Differential Regulation of ER Ca\(^{2+}\) Uptake and Release Rates Accounts for Multiple Modes of Ca\(^{2+}\)-induced Ca\(^{2+}\) Release

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**Abstract**

The ER is a central element in Ca\(^{2+}\) signaling, both as a modulator of cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and as a locus of Ca\(^{2+}\)-regulated events. During surface membrane depolarization in excitable cells, the ER may either accumulate or release net Ca\(^{2+}\), but the conditions of stimulation that determine which form of net Ca\(^{2+}\) transport occurs are not well understood. The direction of net ER Ca\(^{2+}\) transport depends on the relative rates of Ca\(^{2+}\) uptake and release via distinct pathways that are differentially regulated by Ca\(^{2+}\), so we investigated these rates and their sensitivity to Ca\(^{2+}\) using sympathetic neurons as model cells. The rate of Ca\(^{2+}\) uptake by SERCAs (\(\text{J}_{\text{SERCA}}\)), measured as the t-BuBHQ-sensitive component of the total cytoplasmic Ca\(^{2+}\) flux, increased monotonically with [Ca\(^{2+}\)]\(_i\). Measurement of the rate of Ca\(^{2+}\) release (\(\text{J}_{\text{Release}}\)) during t-BuBHQ-induced [Ca\(^{2+}\)]\(_i\) transients made it possible to characterize the Ca\(^{2+}\) permeability of the ER (\(\text{P}_{\text{ER}}\)), describing the activity of all Ca\(^{2+}\)-permeable channels that contribute to passive ER Ca\(^{2+}\) release, including ryanodine-sensitive Ca\(^{2+}\) release channels (RyRs) that are responsible for CICR. Simulations based on experimentally determined descriptions of J\(_{\text{SERCA}}\), \(\text{P}_{\text{ER}}\), and of Ca\(^{2+}\) extrusion across the plasma membrane (\(\text{J}_{\text{pm}}\)) accounted for our previous finding that during weak depolarization, the ER accumulates Ca\(^{2+}\), but at a rate that is attenuated by activation of a CICR pathway operating in parallel with SERCAs to regulate net ER Ca\(^{2+}\) transport. Caffeine greatly increased the [Ca\(^{2+}\)] sensitivity of \(\text{P}_{\text{ER}}\), accounting for the effects of caffeine on depolarization-evoked [Ca\(^{2+}\)]\(_i\) elevations and caffeine-induced [Ca\(^{2+}\)]\(_i\) oscillations. Extending the rate descriptions of J\(_{\text{SERCA}}\), \(\text{P}_{\text{ER}}\), and \(\text{J}_{\text{pm}}\) to higher [Ca\(^{2+}\)]\(_i\) levels shows how the interplay between Ca\(^{2+}\) transport systems with different Ca\(^{2+}\) sensitivities accounts for the different modes of CICR over different ranges of [Ca\(^{2+}\)]\(_i\), during stimulation.

**Key words:** CICR • ER • ryanodine receptors • SERCA • sympathetic neurons

**Introduction**

The ER is an important component in Ca\(^{2+}\) signaling in virtually all nonmuscle cells (Pozzan et al., 1994; Clapham, 1995; Berridge, 1998; Meldolesi and Pozzan, 1998). Net Ca\(^{2+}\) transport by the ER is critical for regulating intraluminal Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_\text{ER}\)) as well as for modulating the dynamics of cytoplasmic free Ca concentration ([Ca\(^{2+}\)]\(_i\)) during and after stimulation. As a result, Ca\(^{2+}\) transport by this organelle is expected to influence the activity of Ca\(^{2+}\)-sensitive processes within the ER and the cytoplasm, as well as in organelles such as mitochondria and the nucleus that undergo secondary changes in intraluminal Ca\(^{2+}\) concentration in response to evoked changes in [Ca\(^{2+}\)]\(_i\) (Gerasimenko et al., 1996; Babcock and Hille, 1998).

