; García and Miller, 1984; Pape et al., 1992; Gillespie and Fill, 2008), the driving force for Ca\(^{2+}\) release should be proportional to [Ca\(^{2+}\)\(_{SR}\)].

Until recently, [Ca\(^{2+}\)\(_{SR}\)] could not be readily measured; instead, [Ca\(^{2+}\)\(_{SR}\)] (and therefore the driving force) was assumed to be proportional to the concentration of total Ca within the SR ([Ca\(_{T}\)\(_{SR}\)]). Accordingly, one commonly used measure of the SR Ca permeability has been the depletion-corrected rate of Ca\(^{2+}\) release (DCRR), defined as the rate of Ca\(^{2+}\) release from the SR (−d[Ca\(_{T}\)\(_{SR}\)]/dt) normalized by [Ca\(_{T}\)\(_{SR}\)] and multiplied by 100 to give percentage of total Ca in the SR released per millisecond (Jacquemond et al., 1991; Jong et al., 1995; see also Pape and Carrier, 1998, and Pizarro and Ríos, 2004).

With recent advances in the ability to measure [Ca\(^{2+}\)\(_{SR}\)] (see below), it is now possible to make a more appropriate estimate of the SR Ca permeability during excitation–contraction coupling, namely −d[Ca\(_{T}\)\(_{SR}\)]/dt normalized by [Ca\(^{2+}\)\(_{SR}\)]. This article denotes this quantity as “release permeability.” As will be shown, studies...
of release permeability channel activation based on estimates of release permeability can lead to different conclusions from those based on the DCRR, particularly in regard to calcium feedback mechanisms that regulate the SR Ca$^{2+}$ release channels. These mechanisms fall into two general categories: CICR and Ca$^{2+}$ inactivation of Ca$^{2+}$ release. Considerable controversy has surrounded the study of both of these mechanisms under physiological or quasi-physiological fiber conditions (Baylor and Hollingworth, 1988; Jacquemod et al., 1991; Hollingworth et al., 1992; Gernoch et al., 1993; Jong et al., 1993; Pape et al., 1993; Pizarro and Rios, 2004); thus, the new estimates of release permeability may help clarify the role of these mechanisms in normal muscle function.

To reliably estimate the release permeability signal and its control by calcium feedback mechanisms, it is useful, if not essential, to have information about the calcium-buffering properties of SR that relate [Ca$^{2+}$]$_{SR}$ to [Ca$^{2+}$]$_{SR}$. Pape et al. (2007), in voltage-clamp experiments on frog cut fibers, simultaneously measured [Ca$^{2+}$]$_{SR}$ and a signal from the absorbance dye tetramethylrhodamine (TMX) that monitors changes in [Ca$^{2+}$]$_{SR}$. The resultant information permitted estimating the full Ca-binding curve for the SR, i.e., [Ca$^{2+}$]$_{SR}$ versus [Ca$^{2+}$]$_{SR}$ with various assumed values for resting [Ca$^{2+}$]$_{SR}$ (denoted as [Ca$^{2+}$]$_{SR}$,R) and the apparent dissociation constant for TMX (denoted as K$_{app}$). The first part of this article more firmly establishes the Ca-buffering properties of the SR based on the recently determined value of 0.256 for the ratio of [Ca$^{2+}$]$_{SR}$/K$_{app}$ in Lamboley and Pape (2011), a value resulting in an approximately linear relationship between the absorbance signal from TMX and [Ca$^{2+}$]$_{SR}$(t). Nonlinearity between [Ca$^{2+}$]$_{SR}$ and [Ca$^{2+}$]$_{SR}$,R more firmly established here, indicates a significant degree of cooperativity likely arising mainly from calasequestrin, a high capacity Ca-binding protein in the SR discovered by MacLennan and Wong (1971).

To our knowledge, this is the second article to report release permeability signals from d[Ca$^{2+}$]/dt and [Ca$^{2+}$]$_{SR}$ signals measured during maintained voltage-clamp depolarization of skeletal muscle fibers. The first was the recent study from Rios’s laboratory (Sztreyte et al., 2011b) on enzyme-dissociated fibers from mice. The [Ca$^{2+}$]$_{SR}$ signal in that study was measured with the recently developed cameleon indicator D4cpv fused into calasequestrin. These measurements were used in combination with a myoplasmic [Ca$^{2+}$] signal measured with the fluorescent indicator X-rhod-1 used to estimate the rate of SR Ca$^{2+}$ release (d[Ca$^{2+}$]/dt) based on the removal model of Melzer et al. (1984, 1987). The release permeability signal of Sztreyte et al. (2011b) in response to a voltage-clamp step displayed an early peak followed by a progressive decrease occurring over several hundred milliseconds. Because the release permeability signal in fibers from calasequestrin-null mice remained constant at about the level of the early peak, they concluded that calasequestrin is involved in a mechanism that closes the SR Ca release channels when the Ca content of the SR falls below a critical level. Results in this article indicate that the decrease in release permeability that takes place in frog fibers in response to a depolarizing voltage-clamp step is not caused by this or any other related termination mechanism involving a luminal sensor of [Ca$^{2+}$]$_{SR}$. Rather, the results indicate a significant degree of recruitment of neighboring SR Ca$^{2+}$ release by CICR during the quasi-steady level of SR Ca$^{2+}$ release during voltage-clamp stimulation, a mechanism likely involved in controlling SR Ca$^{2+}$ release during a train of action potentials.

MATERIALS AND METHODS

Details of the experimental protocols are described in Pape and Carrier (1998). In brief, frogs (Rana temporaria) were decapitated and double pithed with a protocol approved by the Comité d’éthique de l’experimentation animale at the Université de Sherbrooke. Cut fibers from twitch skeletal muscles (semimembranosus or iliofibularis) were mounted in a double Vaseline-gap chamber (Hille and Campbell, 1976; Irving et al., 1987) with sarcomere spacing set at 3.5–3.9 µm. This chamber was then mounted on a setup for simultaneously measuring electrical signals and light absorbance at three wavelengths. The fibers were maintained at a resting potential of −90 mV and a temperature of 16–17°C for all of the experiments except those in Figs. 3 and 4. The temperature for the experiments in Figs. 3 and 4 was maintained at 13–15°C.

Composition of the internal and external solutions

The usual internal solution contained (mM): 45 Cs-glutamate, 20 EGTA, 6.8 MgSO$_4$, 5 CaATP, 20 Cs$_8$-phospho(enol)pyruvate, 5 Cs$_8$-phospho, 5 MOPS, 1.76 calcium, with pH set to 7.0 with CsOH. The estimated values for [Ca$^{2+}$] and [Mg$^{2+}$] were 32 nM and 0.86 mM, respectively, determined as described in Pape et al. (2007).

The central pool solution contained 110 mM TEA-glucuronate, 10 mM MgSO$_4$, 1 mM tetrodotoxin, and 10 mM MOPS, with pH set to 7.1. The suppliers of the chemicals are listed in Pape and Carrier (1998). Because there was no Ca in the external solution, the SR should be the only source of Ca entering the myoplasm during the relatively short period associated with voltage-stimulated Ca$^{2+}$ release (compared with the relatively long time constants associated with exchange with the internal solution in the end pools). Shorty after mounting the fiber, the end pools were permeabilized with a 2-min exposure to internal solution containing 0.01% saponin. Usually ~10 min later, the internal solution in the end pools was exchanged for one containing one or two indicator dyes. Every experiment included the pH indicator phenol red at a nominal concentration of 1 mM. In some experiments, the internal solution also included the Ca$^{2+}$ indicator TMX at a nominal concentration of 2 mM.

Optical measurements

The optical apparatus is the same as that described in Pape and Carrier (1998), modified with dichroic mirrors replacing the beam splitters, as described in Pape et al. (2007). Pape et al. (2007) detail how the dye-related absorbance signals were obtained. In brief, the nominal center wavelengths in nanometers (bandwidths in nanometers) for the interference filters were 480 (10 or 30), 570 (30), and 690 (30). The 690-nm filter was used to monitor the intrinsic absorbance of the fiber, as none of the...
possible dye species present (protonated and unprotonated phenol red and Ca-free and Ca-bound TMX) absorbs in the wavelength range of this filter. The extinction coefficients for the other filters and for all of the possible dye species present are given on page 323 of Pape et al. (2007).

Summary of the EGTA/phenol red method for measuring $\Delta[Ca_{T}]$ and related signals

This section provides a brief summary of the EGTA/phenol red method as first described in Pape et al. (1995). This method relies on a decrease in pH caused by the liberation of two protons for each Ca$^{2+}$ bound to EGTA, given by the reaction Ca$^{2+} + H_{2}EGTA^{2-} \leftrightarrow 2H^{+} + CaEGTA^{2-}$ (Appendix A of Pape et al., 1995). As detailed in Appendix A and summarized on page 304 of Pape et al. (1995), in response to an action potential, EGTA appears to rapidly capture nearly all (~96%) of the total Ca released from the SR, with most of the remaining ~3% being captured by troponin and ~1% captured by parvalbumin. The change in the concentration of CaEGTA referred to myoplasmic volume (denoted as $\Delta(CaEGTA)$) should be very closely approximated by the relationship

$$
\Delta(CaEGTA) = -\frac{\beta}{2} \Delta pH,
$$

(1)

where $\beta$ is the buffering power of myoplasm. The value of $\beta$ is assumed to be 22 mM/pH unit, the value determined in calibration experiments in cut fibers with furca-2 described in Figs. 5 and 6 of Pape et al. (1995).

Previously, $\Delta(CaEGTA)$ was taken to be equal to the change in the concentration of total Ca in the myoplasm in response to stimulation, i.e., $\Delta(Ca_{T}) = \Delta(CaEGTA)$. As introduced in Pape et al. (2007) and explained and justified more fully in Section 1 of the supplemental text for this article, $\Delta pH$ signals during long depolarizations (800 ms or more) often display a small but clear reversal in direction. This reversal is not related to Ca$^{2+}$ entry back into the SR, and it is tentatively attributed to some type of proton loss from myoplasm or some related process. This putative proton loss is well described by a positive rate of change of the $\Delta pH$ signal ($d\Delta pH/dt$) that is proportional to the $\Delta pH$ signal itself. We denote the $d\Delta pH$ signal corrected for this putative proton loss as $\Delta pH_{corr}$, so that $\Delta(Ca_{T})$ is given by

$$
\Delta(Ca_{T}) = -\frac{\beta}{2} \Delta pH_{corr},
$$

(2)

The final level of the $\Delta(Ca_{T})$ signal in response to a “fully depleting stimulation” gives the concentration of total Ca in the SR just before the stimulation (denoted as $[Ca_{T}]_{SR,0}$). The total concentration at any time during the stimulation is given by

$$
[Ca_{T}]_{SR}(t) = [Ca_{T}]_{SR,0} - \Delta(Ca_{T}).
$$

(3)

The rate of SR Ca$^{2+}$ release is given by the negative of the time derivative of $[Ca_{T}]_{SR}(t)$ and the DCRR is given by

$$
DCRR = -100 \frac{d[Ca_{T}]_{SR}}{[Ca_{T}]_{SR}} = 100 \frac{d\Delta(Ca_{T})}{[Ca_{T}]_{SR}}
$$

(4)

which gives the percentage of SR Ca content released per unit time.