In neurons, the role that the ER plays in modulating depolarization-induced [Ca\(^{2+}\)]\(_i\) elevations is complicated, since this organelle may act as either a Ca\(^{2+}\) source or sink, in some cases even in the same cell type (Friel and Tsien, 1992a; Garaschuk et al., 1997; Toescu, 1998; for review see Simpson et al., 1995; Rose and Konnerth, 2001). These distinct forms of net ER Ca\(^{2+}\) transport are expected to have very different functional effects on the activity of intraluminal Ca\(^{2+}\) binding proteins (Corbett and Michalak, 2000). Nevertheless, the conditions of stimulation that determine which form of transport occurs are incompletely understood. The direction and rate of net ER Ca\(^{2+}\) transport depend on the relative rates of Ca\(^{2+}\) uptake and release via distinct transport pathways. Ca\(^{2+}\) uptake is regulated by sarco(endo)plasmic reticulum Ca ATPases (SERCAs; East, 2000), whereas passive Ca\(^{2+}\) release is regulated by Ca\(^{2+}\) release channels that open in response to elevations in [Ca\(^{2+}\)]\(_i\) and contribute to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR; Bezprozvanny et al., 1991; Ehrlich, 1995; for reviews see Kuba, 1994; Verkhratsky and Shmigol, 1996; Usachev and Thayer, 1999). If the rate of Ca\(^{2+}\) uptake exceeds the rate of release, the ER acts as a Ca\(^{2+}\) sink and slows depolarization-evoked [Ca\(^{2+}\)]\(_i\) elevations. If release is faster than uptake, it acts as a source or sink.
Ca^{2+} source, speeding and potentially amplifying these responses.

The main goal of the present study was to understand how differential regulation of ER Ca^{2+} uptake and release rates by Ca^{2+} determines the direction and rate of net ER Ca^{2+} transport during stimulation. Our previous work in sympathetic neurons showed that as evoked [Ca^{2+}], elevations become larger, the ER undergoes a transition from a Ca^{2+} sink to a Ca^{2+} source (Albrecht et al., 2001; Hongpaisan et al., 2001). Specifically, it was found that if [Ca^{2+}], is raised to less than or equal to ~350 nM by weak depolarization, the ER accumulates Ca^{2+}, whereas if global [Ca^{2+}] is raised to 600–800 nM by stronger depolarization, there is little or no net ER Ca^{2+} transport. However, if [Ca^{2+}] rises to higher levels during depolarization, for example, in outer cytoplasmic regions near sites of Ca^{2+} entry, or during inhibition of mitochondrial Ca^{2+} uptake, the ER releases net Ca^{2+}, presumably reflecting net CICR. We proposed a simple explanation for this transition: progressive [Ca^{2+}] dependent activation of a ryanodine-sensitive CICR pathway that operates in parallel with SERCAs to regulate net ER Ca^{2+} transport. According to this idea, small [Ca^{2+}] elevations stimulate Ca^{2+} uptake more effectively than Ca^{2+} release, leading to Ca^{2+} accumulation, whereas large [Ca^{2+}] elevations stimulate release more effectively than uptake, leading to net Ca^{2+} release. We also presented indirect evidence that activation of the CICR pathway is influential even when [Ca^{2+}], is low and the ER acts as a Ca^{2+} sink, causing the rate of ER Ca^{2+} accumulation to be reduced so that the ER becomes a less powerful buffer.

A quantitative model was presented, which showed that this idea is plausible (Albrecht et al., 2001). In terms of the model, differential regulation of ER Ca^{2+} uptake and release rates by Ca^{2+} is the key factor in determining when the ER acts as a Ca^{2+} source or sink. Moreover, we proposed that the relative rates of net ER Ca^{2+} transport and Ca^{2+} clearance by other pathways determine whether net Ca^{2+} release, if it occurs, can be regenerative.

In the present study, we test some of these ideas by characterizing the Ca^{2+} transport pathways responsible for ER Ca^{2+} uptake and release in sympathetic neurons. We sought to determine how Ca^{2+} transport by each pathway is regulated by Ca^{2+} when [Ca^{2+}], is low and the ER acts as a Ca^{2+} sink. It was asked if quantitative differences in the Ca^{2+} dependent regulation of Ca^{2+} uptake and release rates can account for Ca^{2+} accumulation at low [Ca^{2+}], at a rate that is reduced by activation of a ryanodine-sensitive CICR pathway. We also sought to understand how [Ca^{2+}] dependent regulation of ER Ca^{2+} uptake and release rates determines when the ER is a Ca^{2+} source or sink over a wider [Ca^{2+}], range, and how net ER Ca^{2+} transport contributes to multiple modes of CICR.

**Materials and Methods**

**Cell Dissociation and Culture**

Bullfrog sympathetic neurons were dissociated and placed in culture for up to 1 wk, as described previously (Colegrove et al., 2000a). All procedures conform to guidelines established by our Institutional Animal Care and Use Committee.

**Cytosolic Calcium Measurements**

To measure [Ca^{2+}], cells were incubated with 3 μM fura-2 AM in normal Ringer’s solution for 40 min at room temperature with gentle agitation followed by rinsing. The composition of normal Ringer’s solution was the following (in mM): 128 NaCl, 2 KCl, 2 CaCl₂, 10 HEPES, and 10 glucose, pH adjusted to 7.3 with NaOH. Fura-2 AM was dispensed from a 1-mM stock solution in DMSO containing 25% (wt/wt) pluronic F127 (BASF Corporation). Cells were washed with normal Ringer’s solution and placed on the stage of an inverted microscope (Diaphot TMD; Nikon) and superfused continuously (~5 ml/min). Recordings began ~20 min after washing away fura-2 AM, permitting de-esterification of the Ca^{2+} indicator. With this loading procedure, there is little compartmentalization of fura-2 (Albrecht et al., 2001). Solution changes (~200 ms) were made using a system of microcapillaries (20 μl; Drummond microcaps) mounted on a micromanipulator. Fluorescence measurements were performed as described in Colegrove et al. (2000a).

**Ca^{2+} Flux Measurements**

In this study, three different macroscopic Ca^{2+} fluxes were measured: (1) J_{SERCA}, the rate of Ca^{2+} uptake via SERCAs; (2) J_i, the total cytosolic Ca^{2+} flux when SERCAs are inhibited; and (3) J_{pm}, the rate of Ca^{2+} extrusion across the plasma membrane. One additional flux (J_{Release}) was calculated from the difference between two of the measured fluxes (J_i and J_{pm}). Finally, J_{Release} and its integral were used to obtain information about intracellular Ca^{2+} concentration and the Ca^{2+} permeability of the ER (P_{ER}).

Fluxes were measured using the following experimental protocols. To determine J_{SERCA}, cells were rapidly exposed to a saturating concentration (100 μM) of the SERCA inhibitor 2,5-Di-(betyl)-1,4-hydroquinone (t-BuBHQ), using the abrupt change in d[Ca^{2+}]/dt after application of the inhibitor as a measure of the t-BuBHQ-sensitive component of the total cytosolic Ca^{2+} flux (see Fig. 2 A). To measure this change, lines were fit to the linear portions of the [Ca^{2+}], record just before and after the perturbation, and J_{SERCA} was taken as the difference between the final and initial slopes. The total cytosolic Ca^{2+} flux (J_i) during t-BuBHQ-induced [Ca^{2+}], transients (see Fig. 3, A and B) was calculated as the time derivative of [Ca^{2+}], at each sample time t, according to \((d[Ca^{2+}]/dt)_{t} = (t_{i} + \Delta t/2) - [Ca^{2+}],(t_{i} - \Delta t/2)/\Delta t\), where \(\Delta t\) (400–500 ms) is twice the sampling interval. For the first and last sample points, the flux was estimated by computing the slope of a fitted line over the first and last sets of three sample points, respectively. The rate of Ca^{2+} extrusion across the plasma membrane (J_{pm}) was determined by measuring the total cytosolic Ca^{2+} flux during the [Ca^{2+}], recovery after brief high K⁺ depolarizations while cells were continuously exposed to t-BuBHQ, and to FCCP to inhibit mitochondrial Ca^{2+} uptake (see Fig. 3, A–C; Colegrove et al., 2000a). The rate of passive Ca^{2+} release from the ER (J_{leak}) during the t-BuBHQ-induced [Ca^{2+}], transient was then taken as the difference between J_i and J_{pm} at corresponding times (Fig. 3 B). J_{pm} was determined at each point in time during the [Ca^{2+}], transient based on the rate of Ca^{2+} extrusion during the recovery after depolarization at corresponding values of [Ca^{2+}],. This is justified by our previous finding that, in
sympathetic neurons, $J_{pm}$ can be specified at each time by the magnitude of $[Ca^{2+}]$, at that time (Colegrove et al., 2000a). Before calculating $d[Ca^{2+}]/dt$, $[Ca^{2+}]$, measurements were smoothed with a binomial filter. Since measurements were acquired with regular sample intervals, $J$ and $J_{pm}$ were not always measured at identical values of $[Ca^{2+}]$, so to facilitate flux subtraction, linear interpolation was used to approximate each of the measured fluxes at equally spaced values of $[Ca^{2+}]$.