Measurements of $[CaTMX]$ and $[Ca^{2+}]_{SR}$ signals with TMX

The other indicator used in this study, TMX, is a low affinity Ca indicator that distributes in the SR and myoplasmic compartments. Because of its low affinity (Maylie et al., 1987, reported a value of 2.6 mM for the dissociation constant of TMX in free solution), there should be no CaTMX signal from the myoplasm during rest so that all of it should come from the SR. The concentration of free Ca in the SR at rest (denoted as $[Ca^{2+}]_{SR,R}$) is given by the relationship:

$$
[Ca^{2+}]_{SR,R} = \frac{K_{app} f_{TMX}}{1 - f_{TMX}}
$$

(5)

where $K_{app}$ is the apparent dissociation constant of TMX in the SR, and $f_{TMX}$ is the fraction of total TMX in the SR complexed with Ca. Lamboley and Pape (2011) determined the fraction of total TMX in the SR complexed with Ca (denoted as $f_{TMX}$) by permeabilizing the surface membrane of depolarized cut fibers with the detergent saponin, thereby washing out most of the TMX from the myoplasm. The average value of $f_{TMX}$ was 0.204 (columns 2 and 3 of Table 2 in Lamboley and Pape, 2011), which gives a value of 0.256 for the ratio $[Ca^{2+}]_{SR}/K_{app}$. As shown later, this ratio is the main determinant of the time course of $[Ca^{2+}]_{SR}(t)$ derived from the $\Delta A_{TMX}$ signal. Unfortunately, even though $f_{TMX}$ and the ratio $[Ca^{2+}]_{SR}/K_{app}$ are now well established, the actual value of $[Ca^{2+}]_{SR,R}$ is uncertain because of uncertainty in $K_{app}$, which is mainly caused by the likelihood of a significant amount of binding of TMX to sites in the SR. This effect of binding along with a dependence of the $K_{e}$ of TMX in free solution on TMX concentration was used to obtain a range of $K_{app}$ values and associated $[Ca^{2+}]_{SR,R}$ values in Section 3 of the supplemental text. Four combinations of $K_{app}$ and $[Ca^{2+}]_{SR,R}$ are considered in this article, referred to as cases 1–4. For cases 1–3, the ratio $[Ca^{2+}]_{SR}/K_{app}$ was 0.256, the value above obtained by Lamboley and Pape (2011). Cases 2 and 3 correspond to the lower and upper limits of $[Ca^{2+}]_{SR,R}$, 0.26 and 2.35 mM, respectively, given in Section 3 of the supplemental text. For case 1 (the case used for the figures), $K_{app}$ was assumed to be 2.6 mM, the value determined by Maylie et al. (1987); the corresponding value of $[Ca^{2+}]_{SR,R}$ was 0.6656 mM. For case 4, $K_{app}$ and $[Ca^{2+}]_{SR,R}$ = 2.6 and 3.0 mM, respectively, a combination previously considered possible but now ruled out by results in Lamboley and Pape (2011).

The time course of $[Ca^{2+}]_{SR}$ in the SR was determined with Beer’s law given by

$$
[CaTMX]_{SR}(t) = \frac{\Delta A_{TMX}(t) - \Delta A_{TMX}(FD)}{(\epsilon_{TMX} - \epsilon_{TMX,FD}) l_{SR}}.
$$

(6)

where $l_{SR}$, $\epsilon_{TMX}$, and $\epsilon_{TMX,FD}$ are, respectively, the optical path length through the SR water volume and the extinction coefficients for GaTMX and TMX at 480 nm. $\Delta A_{TMX}(FD)$ is the change in TMX absorbance in response to a “fully depleting” stimulation. The determination of $\Delta A_{TMX}(FD)$ is described in Section 2 of the supplemental text. As shown previously with experiments with just TMX, $[CaTMX]_{SR}$ approaches zero with long depolarizations like those used in this study (Table 1 in Pape et al., 2007). The calibrated values on our setup for $[Ca^{2+}]_{SR}$ and $[Ca^{2+}]_{SR,R}$ are considered in this article, referred to as cases 1–4. For cases 1–3, the ratio $[Ca^{2+}]_{SR}/K_{app}$ was 0.256, the value above obtained by Lamboley and Pape (2011). Cases 2 and 3 correspond to the lower and upper limits of $[Ca^{2+}]_{SR,R}$, 0.26 and 2.35 mM, respectively, given in Section 3 of the supplemental text. For case 1 (the case used for the figures), $K_{app}$ was assumed to be 2.6 mM, the value determined by Maylie et al. (1987); the corresponding value of $[Ca^{2+}]_{SR,R}$ was 0.6656 mM. For case 4, $K_{app}$ and $[Ca^{2+}]_{SR,R}$ = 2.6 and 3.0 mM, respectively, a combination previously considered possible but now ruled out by results in Lamboley and Pape (2011).
The derivation of this equation assumes that: (a) the Ca–TMX binding reaction is in instantaneous equilibrium with \([Ca^2+]_{SR}\), a valid assumption for the time scale of interest here given that it appears that any delay would have to be on the order of 1 ms or less (see text after Eq. 21 in Lamboloe and Pape, 2011), and (b) there is no myoplasmic CaTMX signal. In regards to the second assumption, a myoplasmic CaTMX component could affect the signal, though only at higher rates of SR Ca\(^{2+}\) release. \(\Delta A_{TMX}\) from a myoplasmic \(\Delta A_{TMX}\) signal is estimated to be less than or equal to \(\sim 1.5\%\) of \(\Delta A_{TMX}(FD)\) in the voltage-clamp studies in this article, a result consistent with a small myoplasmic \(\Delta A_{TMX}\) signal measured with action potential stimulation (Fig. 11 of Pape et al., 2007).

An important thing to note with Eq. 7 is that the form of the \([Ca^2+]_{SR}(t)\) signal derived from a \(\Delta A_{TMX}\) signal depends on the ratio \([Ca^2+]_{SR}/K_{TMX}\), whereas its amplitude depends on \([Ca^2+]_{SR}\). As indicated above and shown in Lamboloe and Pape (2011), this ratio should be insensitive to factors affecting \(K_{TMX}\). The relatively small value for this ratio (0.256) means that the \([Ca^2+]_{SR}(t)\) signal is approximately linearly related to the \(\Delta A_{TMX}(t)\) signal.

**Summary of method used to determine the \(\Delta pH\) signal with both phenol red and TMX present: Estimation of \([Ca^2+]_{SR}\)**

The methods used to determine the dye-related absorbance signals with both phenol red and TMX (\(\Delta A_{PR}\) and \(\Delta A_{TMX}\), respectively) and the determination of \(\Delta pH\) from the \(\Delta A_{PR}\) signal are given in Pape et al. (2007). Because of spectral overlap of the two dyes, not all of the information needed to determine \(\Delta pH\) was available from resting absorbance values. The procedure adopted involved adjusting the myoplasmic resting pH (pH\(_R\)) to give a value of \([Ca^2+]_{SR}\) that matched the mean value of 2.035 μM from 20 experiments done with just phenol red present. The procedure is described in Appendices A and B of Pape et al. (2007; specifically, see the start of their p. 354 and a verification of this approach on their p. 329). Because the mean value of 2.035 μM was from \(\Delta pH\) signals not corrected for proton loss, the procedure of adjusting pH\(_R\) was done before the correction for proton loss with Eq. 2, so that the actual value of \([Ca^2+]_{SR}\) is somewhat greater in experiments requiring such a correction for proton loss. An important thing to note is that the assumed value of pH\(_R\) has little effect on the time course of \(\Delta pH\) because \(\Delta pH\) is approximately linearly related to the \(\Delta A_{PR}\) signal (p. 327 of Pape et al., 2007).

**Assessment of Ca-binding properties of Ca buffers in the SR**

This section describes how the \([Ca^2+]_{SR}\) and \([Ca^2+]_{TMX}\) signals are used to assess the Ca-binding properties of intrinsic buffers (IB) in the SR. As indicated by the following equation, total Ca in the SR includes free Ca\(^{2+}\), Ca bound to intrinsic buffers (CaIB), and CaTMX:

\[
[Ca_T]_{SR} = [Ca^{2+}]_{IB} + [CaTMX]_{SR} + [CaIB].
\] (8)

The data are well described by cooperative binding (CB) as described by the Hill equation for the fraction of total Ca-binding sites bound with Ca on the intrinsic buffers (\(f_{CB}\)) given by

\[
 f_{CB} = \frac{[Ca^{2+}]_{SR}^{n}}{[Ca^{2+}]_{IB}^{n} + K_{CB}^{n}},
\] (9)

where \(n\) is the Hill coefficient for cooperative binding, and \(K_{CB}\) is the dissociation constant. The concentration of CaIB is then obtained by combining Eqs. 8–10.

\[
[Ca_{IB}]_{fit-1} = f_{CB}[CaIB]_{max},
\] (10)

where \([CaIB]_{max}\) is the concentration of total Ca-binding sites on the intrinsic buffers for this model. The subscript “fit-1” for \([CaIB]_{fit-2}\) is to distinguish it from \([CaIB]_{fit-2}\) associated with a second model for Ca binding described below. The modeled concentration of total Ca bound in the SR (denoted as \([Ca_T]_{SR,model}\)) is given by

\[
[Ca_T]_{SR,model} = [Ca^{2+}]_{SR} + [CaTMX]_{SR} + [CaIB]_{fit-1},
\] (11)

where \([CaIB]_{fit-2}\) could be either \([CaIB]_{fit-1}\) or \([CaIB]_{fit-2}\) determined from the \([Ca^2+]_{SR}(t)\) signal using either Eq. 10 or 12, respectively. Values of the nonlinear parameters \(n\) and \(K_{CB}\) were obtained by manually adjusting their values to give the minimum sum of squares of the differences between the measured \([Ca_{IB}]_{SR}\) and \([Ca_{IB}]_{SR,model}\) data points.