As shown in Appendix A, if $Ca^{2+}$ binding to cytoplasmic buffers equilibrates rapidly compared with changes in $[Ca^{2+}]$, each measured $Ca^{2+}$ flux can be interpreted as the rate of $Ca^{2+}$ transport by the respective system (e.g., $J$ in nmol/s) divided by the product of the cytoplasmic volume ($v_C$) and a buffering factor ($κ$) that gives the change in total cytoplasmic Ca concentration accompanying small changes in $[Ca^{2+}]$, (Necher and Augustine, 1992; Tse et al., 1994; Neher, 1995; Colegrove et al., 2000a). While it was assumed that $v_C$ is constant, $κ$ was treated as a function of $[Ca^{2+}]$, (see next section). Thus, the measured fluxes are expected to show a composite $[Ca^{2+}]$-dependence that reflects $Ca^{2+}$-dependent regulation of the individual transport rates and the $[Ca^{2+}]$-dependence of $κ$.

To determine if the measured fluxes account for the time course of $[Ca^{2+}]$, after various experimental perturbations, quantitative descriptions of $J_{pm}$, $J_{SERCA}$, $P_{in}$, and $κ$ were used as the defining equations in the model described in Appendix A. Quantitative descriptions of the fluxes were of the form $J_i / (v_C κ_i)$, where $J_i$ is represented by a Hill-type equation, or in the case of $J_{pm}$, such an equation plus a leak, $v_C$ is constant and $κ_i$ is a known function of $[Ca^{2+}]$, (see next section). Parameters of these equations were estimated based on the flux versus $[Ca^{2+}]$ measurements. While mechanistically motivated, the equations used to describe the $[Ca^{2+}]$-dependence of transport by the different pathways should be regarded as empirical, since the $[Ca^{2+}]$-range over which measurements were made was not always broad enough to determine unique parameter sets. Nevertheless, as shown in results, given equations that accurately describe the $[Ca^{2+}]$-dependence of the measured fluxes, it is possible to account for $[Ca^{2+}]$-dynamics after various experimental perturbations. Given information about the $[Ca^{2+}]$-dependence of $κ$, it is also possible to make inferences about the $[Ca^{2+}]$-dependence of transport rates and $[Ca^{2+}]$-dynamics in cells without exogenous $Ca^{2+}$ buffers, but this was not the focus of the present study.

To examine qualitative properties of CICR over a wider range of $[Ca^{2+}]$, continuous extensions of the descriptions for $J_{pm}$, $J_{SERCA}$, and $P_{in}$, were used. In this case, there is uncertainty regarding quantitative features of the simulations (e.g., precise values of $[Ca^{2+}]$-thresholds for net CICR), but the qualitative features (e.g., the existence of thresholds and their order relationship) are expected to be reliable. Nevertheless, experimental characterization of the transport systems at higher $[Ca^{2+}]$, levels is an important goal of future experiments.

**Cytoplasmic $Ca^{2+}$ Buffering**

To characterize the $Ca^{2+}$ permeability of the ER, it was necessary to obtain information about the driving force favoring passive $Ca^{2+}$ release during t-BuBHQ-induced $[Ca^{2+}]$, transients (Appendix A). Information about changes in intraluminal $Ca^{2+}$ concentration was obtained by integrating $J_{release}$ over time. However, since $J_{release}$ is a flux per unit (effective) cytoplasmic volume (in nmol/v_C/κ/s), conversion to a flux per unit (effective) intraluminal volume (in nmol/γ_{γCa}κ/s) was required before integration. The appropriate flux was obtained after multiplying $J_{release}$ by (γ_C/γ_{γCa}). It was assumed that (1) $v_C$ is constant, (2) $κ$ is constant, as would be expected if intraluminal $Ca^{2+}$ buffers have low affinity for $Ca^{2+}$, and (3) that $κ$ adjusts instantaneously to changes in total Ca concentration. Evaluation of $κ$ and its $[Ca^{2+}]$-dependence required consideration of fura-2, since this $Ca^{2+}$ indicator must contribute to cytoplasmic $Ca^{2+}$ buffering in our experiments and binds $Ca^{2+}$ with moderately high affinity ($κ_{Fura-2} \sim 224$ nM). To evaluate this potential contribution to the $[Ca^{2+}]$-dependence of $κ$, the experiments illustrated in Fig. 1 were performed.

Fig. 1 (A and B) shows a representative voltage-sensitive $Ca^{2+}$ current and associated $[Ca^{2+}]$, response elicited from a fura-2 AM-loaded sympathetic neuron by weak depolarization under voltage clamp (perforated patch conditions). Using standard methods (Albrecht et al., 2001), we measured the cytoplasmic buffering strength $κ_{ER}$ when $[Ca^{2+}]$, was at its resting level. By progressively reducing the fura-2 AM incubation time, it was possible to estimate the resting value of $κ$, in native, unloaded cells, as well as the component of $κ$, representing fura-2 in our experiments, where the incubation time was 40 min. As expected, $κ$ is smaller if the incubation time is reduced (Fig. 1 C). The dependence of $κ$ on loading time could be described empirically by a third-order polynomial function, and extrapolation to zero incubation time provides an estimate of the endogenous cytoplasmic $Ca^{2+}$ buffering strength at rest ($κ_{Endog} \sim 23$). The strength of buffering ($κ_{Fura-2}$ by fura-2 at the 40-min time point then was calculated as $κ_{Endog} − κ_{Fura-2} \sim 238$. Thus, under our experimental conditions, fura-2 represents the major cytoplasmic $Ca^{2+}$ buffer at rest. This made it possible to describe explicitly the $[Ca^{2+}]$-dependence of $κ$, in our experiments as follows (Fig. 1 D):

\[
κ_i = κ_{Endog} + κ_{Fura-2}
\]

\[
κ_{Endog} = \frac{[Fura-2]κ_{Fura}/(κ_{Fura} + [Ca^{2+}]^2)}{1}
\]

Given $κ_{Endog}$, $κ_{Fura-2}$, and the mean resting $[Ca^{2+}]$, (Fig. 1, legend), Eq. 1 permits calculation of the average cytoplasmic fura-2 concentration ((Fura-2)) after a 40-min incubation, ~80 μM. Thus, assuming that $κ_{Endog}$ is constant, small elevations in $[Ca^{2+}]$, starting from initial values within the range 50–300 nM are associated with changes in total cytoplasmic Ca concentration between ~250× and 100× as large, with most of the $Ca^{2+}$ being bound by fura-2. Based on these measurements and assumptions 1–3 above, (κ_{Fura}/γ_{γCa}) was treated as the product of a constant ($v_C$/γ_{γCa}) and a $[Ca^{2+}]$-dependent term κ, that could be described explicitly by the curve in Fig. 1 D. Note that this treats all cells as if they have identical cytoplasmic $Ca^{2+}$ buffering properties; in the absence of single cell measurements of $κ$ and its $[Ca^{2+}]$-dependence, this seems to be a reasonable simplifying assumption. As described in Appendix A, information about the $[Ca^{2+}]$, dependence of $κ$, was used to convert $J_{release}$ into a flux that could be integrated to give information about changes in intraluminal $Ca^{2+}$ concentration during t-BuBHQ-induced $[Ca^{2+}]$, transients.