The second model of Ca binding includes a linear binding (LB) component (binding proportional to \([Ca^2+]_{SR}\) and a cooperative component, the latter again described by the Hill equation for cooperative binding, Eq. 9. The reason for evaluating the possible contribution of linear buffers is to set an upper limit on the contribution of intrinsic linear Ca–binding proteins in the SR (see Section 5 of the supplemental text and the Discussion). For this model of linear binding,

\[
[CaIB]_{fit-2} = f_{LB}^{-1}[Ca_{IB}]_{SR,R}^{-1} \left[\frac{[Ca^{2+}]_{SR}^{n} - [CaTMX]_{SR}^{n} + f_{CA}[CaCB]_{max}}{[Ca^{2+}]_{IB}^{n} + K_{CB}^{n}}\right],
\] (12)

where \(f_{LB}\) is the fraction of total Ca in the SR at rest that displays linear binding, and \([CaCB]_{max}\) is the concentration of Ca-binding sites on the portion of the intrinsic buffers displaying cooperative binding. For a particular fixed value of \(f_{LB}\), the determined values of the parameters \(n\), \(K_{CB}\), and \([CaCB]_{max}\) again provide the best match of the \([Ca_T]_{SR,model}\) to the measured \([Ca_T]_{SR}\) signal as described above.

**Conversion between \([Ca^2+]_{SR}\) and \([Ca_T]_{SR}\) and vice versa**

This section shows how the information about Ca binding to intrinsic buffers from the previous section can be used to estimate the concentration of total calcium in the SR ((\([Ca_T]_{SR}\)) from a value of \([Ca^2+]_{SR}\) or vice versa. The following assumptions are made: (a) the calcium-binding reactions of the intrinsic buffers are fast (as expected for the primary Ca buffer, calsequestrin; see Prieto et al., 1994), and (b) there are no spatial gradients of \([Ca^2+]\) within the SR. In regards to the second assumption, because of diffusional delays, the spatially averaged value of \([Ca^2+]_{SR}\) in the SR is expected to be delayed on the order of 7 ms with respect to changes in \([Ca^2+]_{SR}\) at moderate to high rates of SR Ca\(^{2+}\) release (see Fig. 12 and associated text in Pape et al., 2007). For the purposes of this article, these delays can be ignored, as the processes considered here tend to be associated with changes in \([Ca^2+]_{SR}\) or \([Ca^2+]_{SR}\) occurring over hundreds of milliseconds. With these assumptions and the assumption of a single intrinsic buffer component displaying cooperative binding,

\[
[Ca_{IB}]_{SR} = \left[\frac{[Ca^2+]_{SR}^{n} - [CaTMX]_{SR}^{n} + [CaCB]_{max}}{[Ca^{2+}]_{IB}^{n} + K_{CB}^{n}}\right]^{1/n} + [CaTMX]_{SR},
\] (13)

obtained by combining Eqs. 8–10.

Possible combinations of values for \([CaCB]_{max}\), \(n\), and \(K_{CB}\) in Eq. 13 include the valid cases (cases 1–3) tabulated in Table 1. In an experiment in which TMX is the only indicator, \([Ca^2+]_{SR}(t)\) and \([CaTMX]_{SR}(t)\) are determined as described above with Eqs. 7 and 6, respectively, using the values of \([Ca^2+]_{SR}\) and \([CaTMX]_{SR}\) associated with the particular case, thereby giving all of the information needed to calculate \([Ca_{IB}]_{SR}(t)\) with Eq. 13. As described...
below, it is also of interest to derive a \([Ca^{2+}]_{SR}\) signal from a measured \([CaT]_{SR}\) signal in an experiment with no TMX (in which case the last term in Eq. 13 is zero). The brute-force approach adopted to determine \([Ca]^{2+}\) in this case is to step through values of \([Ca^{2+}]_{SR}\) until the \([CaT]_{SR}\) value determined with Eq. 13 matched the measured value with an error of <0.1%.

Online supplemental material
The online supplemental material has five sections. Section 1 reviews and provides additional justification of a previously introduced modification of the EGTA/phenol red method to correct for proton loss (or its equivalent) to give \(\Delta pH_{cor}\) used in Eq. 2. Section 2 describes the estimation of \(\Delta pH_{ axial}\) that would be reached at full depletion of Ca from the SR or \(\Delta pH_{MD}\) (FD). Section 3 starts with a correction of the ratio of myoplasmic to SR water volume \((V_{myo}/V_{SR})\) followed by updates of values in Lambloley and Pape (2011) with this corrected ratio, in particular, values of \(K_{pp}\) and \([Ca^{2+}]_{SR}\) used in this article. Section 4 reviews the method for imposing the constraints of matching slope and value at the end of the final pulse for the \([CaT]_{SR}\) and \([Ca^{2+}]_{SR}\) signals. Section 5 evaluates the possibility of a linear binding component of intrinsic Ca buffers in the SR. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201110730/DC1.

RESULTS
\([CaT]_{SR}(t)\) and \([Ca^{2+}]_{SR}(t)\) in response to a fully depleting stimulation
Fig. 1 shows \([CaT]_{SR}\) and \([Ca^{2+}]_{SR}\) signals measured with the EGTA/phenol red method and TMX. The top trace in Fig. 1 A shows the measured voltage. The next two traces (from top to bottom) are the dye-related absorbance changes for phenol red at 570 nm and TMX at 480 nm, \(\Delta APR\) (570) and \(\Delta ATPMX\) (480), respectively. The \(\Delta ATPMX\) signal in the top pair of traces was scaled by 1.8 so that the two signals have the same heights at the start to highlight the nonlinearity of the two signals. The next pair of traces includes the same \(\Delta ATPMX\) (480) signal (calibration bar not depicted) and the \([Ca^{2+}]_{SR}\) signal determined from the \(\Delta ATPMX\) signal using Eq. 7 in Materials and methods with the parameters for case 1 ([Ca]^{2+})_{SR} and \(K_{pp}\) values of 0.6656 and 2.6 mM, respectively. The dashed line indicates the final level approached by the \(\Delta ATPMX\) signal, \(\Delta ATPMX(\infty)\).

The next trace in Fig. 1 A is \(\Delta pH\) obtained from the \(\Delta APR\) signal. Because the \(\Delta pH\) signal declined to a steady level with no reversal in the direction during the pulse to \(-30\) mV, the correction of the final slope based on a putative loss of protons from the myoplasm was not done, so that \(\Delta pH_{cor} = \Delta pH\) in Eq. 2. The \([CaT]_{SR}\) signal in the bottom pair of traces was obtained from the \(\Delta pH_{cor}\) signal with Eqs. 2 and 3. As seen with these equations, the \([CaT]_{SR}\) signal is linearly related to the \(\Delta pH_{cor}\) signal. The \([CaT]_{SR}\) signal is plotted with the \([Ca^{2+}]_{SR}\) signal determined above; their calibration bars are shown on the left and right sides, respectively. Because the time courses of the \([CaT]_{SR}\) and \([Ca^{2+}]_{SR}\) signals are now well determined, these results indicate nonlinear buffering of \([Ca^{2+}]_{SR}\) in the SR.

Calcium binding by intrinsic Ca buffers in the SR can be characterized by a single cooperative binding reaction
The black points in Fig. 1 B plot \([CaT]_{SR}\) Versus \([Ca^{2+}]_{SR}\) using all of the points in the signals shown in the bottom pair of traces in Fig. 1 A up to the end of the long pulse. The \([Ca^{2+}]_{SR}\) signal was determined from the \(\Delta ATPMX\) signal using Eq. 7 with \([Ca^{2+}]_{SR} = 0.6656\) mM, the ratio \([Ca^{2+}]_{SR}/K_{pp} = 0.256\) (the \([Ca^{2+}]_{SR}\) and \(K_{pp}\) values are those for case 1 in Table 1). The next trace shows the \(\Delta pH\) signal determined from the \(\Delta APR\) signal. In the bottom pair of traces, the \([Ca^{2+}]_{SR}\) signal is the same one shown above, and the \([CaT]_{SR}\) signal was determined from the \(\Delta pH\) signal. (See text for details). (B) The black dots plot values of \([CaT]_{SR}\) versus \([Ca^{2+}]_{SR}\) from the signals in A. The blue line plots \([Ca^{2+}]_{SR}\) and the red curve plots the sum of \([Ca^{2+}]_{SR}\) and \([Ca^{2+}]_{MX}\). The \([Ca^{2+}]_{MX}\) component of this sum was determined from the \(\Delta ATPMX\) signal using Eq. 6. (C) The black dots plot the estimated concentration of Ca in the SR bound to intrinsic buffers, \([CaB]\); they are given by \([CaT]_{SR} - ([Ca^{2+}]_{SR} - [CaTMX])\). The red curve is a least-squares best fit of a cooperative binding function (Eqs. 9 and 10) to the \([CaB]\) data. A description of the fit and the best-fit parameters is given in the text. Fiber reference, 724011; time after saponin treatment, 77 min; time after adding dye, 57 min; sarcomere length, 3.9 µm; fiber diameter, 84 µm; holding current, \(-50\) nA; 17.0°C.

Figure 1. Phenol red and TMX absorbance signals and cooperative Ca^{2+}–calsequestrin binding reaction. (A) The top trace shows voltage measured in the V1 end pool. The top pair of traces shows the \(\Delta APR\) and \(\Delta ATPMX\) signals determined from absorbance signals measured at the same time as the voltage trace. This same \(\Delta ATPMX\) signal (without a scale bar) is shown with its associated \([Ca^{2+}]_{SR}\) signal in the next pair of traces (from top to bottom). The \([Ca^{2+}]_{SR}\) signal was determined from the \(\Delta ATPMX\) signal using Eq. 7 with \([Ca^{2+}]_{SR} = 0.6656\) mM, the ratio \([Ca^{2+}]_{SR}/K_{pp} = 0.256\) (the \([Ca^{2+}]_{SR}\) and \(K_{pp}\) values are those for case 1 in Table 1). The next trace shows the \(\Delta pH\) signal determined from the \(\Delta APR\) signal. In the bottom pair of traces, the \([Ca^{2+}]_{SR}\) signal is the same one shown above, and the \([CaT]_{SR}\) signal was determined from the \(\Delta pH\) signal. (See text for details). (B) The black dots plot values of \([CaT]_{SR}\) versus \([Ca^{2+}]_{SR}\) from the signals in A. The blue line plots \([Ca^{2+}]_{SR}\) and the red curve plots the sum of \([Ca^{2+}]_{SR}\) and \([Ca^{2+}]_{MX}\). The \([Ca^{2+}]_{MX}\) component of this sum was determined from the \(\Delta ATPMX\) signal using Eq. 6. (C) The black dots plot the estimated concentration of Ca in the SR bound to intrinsic buffers, \([CaB]\); they are given by \([CaT]_{SR} - ([Ca^{2+}]_{SR} - [CaTMX])\). The red curve is a least-squares best fit of a cooperative binding function (Eqs. 9 and 10) to the \([CaB]\) data. A description of the fit and the best-fit parameters is given in the text. Fiber reference, 724011; time after saponin treatment, 77 min; time after adding dye, 57 min; sarcomere length, 3.9 µm; fiber diameter, 84 µm; holding current, \(-50\) nA; 17.0°C.
pulse to −30 mV. The blue line in Fig. 1 B plots [Ca\(^{2+}\)]\(_{SR}\) and the red curve plots the sum of [Ca\(^{2+}\)]\(_{SR}\) and [CaTMX]\(_{SR}\), the latter obtained from the ΔATMX signal using Eq. 6. It is clear that the contributions of free Ca\(^{2+}\) and CaTMX to total SR Ca are relatively small until the SR approaches full depletion.