**Inhibition of CICR**

To inhibit CICR, cells were exposed to ryanodine (1 μM) and then transiently to caffeine (10 mM) in the continued presence of ryanodine. Under these conditions, caffeine elicits a transient rise in $[Ca^{2+}]$, like that observed in control cells, but unlike control cells, responsiveness to caffeine is not restored after caffeine is removed (Thayer et al., 1988). Caffeine opens RyRs by increasing their sensitivity to $[Ca^{2+}]$, (Rousseau et al., 1988), and ryanodine is thought to inhibit caffeine responsiveness by irreversibly modifying RyRs so that they are insensitive to $Ca^{2+}$ (Rousseau et al., 1987) or have greatly increased $Ca^{2+}$ sensitivity (Masumiya et al., 2001). Ryanodine was used in conjunction with caffeine because ryanodine preferentially interacts with the open channel, causing ryanodine-induced RyR modifications to be use-dependent.
Population results are expressed as mean ± SEM, and statistical significance was assessed using t test. Fura-2 AM was obtained from Molecular Probes, ryanodine was obtained from RBI, and t-BuBHQ was purchased from Calbiochem. All other compounds were obtained from Sigma-Aldrich.

Simulations

Rate equations describing Ca$^{2+}$ extrusion across the plasma membrane (Colegrove et al., 2000b) and Ca$^{2+}$ uptake and release by the ER were incorporated into a system of differential equations (see appendix b) that was solved numerically using a fourth-order Runge-Kutta routine (Boyce and DiPrima, 1969) written in Igor Pro (Wavemetrics, Inc.). Step size was 50 ms; further reductions in step size did not noticeably alter the results.

RESULTS

In the following, we will describe measurements of the rate of ER Ca$^{2+}$ uptake by SERCAs ($J_{\text{SERCA}}$), illustrating how it varies with [Ca$^{2+}$]$_i$. We will describe measurements of the rate of passive Ca$^{2+}$ release by the ER ($J_{\text{Release}}$), showing how it depends on a [Ca$^{2+}$]$_i$-sensitive permeability ($P_{\text{ER}}$) that is influenced by the activity of RyRs. We then show how these rate descriptions, when taken together with a description of Ca$^{2+}$ extrusion across the plasma membrane, account for several interesting features of Ca$^{2+}$ dynamics described previously, including [Ca$^{2+}$]$_i$-dependent attenuation of ER Ca$^{2+}$ accumulation during depolarization, caffeine-induced [Ca$^{2+}$]$_i$ oscillations, and multiple modes of CICR.