The black points in Fig. 1 C plot [CaIB] versus [Ca\(^{2+}\)]\(_{SR}\), where [CaIB] is given by difference of the black points and red curve in Fig. 1 B. The red curve in Fig. 1 C is the least-squares best fit to the [CaIB] data of CaIB based on the [Ca\(^{2+}\)]\(_{SR}\) signal and the cooperative binding function given by Eqs. 9 and 10. It is clear that this model of cooperative binding fits the [CaIB] data very well. The least-squares best-fitted values of [CaIB]\(_{max}\), the Hill coefficient (n), and K\(_{CB}\) were 36.0 mM, 3.95, and 0.73 mM, respectively. The two hashed lines plot the first term of Eq. 12, which gives the amount of Ca bound to linear buffers (Ca bound proportional to [Ca\(^{2+}\)]\(_{SR}\)) with assumed fractions of [CaT]\(_{SR,R}\) displaying linear binding (f\(_{LB}\)) of 0.1 and 0.2, as indicated. As clearly seen, the curve with f\(_{LB}\) = 0.2 is above the measured data at the start of the binding curve (see arrow), which would indicate that <20% of the intrinsic buffers display linear binding. However, as indicated later, the data from this experiment would, in fact, be consistent with f\(_{LB}\) = 0.2 (but not 0.3) if proton loss had obscured a linear [CaT]\(_{SR}\) component as full depletion of the SR was approached.

Fig. S3 shows results from another experiment with the same format as that shown in Fig. 1, except that full depletion was achieved in multiple steps. As seen with Fig. S3, the ΔpH signal displayed a reversal in direction during depolarizing steps so that the correction for the putative loss of protons was applied to obtain the corrected ΔpH signal (ΔpH\(_{cor,1}\)) having a slope of zero at the end of the last pulse to −20 mV. Fig. 2 shows results with this same multistep experiment illustrating the main approach used to evaluate Ca buffering in the SR in this study. The top trace in Fig. 2 A shows the voltage signal. The middle set of traces shows [Ca\(^{2+}\)]\(_{SR}\) (blue), [CaTMX]\(_{SR}\) (green), [CaT]\(_{SR}\) (black; modified slightly as described below), and [CaT]\(_{SR, model}\) (red). The [CaT]\(_{SR, model}\) signal was given by the model of a single component displaying cooperative binding (Eqs. 9–11). The best-fit values of the parameters for the cooperative binding reaction ([CaIB]\(_{max}\), n, and K\(_{CB}\)) involved minimizing the sum of squares of the difference between the [CaT]\(_{SR, model}\) and [CaT]\(_{SR}\) data using all of the points up to the end of the last pulse (all of the points shown here). It is clear that the [CaT]\(_{SR, model}\) trace closely matches the [CaT]\(_{SR}\) trace, indicating that this model accounts well for the nonlinearity between the underlying ΔATMX and ΔAPR traces.

The slight modification of the [CaT]\(_{SR}\) signal involved matching the final slopes and final values at the end of the pulse to −20 mV of the [CaT]\(_{SR}\) and [CaT]\(_{SR, model}\) signals as described previously in Pape et al. (2007) and summarized in Section 4 of the supplemental text. The fact that this modification was slight is seen by almost no
difference between the underlining $\Delta p$H signals shown at the bottom of Fig. 2 A (the black trace labeled “$\Delta p$H” is with the imposed constraints, and the red trace is the same $\Delta p$Hcor,1 signal in Fig. S3 A). Although the imposition of the constraints does not have much effect for the model of a single component displaying cooperative binding shown here, it does have a significant effect when assessing the possibility of linear binding as described later. All of the results in the rest of the article were obtained with the constraints of matching slopes and values at the end of the final stimulation. As indicated in Materials and methods, four combinations of $[Ca^{2+}]_{SR,R}$ and $K_{app}$ for TMX in the SR are considered in this article. As for case 1, the $[CaT]_{SR,model}$ signal closely matched the $[CaT]_{SR}$ signal for cases 2–4, giving least-squares fits very similar to that in Fig. 2 A (the minimized sum of squares in order of cases 1–4 were: 83.76, 84.59, 84.33, and 81.32 mM²). Therefore, all four cases can account equally well for the nonlinearity between the underlying $\Delta A_{TMX}$ and $\Delta A_{PR}$ traces.

Table 1

| Fiber  | $K_{CB}$ mM | $n$ | $[CaIB]_{max}$ mM | $f_{CB,R}$ | $[CaIB]_{R}$ mM | r | q |
|-------|----------|-----|------------------|----------|----------------|---|---|
| Case 1: $[Ca^{2+}]_{SR,R} = 0.6656$ mM, $K_{app} = 2.6$ mM |
| 724011 | 0.75     | 3.79 | 38.2             | 0.389    | 14.9           | 24.9 | 51.7 |
| 211021 | 0.95     | 2.29 | 58.5             | 0.304    | 17.8           | 28.6 | 42.1 |
| 423021 | 0.64     | 2.86 | 35.5             | 0.528    | 18.7           | 30.1 | 38.0 |
| Mean  | 0.78     | 2.98 | 44.0             | 0.407    | 17.1           | 27.9 | 43.9 |
| SEM   | 0.09     | 0.44 | 7.3              | 0.065    | 1.1            | 1.5  | 4.1  |
| Case 2: $[Ca^{2+}]_{SR,R} = 0.26$ mM, $K_{app} = 1.0$ mM |
| 724011 | 0.30     | 3.66 | 40.8             | 0.372    | 15.2           | 68.3 | 134.2 |
| 211021 | 0.38     | 2.24 | 60.7             | 0.299    | 18.2           | 78.1 | 109.6 |
| 423021 | 0.25     | 2.83 | 36.2             | 0.528    | 19.1           | 76.9 | 98.1  |
| Mean  | 0.31     | 2.91 | 45.9             | 0.400    | 17.5           | 71.1 | 114.0 |
| SEM   | 0.04     | 0.41 | 7.5              | 0.068    | 1.2            | 4.1  | 10.6 |
| Case 3: $[Ca^{2+}]_{SR,R} = 2.35$ mM, $K_{app} = 9.1$ mM |
| 724011 | 2.34     | 4.68 | 26.6             | 0.505    | 13.4           | 7.1  | 13.2 |
| 211021 | 2.95     | 2.51 | 45.0             | 0.361    | 16.2           | 8.2  | 11.1 |
| 423021 | 2.15     | 3.12 | 30.3             | 0.569    | 17.2           | 8.6  | 9.9  |
| Mean  | 2.48     | 3.44 | 34.0             | 0.478    | 15.6           | 8.0  | 11.4 |
| SEM   | 0.24     | 0.65 | 5.6              | 0.062    | 1.1            | 0.4  | 1.0  |
| Case 4: $[Ca^{2+}]_{SR,R} = 3.0$ mM, $K_{app} = 2.6$ mM |
| 724011 | 2.58     | 3.44 | 20.2             | 0.629    | 12.7           | 5.6  | 5.4  |
| 211021 | 2.55     | 2.05 | 26.7             | 0.583    | 15.5           | 6.4  | 4.4  |
| 423021 | 2.08     | 2.48 | 23.2             | 0.713    | 16.5           | 6.7  | 3.9  |
| Mean  | 2.49     | 2.66 | 23.4             | 0.642    | 14.9           | 6.2  | 4.6  |
| SEM   | 0.16     | 0.41 | 1.9              | 0.038    | 1.1            | 0.3  | 0.4  |

Column 1 gives the fiber reference. See text for an explanation of the values in columns 2–8. For reference 724011, 211021, and 423021, respectively, the ranges of values of $k$ for proton loss (Eqs. S1 and S2 and associated supplemental text) used to impose the constraints of matched slopes and values of the $[CaT]_{SR}$ and $[CaT]_{SR,model}$ signals for cases 1–4 were 0.8–1.7, 8.3–8.6, and 10.9–11.5 × 10⁻⁵ pH units/ms. The corresponding ranges of pHmin were $−0.187$ to $−0.190$, $−0.215$ to $−0.216$, and $−0.222$ to $−0.225$. Additional details for these fibers are given in the legends (for Fibers 724011, 211021, and 423021, respectively) of Tables 2 and 3 of Pape et al. (2007).
Properties of intrinsic Ca\(^{2+}\) buffers in the SR assumed to arise from a single population displaying cooperative binding

Table 1 gives values of parameters obtained for the four cases above. Columns 2, 3, 4, and 5 give values of \(K_{CB}, n, [CaIB]_{max}\), and \(f_{CB,R}\), respectively. Column 6 gives the concentration of calcium bound to the intrinsic buffers in the resting state (denoted as \([CaIB]_R\)), given by the product \([CaIB]_{max} \times f_{CB,R}\) (column 4 times column 5). Columns 7 and 8 give parameters related to the importance of Ca\(^{2+}\) buffering by the intrinsic buffers. Column 7 is the ratio \([CaIB]_R/[CaT]_{SR,R}\) (denoted as \(r\)). Column 8 gives \(\eta\), the ratio of the number of Ca\(^{2+}\) ions originating from the intrinsic buffers as opposed to the pool of free Ca\(^{2+}\) in the SR at the start of Ca\(^{2+}\) release with a nominally physiological resting calcium load in the SR. This parameter is given by the equation

\[
q = r \cdot \frac{d\left(\frac{f_B}{f_{CB,R}}\right)}{d\left(\frac{Ca^{2+}_{SR}}{Ca^{2+}_{SR,R}}\right)}.
\]

(14)

evaluated at the start of release. The second factor is the initial slope of the normalized Ca-binding curve as shown in Fig. 2 B, i.e., the slope at point (1, 1). It is clear from all of the possible values of \(q\) (cases 1–3) that intrinsic buffers are the source of most of the Ca\(^{2+}\) released from the SR.

Release permeability versus DCRR at \(-20\) mV

Fig. 3 shows signals associated with an 800-ms voltage-clamp pulse to \(-20\) mV using the EGTA/phenol red method to monitor \([CaT]_{SR}\) (TMX was not present). The top trace in Fig. 3 A shows voltage. The next trace shows the \(\Delta Ca_{SR}\) (570) signal. In the next pair of traces, the black and blue \([CaT]_{SR}\) signals were based, respectively, on the \(\Delta pH\) signal not corrected and corrected for proton loss to give a final slope of zero at the end of the pulse to \(-20\) mV.

In the next pair of traces, the trace labeled \([Ca^{2+}]_{SR}\) (the black trace) was determined from the corrected \([CaT]_{SR}\) trace using the brute-force approach with Eq. 13 and the average parameters for \(K_{CB}, n, [CaIB]_{max}\) for case 1 in Table 1. The blue trace is the same corrected \([CaT]_{SR}\) trace normalized to have the same starting height as the \([Ca^{2+}]_{SR}\) trace. As expected, cooperative binding results in a slower rate of decline of \([Ca^{2+}]_{SR}\) compared with \([CaT]_{SR}\).

The bottom three traces in Fig. 3 A, shown on an expanded time scale, were derived from the \([CaT]_{SR}\) and \([Ca^{2+}]_{SR}\) signals. The top one is the rate of SR Ca\(^{2+}\) release, i.e., \(-[CaT]_{SR}/dt\). The next trace is the DCRR given by Eq. 4 in Materials and methods. The final part of the \([CaT]_{SR}\) signal is well described by an exponential function (not depicted) with a time constant, \(\tau\), of 56.43 ms; the red line with the DCRR signal is 100 divided by this value of \(\tau\) or 1.77% per ms. The DCRR signal is typical of that observed previously. After the initial peak, the DCRR signal declines rapidly to an approximately constant level in some cases or shows a subsequent modest rise in the case of the signal in Fig. 3 A or an even greater rise in the case of Pizarro and Ríos (2004).

As indicated in the Introduction, release permeability, given by

\[
\text{release permeability} = \frac{-d[CaT]_{SR}/dt}{[Ca^{2+}]_{SR}}
\]

(15)
is a more appropriate measure of the extent of activation of SR Ca\(^{2+}\) release channels. In contrast to the DCRR, the associated release permeability shown at the bottom of Fig. 3 A decreases significantly with time as the SR becomes depleted of calcium. The peak of the release permeability had a value of 0.556 ms\(^{-1}\). After this peak, the signal rapidly declined (exponential time constant of 3.70 ms) to a quasi-steady level of 0.312 ms\(^{-1}\) for \(-50\) ms. This level is indicated by the first red line segment (line segments like this plot the average value of the points between the ends of the line segment). The rapid decline is caused by calcium inactivation of Ca\(^{2+}\) release (see e.g., Jong et al., 1995). After the quasi-steady level, release permeability decreased over the next \(-100\) ms to a level of 0.0875 ms\(^{-1}\) (indicated by the second red line segment), which corresponds to a 3.6-fold decrease (0.312 \div 0.0875) from the quasi-steady level. Because intramembranous charge movement continued to move slowly during the period of this 3.6-fold decrease (not depicted), and because such additional intramembranous charge movement appears to activate additional SR Ca\(^{2+}\) release (see Fig. 4 and associated text in Pape et al., 1996), the factor of 3.6 likely underestimates the change in whatever mechanism(s) is responsible for the decrease.

The extent of the late decrease in release permeability is relatively insensitive to the assumed value of \([Ca^{2+}]_{SR,R}\) and the presence of a linear Ca-binding component

Fig. 3 B compares release permeability signals obtained with different sets of parameters used in the Ca-binding models. The top trace shows the start of the same voltage pulse shown in Fig. 3 A. The black trace in each of the four pairs of release permeability signals is the same release permeability shown in Fig. 3 A (associated with the case 1 parameters). The red trace in each pair of traces (not visible for the top pair of traces because they are almost identical) is associated with a different set of conditions. All of the release permeability signals are normalized by their peak values given in the figure legend. The line segments give the average values for the quasi-steady level and the late
The red traces in the third and fourth pairs of release permeability signals in Fig. 3 B were obtained with the model including linear Ca binding (Fig. S4 and associated text in the supplemental material) with values of \( f_{LB} \) of 0.1 and 0.2, respectively, as indicated. The values for the ratio of the quasi-steady level to late level of the release permeability signal with \( f_{LB} = 0.1 \) and 0.2 were 3.4 and 2.6. In summary, the only condition that significantly affected the ratio of the quasi-steady level to late level of the release permeability signal was with the 20% upper limit for linear binding, a condition that produced only a modest (28%) decrease in the estimated decline in release permeability compared with that obtained with case 1 (28% = 100 × (3.6–2.6)/3.6).

DCRR and release permeability at −60 and −45 mV as functions of [Ca\(^{2+}\)]\(_{SR}\) or [Ca\(^{2+}\)]\(_{SR}\)

Fig. 4 A plots the DCRR as a function of [Ca\(^{2+}\)]\(_{SR}\) using the DCRR and corrected [Ca\(^{2+}\)]\(_{SR}\) signals in Fig. 3 A (data from case 1). The idea behind this type of plot is...
that the local increase in \([\text{Ca}^{2+}]\) on the myoplasmic side of an open SR \(\text{Ca}^{2+}\) release channel should be proportional to the flux of \(\text{Ca}^{2+}\) ions through the channel (based on solutions of the diffusion equation in Neher, 1986, Stern, 1992, and Pape et al., 1995). Assuming that the driving force for SR \(\text{Ca}^{2+}\) release is proportional to \([\text{Ca}_1]\_\text{SR}\), a plot of DCRR versus \([\text{Ca}_1]\_\text{SR}\) would be expected to give information about the extent of activation of \(\text{Ca}^{2+}\) release channels as a function of a value proportional to \([\text{Ca}^{2+}]\) at a Ca regulatory site on the myoplasmic side of the channel. The horizontal scale on the bottom gives \([\text{Ca}_1]\_\text{SR}\) referred to the SR volume, whereas the horizontal scale on top gives \([\text{Ca}_1]\_\text{SR}\) referred to myoplasmic volume. The latter is shown here to help with the comparisons below with earlier studies. 

Fig. 4 B plots release permeability versus \([\text{Ca}^{2+}]_\text{SR}\) from the same experiment, obtained from the release permeability signal and \([\text{Ca}^{2+}]_\text{SR}\) signals in Fig. 3 A. Because the driving force for SR \(\text{Ca}^{2+}\) release should be given by \([\text{Ca}^{2+}]_\text{SR}\) instead of \([\text{Ca}_1]\_\text{SR}\) this plot (as opposed to Fig. 4 A) should provide the correct dependence of the extent of activation of SR \(\text{Ca}^{2+}\) release channels versus a value proportional to \([\text{Ca}^{2+}]\) at any myoplasmic Ca regulatory site within the vicinity of an open SR \(\text{Ca}^{2+}\) release channel. The arrow shows the start of the so-called quasi-steady level. The higher and lower red line segments plot the same constant values for the quasi-steady and late levels, respectively, plotted with the release permeability signals for case 1 in Fig. 3.

Fig. 4 C plots DCRR versus \([\text{Ca}_1]\_\text{SR}\) for pulses to \(-60\) mV (black circles) and \(-45\) mV (red circles). (The scale bar labels of 1.0 and 0.1%/ms correspond to the \(-45\)- and \(-60\)-mV data, respectively.) This data are a subset of the same data plotted in Fig. 5 A of Pape et al. (2002). In brief, in contrast to the data in Fig. 4 (A and B) of this article in which the variation in \([\text{Ca}_1]\_\text{SR}\) was achieved during a single stimulation, the data in Fig. 4 C was obtained by varying \([\text{Ca}_1]\_\text{SR, R}\), i.e., the SR Ca load before

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**Figure 4.** DCRR versus \([\text{Ca}_1]\_\text{SR}\) and release permeability versus \([\text{Ca}^{2+}]_\text{SR}\). (A) DCRR versus \([\text{Ca}_1]\_\text{SR}\) obtained from the DCRR and \([\text{Ca}_1]\_\text{SR}\) versus time signals in Fig. 3 A. (B) Release permeability versus \([\text{Ca}^{2+}]_\text{SR}\) also determined from the corresponding signals in Fig. 3 A. Details for the experiment in A and B are given in the legend of Fig. 3. Details for the experiment in C and D are given at the end of this legend. (C) The red circles plot versus \([\text{Ca}_1]\_\text{SR}\) the values at the peaks of DCRR signals in response to voltage-clamp steps to \(-45\) mV. Each point in this case is from a different stimulation done 5 min apart. The variation of \([\text{Ca}_1]\_\text{SR}\) in this case was achieved by varying the resting SR Ca load, \([\text{Ca}_1]\_\text{SR, R}\). Each black circle plots the steady level of DCRR reached during a 300-ms voltage-clamp step to \(-60\) mV measured 400 ms before the pulse to \(-45\) mV. (D) Release permeability versus \([\text{Ca}^{2+}]_\text{SR}\) obtained from the data in C. Values for \([\text{Ca}^{2+}]_\text{SR}\) on the abscissa were obtained by converting the values of \([\text{Ca}_1]\_\text{SR}\) in A with Eq. 13 using the average values for \([\text{Ca}_1]\_\text{SR, R}\), \(n\), and \([\text{Ca}^{2+}]_\text{SR, max}\) for case 1 in Table 1. Each release permeability value was obtained by multiplying the corresponding DCRR value in C by the ratio \([\text{Ca}_1]\_\text{SR}\)/\([\text{Ca}^{2+}]_\text{SR}\) for that point. The fiber reference for the experiment in C and D is 510971; details for this experiment are given in the legends of Fig. 2 in Pape and Carrier (1998) and Fig. 5 of Pape et al. (2002). The internal and external solutions were the same as those given in Materials and methods for the two points with the highest concentrations of Ca. After these two points were obtained, the internal solution in the end pools was exchanged for the same Cs internal solution except that there was no Ca present and all of the other points were obtained with this Ca-free internal solution (see Pape and Carrier, 1998).
each stimulation. This variation was achieved by giving fully depleting stimulations every 5 min (the interval of time between neighboring points in Fig. 4 C) in the presence of Ca-free internal and external solutions.