Characterization of the ER Ca$^{2+}$ Uptake Pathway

We begin by summarizing evidence that in sympathetic neurons, Ca$^{2+}$ uptake by the ER is controlled by SERCAs. Three observations support this conclusion. First, specific SERCA inhibitors such as thapsigargin (Tg) and t-BuBHQ elicit transient [Ca$^{2+}$]$_i$ elevations in the absence of extracellular Ca$^{2+}$ (no added Ca$^{2+}$, 0.2 mM EGTA), indicating that they release Ca$^{2+}$ from an intracellular store (unpublished data), presumably by unmasking ongoing passive Ca$^{2+}$ release that discharges the store. Second, when used at saturating concentrations, Ca$^{2+}$ buffering strength. Mean $k_{\text{Endog}}$ values were fit with a third-order polynomial that was extrapolated to zero incubation time, giving an estimated endogenous buffering strength ($k_{\text{Endog}}$) of 24.75. The shaded region describes Ca$^{2+}$ buffering attributable to fura-2 for the different incubation times. Numbers of cells for each incubation time (in min) were as follows: 3 (7), 2 (10), 3 (20), and 4 (40). Crosses indicate single cell measurements and squares give mean values. (D) Calculated [Ca$^{2+}$]$_i$ dependence of $k_i$ in the case of a 40-min incubation period like that used in the present study. Dotted lines indicate $k_i$ when [Ca$^{2+}$]$_i$ = 50 and 300 nM. Inset shows predicted Ca$^{2+}$ binding by fura-2 as a function of [Ca$^{2+}$]. The average fura-2 concentration ([Fura-2]) was determined by solving Eq. 1 under resting conditions ([Ca$^{2+}$]$_i$ = 49 ± 5.8 nM; N = 4) assuming that $K_{d,Fura-2}$ = 224 nM, giving [Fura-2] = 79.7 μM.
tions, pretreatment with one inhibitor occludes responses to the others, arguing that they have a common site of action (unpublished data). Third, pretreatment with a SERCA inhibitor (e.g., Tg) at maximally effective concentrations abolishes responsiveness to other agents that stimulate passive Ca\(^{2+}\)/H\(^{+}\) release in naive cells by different means (e.g., caffeine; Friel, 1995), indicating that the inhibitors effectively dissipate the gradient favoring Ca\(^{2+}\)/H\(^{+}\) release, and that SERCAs represent the major, if not the only, pathway for energy-dependent Ca\(^{2+}\)/H\(^{+}\) uptake by the store. At the concentrations tested, neither of these inhibitors systematically influenced resting [Ca\(^{2+}\)]\(_i\), indicating that depletion of stores does not elicit capacitative Ca\(^{2+}\) entry, in contrast to many nonexcitable cells (Lewis, 1999). Direct measurement of changes in total Ca concentration within the ER and other cellular compartments accompanying Tg- and caffeine-induced [Ca\(^{2+}\)]\(_i\) transients indicate that the Tg- and caffeine-sensitive store in these cells is the ER (Hongpaisan et al., 2001).

To characterize the rate of ER Ca\(^{2+}\) uptake and its regulation by [Ca\(^{2+}\)]\(_i\), the following protocol was used. Cells were exposed to t-BuBHQ to inhibit Ca\(^{2+}\) uptake, and the resulting change in [Ca\(^{2+}\)]\(_i\) was observed (Fig. 2). The inhibitor was applied rapidly (within ~200 ms) and at a high concentration (100 \(\mu\)M; see Fig. 2 C, inset) to minimize the delay between exposure and cessation of uptake. With this concentration, the delay to the first detectable [Ca\(^{2+}\)] increase was within one sample interval (200–250 ms), indicating that t-BuBHQ reached its site of action within this time. Moreover, reducing [t-BuBHQ] from 100 to 50 \(\mu\)M, which would be
Figure 3. Characterization of the ER Ca\(^{2+}\) release pathway. (A) Experimental protocol used to characterize the pathway responsible for passive Ca\(^{2+}\) release from the ER. During continuous exposure to 1 \(\mu\)M FCCP, cells were exposed rapidly to 100 \(\mu\)M t-BuBHQ, and after \([\text{Ca}^{2+}]_{i}\), returned to its prestimulation level, they were depolarized briefly by exposure to 50 mM K\(^{+}\) in the continued presence of t-BuBHQ and FCCP. (B) Time course of the Ca\(^{2+}\) fluxes underlying the t-BuBHQ-induced \([\text{Ca}^{2+}]_{i}\), transient on the same time scale as in A. \(J_{\text{i}}\) is the total cytoplasmic Ca\(^{2+}\) flux representing the imbalance between plasma membrane Ca\(^{2+}\) extrusion (\(J_{\text{pm}}\)) and passive Ca\(^{2+}\) release from the ER (\(J_{\text{release}}\)). \(J_{\text{pm}}\) was calculated based on the recovery after high K\(^{+}\)-induced depolarization, and \(J_{\text{release}}\) was calculated as the difference between \(J_{\text{i}}\) and \(J_{\text{pm}}\) at corresponding times (shaded region). (C) \(J_{\text{pm}}\) was measured by taking the time derivative of \([\text{Ca}^{2+}]_{i}\) during the recovery after the high K\(^{+}\)-induced \([\text{Ca}^{2+}]_{i}\) elevation in A and plotting against \([\text{Ca}^{2+}]_{i}\). Smooth curve represents a fit to Eq. B6. Also shown is \(J_{\text{i}}\) versus \([\text{Ca}^{2+}]_{i}\) during the t-BuBHQ-induced transient. For reference, \(t_{1}\)–\(t_{4}\) correspond to times indicated in A. \(J_{\text{pm}}\) was determined at each point in time \(t\) during the t-BuBHQ-induced \([\text{Ca}^{2+}]_{i}\) transient by calculating the value of the fitted \(J_{\text{pm}}\) curve at \([\text{Ca}^{2+}]_{i}(t)\). \(J_{\text{release}}\) was determined at each point in time in B as \(J_{\text{i}}(t) - J_{\text{pm}}([\text{Ca}^{2+}]_{i}(t))\). (D) Plot of \(\Delta[\text{Ca}^{2+}]_{\text{ER}}(t)\), the integral of \(J_{\text{release}}\) from the instant of t-BuBHQ addi-
expected to cause the intracellular concentration of the inhibitor to increase more slowly, did not reduce the size of evoked \([\text{Ca}^{2+}]\), transients (Fig. 2 C, inset), supporting the conclusion that SERCA activity is rapidly inhibited in these experiments.