As \([\text{Ca}_{\text{T}}]_{\text{SR}}\) increased from near zero to \(\sim 250 \mu\text{M}\), the DCRR values at \(-60\) mV increased from near zero to its peak value near \(0.13\% /\text{ms}\). This increase in DCRR was attributed to CICR. The large decrease in DCRR at \(-60\) mV when \([\text{Ca}_{\text{T}}]_{\text{SR}}\) increased to \(1,000 \mu\text{M}\) was attributed to Ca inactivation. Because there was no effect of 8 mM BAPTA on the release permeability at \(-60\) mV at the peak near \(250 \mu\text{M}\) or on the steady level of release permeability at \([\text{Ca}_{\text{T}}]_{\text{SR}}\) values \(>1,000 \mu\text{M}\), the CICR and Ca inactivation processes were interpreted as acting in an autoregulatory manner (Fénelon and Pape, 2002). (The term “autoregulatory” refers to the regulation of the activity of a channel by Ca\(^{2+}\) released from the same channel via binding to a site on the channel.) In brief, the 8-mM concentration of BAPTA should produce a fourfold decrease in the value of \([\text{Ca}^{2+}]_{\text{SR}}\) compared with the value in the absence of BAPTA, at a putative CICR regulatory site on a Ca\(^{2+}\) release channel that is an immediate neighbor of an open SR Ca\(^{2+}\) release channel (spaced 30 nm apart), while having little effect on the open channel itself. Inhibition of release permeability by BAPTA or a similar type of fast Ca buffer would indicate that recruitment is involved, although not necessarily ruling out some autoregulatory CICR process, whereas a negative effect of BAPTA would indicate that recruitment of neighboring channels is not involved.

The forms of the DCRR data at \(-45\) and \(-60\) mV in Fig. 4 C are very similar up until the peak near \(250 \mu\text{M}\). The 10-fold greater values at \(-45\) mV compared with \(-60\) mV are attributable to the relative degrees of voltage activation as determined from measurements of DCRR and the Q\(_{\text{SDP}}\) component intramembranous charge movement (thought to reflect the movement of voltage sensors for SR Ca\(^{2+}\) release in the T-system) at low SR Ca contents (Pape and Carrier, 2002; see their Table 2; see also Fig. 5 C of Pape et al., 2002). As a result, the Ca-feedback mechanism responsible for the increasing DCRR with increasing \([\text{Ca}_{\text{T}}]_{\text{SR}}\) from near zero to \(250 \mu\text{M}\) must almost certainly act in the same way at both \(-60\) and \(-45\) mV. After the peak at \(250 \mu\text{M}\), DCRR at \(-45\) mV compared with that at \(-60\) mV shows much less of a progressive decline with increasing \([\text{Ca}_{\text{T}}]_{\text{SR}}\). This discrepancy was interpreted as some type of extra activation component of Ca\(^{2+}\) release at higher values of \([\text{Ca}_{\text{T}}]_{\text{SR}}\) present at \(-45\) mV but not at \(-60\) mV. In contrast to the lack of effect of 8 mM BAPTA at \(-60\) mV, the values of the peak of the DCRR signal at \(-45\) mV at the larger values of \([\text{Ca}_{\text{T}}]_{\text{SR}}\) were attenuated somewhat by 8 mM BAPTA (Pape et al., 2002). These results were considered as evidence that neighboring channels are recruited by CICR at the peak of the DCRR signal at \(-45\) mV at the higher (and physiological) levels of \([\text{Ca}_{\text{T}}]_{\text{SR}}\), producing an enhanced rate of Ca\(^{2+}\) release at \(-45\) mV greater than sixfold that expected from voltage activation alone.

Because the interpretations of the results in Fig. 4 C and related experiments were based on the DCRR signal, it is of interest to know whether or not they hold for the relationship between release permeability versus \([\text{Ca}^{2+}]_{\text{SR}}\), the more appropriate signals for assessing Ca-feedback processes. Fig. 4 D plots release permeability versus \([\text{Ca}^{2+}]_{\text{SR}}\) obtained from the data in Fig. 4 C using the procedures described with Eq. 13 in Materials and methods for estimating \([\text{Ca}^{2+}]_{\text{SR}}\) values from \([\text{Ca}_{\text{T}}]_{\text{SR}}\) values and for converting DCRR signals to release permeability signals. The parameters used for the conversion were again those given for case 1 in Table 1. The forms of the release permeability versus \([\text{Ca}^{2+}]_{\text{SR}}\) plots in Fig. 4 D are significantly different than the DCRR versus \([\text{Ca}_{\text{T}}]_{\text{SR}}\) plots in Fig. 4 C. For the \(-60\)-mV data, there is a significantly smaller fractional decrease from the peak to the approximately steady level of release permeability when \([\text{Ca}^{2+}]_{\text{SR}}\) was between \(\sim 0.5\) and \(0.66 \mu\text{M}\). For the \(-45\)-mV data, there is no longer a peak; rather, the data increases monotonically with increasing \([\text{Ca}^{2+}]_{\text{SR}}\). Despite these differences, the relationship between the two sets of data remained about the same. In particular, as with the DCRR results, the form of the plots at \(-45\) and \(-60\) mV are the same up to a certain level—\([\text{Ca}^{2+}]_{\text{SR}}\) near \(0.3 \mu\text{M}\)—and then they diverge. Importantly, the ratio of the release permeability at \(-60\) mV to that at \(-45\) mV at the highest SR Ca contents (0.326; evaluated with the approximately constant levels at the larger SR Ca contents given by the red line segments) is within 5% of the comparable ratio of the DCRR in Fig. 4 C (0.343). Because of this similarity, there is no need to modify the interpretation and quantitative estimate above of a greater than sixfold enhancement of Ca\(^{2+}\) release at \(-45\) mV at higher SR Ca contents relative to that expected from voltage activation alone.

The quasi-steady level of release permeability at \(-20\) mV is greater than expected from voltage activation

Interestingly, the shape of the release permeability versus \([\text{Ca}^{2+}]_{\text{SR}}\) plot of the peaks of the release permeability signal at \(-45\) mV in Fig. 4 D is very similar to that of the data at \(-20\) mV for values of \([\text{Ca}^{2+}]_{\text{SR}}<0.55 \mu\text{M}\) (to the left of the arrow in Fig. 4 B), the point at which the rapid decline from the peak of the release permeability signal ends and the quasi-steady level begins. This similarity suggests that release during the quasi-steady level at \(-20\) mV is also significantly enhanced beyond that predicted by voltage activation alone. Given that release permeability at \(-60\) mV involves autoregulation by CICR and Ca inactivation, one can use the combined results at \(-20\) and \(-60\) mV to account for these components, thereby giving an estimate of the increase in release permeability associated with the recruitment of
neighboring by CICR, as done previously for the combined \(-45\)- and \(-60\)-mV data as summarized above. The assessment in this case uses a point of reference given by the plus symbol in Fig. 4 B, which corresponds to the peak of the release permeability data at \(-60\) mV. The release permeability at the quasi-steady level of release at \(-20\) mV (higher red line segment in Fig. 4 B) is 0.312 ms\(^{-1}\), 1.60 fold greater than the value of 0.195 ms\(^{-1}\) at the point of reference. The steady-state release permeability at \(-60\) mV at the plateau level (indicated by the lower red line segment in Fig. 4 D) was 0.00541 ms\(^{-1}\), 0.49 times less than the value of 0.0111 ms\(^{-1}\) at the point of reference. From these values, it is estimated that quasi-steady level of release permeability at \(-20\) mV is enhanced as a result of recruitment of neighboring channels by CICR (or its equivalent) by a factor of 3.3 (3.3 = 1.60 \(\div\) 0.49). Because not much extra intramembranous charge had moved between the point of reference and the quasi-steady level of release (not depicted), a difference in the degree of voltage activation is not considered here, although it is noted that it would increase the factor of 3.3 somewhat. In support of a recruitment of neighboring SR Ca\(^{2+}\) release channels being involved, release permeability during the quasi-steady level of release at \(-20\) mV is significantly reduced by BAPTA, although at least some of this reduction was associated with long-term irreversible changes apparently caused by higher concentrations of BAPTA. Results (not depicted) indicated a reduction in release permeability of at least \(~15\%\) that was not associated with the long-term irreversible effects.

**DISCUSSION**

The first part of this article reassesses the properties of intrinsic Ca\(^{2+}\) buffers in the SR of twitch skeletal muscle fibers from frog based on measurements of [Ca\(^{2+}\)]\(_{\text{SR}}\) versus [Ca\(^{2+}\)]\(_{\text{SR}}\). The first part of the Discussion further evaluates these results, including an assessment of the degree to which the intrinsic buffering is likely caused by calsequestrin.

The value of [Ca\(^{2+}\)]\(_{\text{SR, R}}\) is likely to be close to that given by case 1 in Table 1

Although the time course of the [Ca\(^{2+}\)]\(_{\text{SR}}\)(t) signal can now be reliably determined from the absorbance signal from TMX, the magnitude of [Ca\(^{2+}\)]\(_{\text{SR}}\)(t) remains uncertain. Given the large range of possible [Ca\(^{2+}\)]\(_{\text{SR,R}}\) values obtained with TMX (0.26–2.35 mM), it would be helpful if a more likely value could be identified. This section argues that the most likely actual physiological value of [Ca\(^{2+}\)]\(_{\text{SR}}\) is close to that for case 1 in Table 1, namely 0.67 mM. This assessment assumes that (a) calsequestrin is the primary buffer of Ca in the SR (as supported by results discussed below), and (b) the dissociation constant for calsequestrin (K\(_{\text{CSQ}}\)) in cut muscle fibers is the same as that determined with disrupted preparations. Results in column 2 of Table 1 indicate a close correspondence between [Ca\(^{2+}\)]\(_{\text{SR,R}}\) (0.31–2.47 mM) and K\(_{\text{CSQ}}\) (0.26–2.35 mM), the latter assumed to be nearly equal to K\(_{\text{CSQ}}\) following from the first assumption above. The reason for favoring case 1 is that the average value of 0.78 mM for K\(_{\text{CSQ}}\) for this case in Table 1 is similar to estimates for K\(_{\text{CSQ}}\) in “physiological solutions” (100 or 150 mM KCl; pH 7.5) in disrupted preparations that range from 0.79 to \(~1\) mM (Ostwald and MacLennan, 1974; Aaron et al., 1984; Cozens and Reithmeier, 1984; Krause et al., 1991; Volpe and Simon, 1991; the latter reference from frog gave a value of 0.9 mM; the other references were from rabbit). In addition, the apparently best estimate for the average value of [Ca\(^{2+}\)]\(_{\text{SR,R}}\) in skeletal muscle fibers from mice is 0.42 mM, determined recently with the FRET-based indicator D4cprv (Sztretye et al., 2011a). Although [Ca\(^{2+}\)]\(_{\text{SR,R}}\) in frog and mice might be very different, this result would tend to favor a value of [Ca\(^{2+}\)]\(_{\text{SR,R}}\) similar to that for case 1 of 0.67 mM versus the high value of 2.35 mM given by case 3.

It is also noted that the lower limit of 0.26 mM for [Ca\(^{2+}\)]\(_{\text{SR,R}}\) is unlikely because it corresponds to the unlikely possibility that TMX does not bind to sites in the SR. We refer to [Ca\(^{2+}\)]\(_{\text{SR,R}}\) and values derived with case 1 as the “most likely” values below.

**Properties of Ca-intrinsic buffers in the SR of frog**

The most likely average resting concentration of CaIB, [CaIB\(_{\text{SR}}\)] was 17.1 mM (concentration referred to SR water volume; column 6 of Table 1), and the most likely average value of \(r\), the ratio of bound to free Ca in the SR at rest, is 27.9 (column 7 of Table 1). Column 8 in Table 1 gives a related parameter, \(q\), the fraction of released Ca\(^{2+}\) ions originating from intrinsic Ca\(^{2+}\) buffers as opposed to the pool of free calcium in the SR at the start of SR Ca\(^{2+}\) release when [Ca\(^{2+}\)]\(_{\text{SR}}\) is near its nominal physiological resting level and the buffering capacity of the intrinsic buffers is maximal. The most likely average value of \(q\) was 43.9, almost twice the value of \(r\), owing to the curvature in the Ca-binding curve of calsequestrin. This value of \(q\) is \(~10\)-fold greater than the average value of 4.6 given for case 4 in Table 1, a case (as noted above) previously considered possible but now ruled out by results in Lamboley and Pape (2011). In summary, the results in this article more firmly establish the strong buffering properties of intrinsic Ca buffers in the SR of frog skeletal muscle.

Most of the total Ca in the SR at rest appears to be bound to calsequestrin

As seen by the results in Figs. 1 C and 2 C, the Ca-binding curves (concentration of bound Ca in the SR vs. [Ca\(^{2+}\)]\(_{\text{SR}}\)) do not display any clear inflection points that would be indicative of two underlying cooperative-binding components with different binding properties. This suggests
that the cooperative-binding component might be caused by a single Ca buffer, namely calsequestrin. The likelihood that it is all or almost all caused by calsequestrin depends much more strongly on results from mammalian preparations as discussed next.

As noted in Section 5 of the supplemental text, two other proteins in the SR, a 160-kD protein (Orr and Shoshan-Barmatz, 1996) and sarcalumenin (Leberer et al., 1990), display cooperative binding similar to calsequestrin. In contrast to calsequestrin, which is located in the terminal cisternae tethered to the Ca\textsuperscript{2+} release channels, these proteins are distributed throughout the SR. Their capacities for Ca\textsuperscript{2+} buffering relative to that of calsequestrin are unknown, although they are apparently much less than that of calsequestrin. These results indicate that any contribution they make to the Ca\textsuperscript{2+}-buffering properties would show up as part of the intrinsic buffer modeled with cooperative binding and that the contribution is minor compared with that of calsequestrin. As also noted in the supplemental text, two other Ca-binding proteins in the SR were identified at about the same time as the discovery of calsequestrin, namely calreticulin and a set of low molecular weight proteins (Ostwald and MacLennan, 1974), both of which displayed approximately linear binding in the disrupted preparations. Assuming that Ca binding is also linear binding under physiological conditions, Ca binding to these proteins should only have shown up in the linear binding component, which was shown to be ~20% of total Ca binding (Section 5 of the supplemental text). Assuming there are no other Ca-binding proteins in frog muscle, results in this article would indicate that calsequestrin is responsible for at least 80% of the total bound Ca in the SR under physiological conditions. Because some of the linear binding component could be caused by calsequestrin, the percentage bound to calsequestrin could be higher.

The strongest evidence in support of calsequestrin being the dominant Ca buffer in the SR comes from a recent study by Murphy et al. (2009). They found that the skeletal muscle isoform of calsequestrin (CS1) accounted for essentially all of the bound Ca\textsuperscript{2+} in fast-twitch skeletal muscle (extensor digitorum longus) from rat. As described here, their estimate of the maximal Ca binding of CS1, 2.9 mM (millimoles per liter of fiber volume), agrees reasonably well with the maximal Ca-binding capacity in the SR of twitch fibers from frog. In frog, an estimate of these values is given by [Ca\textsubscript{B}]\textsubscript{max} in column 4 of Table 1, assuming that the extrapolated value of the fitted cooperative binding curve to infinite [Ca\textsuperscript{2+}]\textsubscript{lab} corresponds to the maximal amount of bound Ca. The most likely average value of [Ca\textsubscript{B}]\textsubscript{max} was 44.0 mM (millimoles Ca per liter of SR water; range of possible values was 34.0–45.9 mM from cases 2 and 3). With a value of 0.0875 for the ratio of the volume of water in the SR to fiber volume (see text after Eq. 6 in Materials and methods), the value of 2.9 mM above corresponds to a maximal binding capacity of 33.1 mM (millimoles per liter of SR water volume; 33.1 = 2.9 x 8 / 0.7), which agrees reasonably well with the most likely value of 44.0 mM for the maximal Ca-binding capacity of intrinsic buffers in the SR of frog fibers. This close correspondence along with the results discussed above in this section strongly suggest that calsequestrin accounts for almost all of the Ca buffering (>80%) in the SR under physiological conditions in frog twitch muscle, as concluded by Murphy et al. (2009) for rat fast-twitch muscle.

Apparently conflicting results indicating that total SR Ca is not greatly reduced in skeletal muscle from mice lacking calsequestrin
One approach to study the role of calsequestrin in fast-twitch skeletal muscle from mice has been to prevent its expression with silencing RNA or with knockout of the skeletal muscle isoform of calsequestrin, CS1. Unexpectedly, although the time course of the tension response was delayed, the peak of the tension response was somewhat enhanced in CS1-null muscle fibers (Paolini et al., 2007). Myoplasmic Ca\textsuperscript{2+} transients measured with high affinity indicators were either about the same (Wang et al., 2006) or lower by 30% on average (Paolini et al., 2007) in CS1-null compared with that measured in wild-type (wt) fibers. In regards to the total Ca content in the SR at rest (predicted to be greatly reduced in CS1-null fibers if calsequestrin is the major SR Ca buffer, as indicated in the preceding section), some results are consistent with a significantly lower SR Ca content in CS1-null fibers (Fig. 8 E of Paolini et al., 2007; Canato et al., 2010), whereas results of Sztretye et al. (2011b) appear to indicate only a modest change in the total Ca in the SR (from their Table 1, the average “total releasable Ca” in CS1-null fibers from mice was only 25% less than that in wt fibers). If the SR Ca content of CS1 null is, in fact, not greatly reduced, one likely explanation would be that various adaptive changes in the CS1-null mice make up for the loss of calsequestrin. These might involve some adaptive changes already shown to occur (Paolini et al., 2007) and likely other unknown changes perhaps involving the expression of other Ca-binding proteins in the SR. It is noted that the lack of a significant loss of function in the CS1-null mouse caused by such adaptive changes would not rule out an important role of calsequestrin under normal physiological conditions.

The degree of cooperativity of Ca binding: Comparison with values from disrupted preparations
Sections above indicate or suggest reasonably close correspondences between the maximum binding capacities and the dissociation constant of calsequestrin in disrupted preparations with related values determined
for frog twitch muscle in this study. This section makes a similar comparison for a third property associated with SR Ca buffering, namely the degree of cooperativity as indicated by the Hill coefficient (n in Eq. 9). The results in Table 1 indicate a most-likely value for n of 3 (possible average values ranging from 2.9 to 3.4). Information from calsequestrin isolated from rabbit skeletal muscle has produced mixed results concerning the cooperativity of Ca binding. Aaron et al. (1984) measured the calcium dependence of conformational changes of calsequestrin (detected with circular dichroism and 1H NMR) and found a value for n of 2.4–2.9 (with \( K_{CSQ} = 0.21–0.25 \text{mM} \) in the absence of KCl and 1.65 (with \( K_{CSQ} = 0.87 \text{mM} \) in the presence of 100 mM KCl. In the presence of 150 mM KCl at pH 7.5, Krause et al. (1991) found no cooperativity of Ca binding to purified calsequestrin. Measurements by Hidalgo et al. (1996) of the Ca-binding dependence of an intrinsic fluorescence change of calsequestrin, again in the presence of 150 mM KCl at pH 7.5, gave a value for n of 2.9 (\( K_{CSQ} = 0.57 \text{mM} \); their value of n depended on pH). Because results in this article indicate significant cooperativity in a relatively intact preparation, and because the degree of cooperativity with purified preparations depends on the experimental conditions, it seems reasonable to conclude that lack of cooperativity observed by Krause et al. (1991) was caused by a loss of cooperativity associated with the purification process and/or experimental conditions. Because values of n from Hidalgo et al. (1996) and Aaron et al. (1984) under some conditions do correspond closely to that measured in this study, it seems reasonable to suggest that their signals may actually reflect total Ca bound to calsequestrin.

It is noted that the degree of cooperativity indicated in this study does not necessarily imply specific molecular interactions, particularly because Ca\(^{2+}\) binding to calsequestrin appears to mainly involve ionic interactions at negatively charged portions of calsequestrin as opposed to covalent binding at specific sites (Ohnishi and Reithmeier, 1987).

**Steady-state Ca\(^{2+}\) release at −60 mV and evidence against luminal regulatory mechanisms in frog muscle**

Several researchers have suggested that Ca\(^{2+}\) release is modulated by Ca\(^{2+}\) regulatory sites in the lumen of the SR rather than, or in addition to, sites on the myoplasmic side (Donoso et al., 1996; Sitsapesan and Williams, 1997; Sztretye et al., 2011b; see the Introduction for a discussion of the latter reference). An argument against this is based on the similarity of the bell-shaped relationship of release permeability versus \([\text{Ca}^{2+}]_{\text{SR}}\) at −60 mV in Fig. 4 D with the bell-shaped relationship for the open probability \(P_{o}\) of reconstituted SR Ca\(^{2+}\) release channels in bilayers versus Ca\(^{2+}\) flux through the channel observed by Tripathy and Meissner (1996). (As reviewed in Results, \([\text{Ca}^{2+}]\) at a regulatory site near the mouth of an open SR Ca\(^{2+}\) release channel on the myoplasmic side should be proportional to the Ca\(^{2+}\) flux through the channel that should, in turn, be proportional to \([\text{Ca}^{2+}]_{\text{SR}}\).) The variation in \(P_{o}\) with Ca\(^{2+}\) flux resulted from differences in the mean open time of the SR Ca\(^{2+}\) release as opposed to the conductance of the channel. The dependence of \(P_{o}\) on Ca\(^{2+}\) flux rather than \([\text{Ca}^{2+}]_{\text{SR}}\) was determined by varying Ca\(^{2+}\) flux independently of \([\text{Ca}^{2+}]_{\text{SR}}\). Given the same type of bell-shaped relationships between channel activation versus Ca\(^{2+}\) flux (or its equivalent) for the results at −60 mV in Fig. 4 D and those with isolated SR Ca\(^{2+}\) release channels, it seems reasonable to suppose that the same Ca−feedback mechanisms are involved, namely CICR for the initial rising phase and Ca inactivation for the falling phase of the bell-shaped relationships. The apparent correspondence also strongly suggests, although does not prove, that release at −60 mV does not depend on luminal regulatory sites.

As indicated in the Introduction, Sztretye et al. (2011b) recently concluded from results with wt and CS1-null fibers from mice that a decrease in \([\text{Ca}^{2+}]_{\text{SR}}\) sensed by calsequestrin terminates SR Ca\(^{2+}\) release. Such a mechanism would be consistent with the decrease in release permeability at −20 mV in Fig. 4 B, as \([\text{Ca}^{2+}]_{\text{SR}}\) decreased from 0.4 to 0.1 mM. However, because release permeability at −60 mV increases rather than decreases over the first part of this range (from 0.4 to 0.3 mM), this mechanism clearly does not occur in frog fibers, at least in this range of SR contents ending at ~10% of the resting SR Ca content (as seen from the abscissa in Fig. 4 C). As explained above, the decrease in release permeability when \([\text{Ca}^{2+}]_{\text{SR}}\) varies from 0.3 mM to near zero is likely caused by a decreasing CICR mechanism involving a myoplasmic regulatory site, although a luminal regulatory mechanism cannot be ruled out.

The quasi-steady level of release at −20 mV is enhanced by recruitment of two to three channels in a calcium release site, or an equivalent effect

As shown in Fig. 14 in Pape et al. (1995), steady-state SR Ca\(^{2+}\) release is steeply voltage dependent between −72 and −57 mV (mean \(e\) fold factor of 3.5 mV), consistent with the voltage steepness of the \(Q_{\text{s}}\) component of intramembranous charge movement (average voltage steepness factor of 2.9 mV in Hui and Chandler, 1990). Based on this correspondence and the low density of Ca\(^{2+}\) release sites activated in this voltage range and the buffering action of 20 mM EGTA, Ca\(^{2+}\) release at −60 mV and nearby voltages in Pape et al. (1995) was considered to be the summation of release from isolated Ca\(^{2+}\) release sites, with each site composed of a single SR Ca\(^{2+}\) release channel activated by its associated voltage sensor (dihydropyridine receptor) and possibly neighboring SR Ca\(^{2+}\) release channels recruited by CICR. Given the insensitivity of release permeability versus \([\text{Ca}^{2+}]_{\text{SR}}\) data at −60 mV
to BAPTA (reviewed in Results), recruitment of neighboring channels by CICR does not appear to occur at this voltage.

The following summarizes our hypotheses of local control processes regulating SR Ca\textsuperscript{2+} release for twitch frog fibers in the presence of 20 mM EGTA based on results and logic described above, progressing from low SR Ca contents to near physiological resting levels. The voltage dependence of Ca\textsuperscript{2+} release (over the full voltage range) for [Ca\textsubscript{T}]\textsubscript{SR} values up to \(\sim 250 \mu M\) (which corresponds to [Ca\textsubscript{G}]\textsubscript{SR} values up to \(\sim 0.28 \mu M\)) can be explained by the summation of a voltage-dependent number of isolated Ca\textsuperscript{2+} release sites open at any one time, with each site composed of a single SR Ca\textsuperscript{2+} release channel activated by its associated voltage sensor, and with its mean open time controlled by an autoregulatory CICR mechanism. By using release at \(-60 \text{ mV}\) as a reference, it was found in Results that the release permeability at higher voltages of [Ca\textsubscript{G}]\textsubscript{SR} during the quasi-steady level of release at \(-20 \text{ mV}\) is 3.3-fold on average greater than that expected from the simple summation of Ca\textsuperscript{2+} release sites. This could be caused by the summation of the same number of Ca\textsuperscript{2+} release sites having the same mean open time but with 3.3 open channels on average per site instead of one. The observation that the extra component of release occurs only at higher voltages is explained by certain elements of the model of Shirokova et al. (1996) for the regulation of SR Ca\textsuperscript{2+} release by voltage and CICR, adapted by Pape et al. (2002; see their Fig. 8 and associated text). The adapted elements involve the idea that a SR Ca\textsuperscript{2+} release channel not coupled to a voltage sensor (dihydropyridine receptor) can be activated by CICR alone as a result of the summation of \(\Delta[Ca^{2+}]\textsubscript{myo}\) from two nearby voltage-activated SR Ca\textsuperscript{2+} release channels, the summation increasing [Ca\textsubscript{G}]\textsubscript{myo} above the threshold for activation by CICR. The voltage dependence of the extra component arises from the greater probability at higher voltages of having two neighboring channels activated by voltage. The dependence on [Ca\textsubscript{G}]\textsubscript{SR} is caused by a requirement for higher Ca\textsuperscript{2+} fluxes through the channels. Pape et al. (2002) modified this idea with the starting situation of two neighboring voltage-activated channels replaced by one voltage-activated channel with the second activated by a combination of voltage and calcium. This modification was needed to account for the extra component of Ca\textsuperscript{2+} release at the peak of the release permeability signal at \(-45 \text{ mV}\), as only a small portion of the steady-state intramembranous charge had moved by this point. One possibility is that some of the 3.3-fold greater enhancement is caused by an increased mean open time of Ca\textsuperscript{2+} release sites rather than recruited channels. In any case, the enhancement can be considered to result from the equivalent of SR Ca\textsuperscript{2+} release sites composed of 3.3 open channels on average instead of just one.

It is noted that by using release at \(-60 \text{ mV}\) as a reference to derive the factor of 3.3, the 3.3-fold enhancement of the quasi-steady level of release at \(-20 \text{ mV}\) is only a net effect of CICR. In fact, the monotonic increase in release permeability as [Ca\textsubscript{G}]\textsubscript{SR} increases near zero up to the start of the quasi-steady level of release obscures an increasing Ca inactivation acting to progressively decrease release permeability as [Ca\textsubscript{G}]\textsubscript{SR} increases over this range. As a result, the total influence of CICR including autoregulation should be much greater because Ca inactivation is very pronounced during the quasi-steady level of release. This follows from the conclusions of Jong et al. (1995) that \(\geq 90\%\) of the SR Ca\textsuperscript{2+} release channels are inactivated by the peak of the SR Ca\textsuperscript{2+} release signal at \(-20 \text{ mV}\), even in the presence of 20 mM EGTA that reduces Ca inactivation severalfold. The fraction of channels inactivated during the quasi-steady level of release should be even greater than that at the peak of release. Collectively, these results indicate that the quasi-steady level of release is determined by powerful local control mechanisms, namely CICR and Ca inactivation.

**Physiological relevance of quasi-steady level of release**

In response to voltage-clamp stimulation to a potential greater than about \(-50 \text{ mV}\), the rate of SR Ca\textsuperscript{2+} release signal \((d\Delta[Ca_{T}]/dt)\) and related signals measured with a variety of methods generally displays an early peak followed by a decline to a steady level (e.g., Schneider et al., 1987; Schneider and Simon, 1988; Jong et al., 1995; Pizarro and Rios, 2004; Fig. 3 of this article). The potential physiological importance of the peak and quasi-steady level with voltage-clamp stimulation follows from the similar behavior obtained with trains of action potentials with SR Ca\textsuperscript{2+} release derived from the myoplasmic [Ca\textsubscript{G}] transient measured with a variety of indicators in both intact and cut muscle fibers (Baylor et al., 1983; Maylie et al., 1987; Baylor and Hollingworth, 1988; Pape et al., 1995). In brief, the rate of SR Ca\textsuperscript{2+} release in a train of closely spaced action potentials (10–20 ms apart) is greatly reduced (by about fivefold) in the second and subsequent action potentials compared with that produced by the first action potential in the train. Moreover, the rate of release during the second and subsequent action potentials tends to remain relatively constant for at least 10 action potentials. Given this background, it seems reasonable to suppose that the local control mechanisms involved in the quasi-steady level of release with voltage-clamp stimulation are the same or similar to those controlling release in the second and subsequent action potentials in a closely spaced train of action potentials. To our knowledge, this is the first report indicating the involvement of CICR during the quasi-steady level of release.

Two very distinct models have been developed to account for the early peak of SR Ca\textsuperscript{2+} release followed by
the decline to the quasi-steady level in voltage-clamp experiments in frog twitch muscle. In one (Jong et al., 1995), these features as well as properties of Ca inactivation are accounted for by the properties of voltage activation and Ca inactivation alone (while not necessarily excluding CICR). In the other, the early peak is attributed to activation by CICR of SR Ca$^{2+}$ release channels not coupled to voltage sensors, whereas the steady level of release is caused exclusively by voltage activation of coupled SR Ca$^{2+}$ release channels (Shirokova et al., 1996). Although the 3.3-fold enhancement of the quasi-steady level of release at −20 mV described in this article is evidence against one of the basic assumptions of the second model, certain elements of this model may contribute to recruitment of neighboring channels by CICR. We hypothesize that the actual situation accounting for the peak to steady level of release and also the properties of Ca inactivation, voltage activation, and CICR involve mainly the first model modified to account for regulation by CICR with elements of the second model along with the added possibility of coupled SR Ca$^{2+}$ release channels being activated by a combination of voltage and CICR.

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