When t-BuBHQ was applied to resting cells (Fig. 2 A), \([\text{Ca}^{2+}]\), rose at an initial rate of \(6.0 \pm 0.6 \text{ nM/s}\); resting \([\text{Ca}^{2+}]\), in this set of experiments was \(43.6 \pm 2.2 \text{ nM}\) (28 responses in six cells). The initial rate of rise provides a measure of the rate of passive \(\text{Ca}^{2+}\) release just before the perturbation, and of the rate of \(\text{Ca}^{2+}\) uptake that balances release under basal conditions (Fig. 2 A, diagrams). The same idea applies under nonsteady-state conditions (e.g., during the recovery after a depolarization-evoked \([\text{Ca}^{2+}]\), elevation; Fig. 2 B), leading to the generalization that the rate of \(\text{SERCA}\)-dependent \(\text{Ca}^{2+}\) uptake is given by the change in slope after rapid \(\text{SERCA}\) inhibition.

Collected results showing how the rate of \(\text{Ca}^{2+}\) uptake \((J_{\text{SERCA}})\) varies with \([\text{Ca}^{2+}]\), are presented in Fig. 2 C. Over the range examined (up to \(\sim 800 \text{ nM}\)), \(J_{\text{SERCA}}\) increases monotonically with \([\text{Ca}^{2+}]\). Although it is difficult to exclude a functional dependence of \(J_{\text{SERCA}}\) on intraluminal \(\text{Ca}^{2+}\) concentration, we found that after treatment with ryanodine, which reduces intraluminal total \(\text{Ca}\) concentration by \(\sim 60\%\) (Hongpaisan et al., 2001) and presumably causes a significant reduction in intraluminal free \(\text{Ca}\) concentration, the resting value of \(J_{\text{SERCA}}\) was unchanged. This suggests that \([\text{Ca}^{2+}]\), is the most important variable controlling \(J_{\text{SERCA}}\) in these experiments, at least under resting conditions. The smooth curve in Fig. 2 C, obtained as described in MATERIALS AND METHODS, provides a description of the composite \([\text{Ca}^{2+}]\), dependence of \(J_{\text{SERCA}}\) from basal levels up to \(\sim 800 \text{ nM}\).

**Characterization of the ER \(\text{Ca}^{2+}\) Release Pathway**

Pathways that may contribute to passive, or energetically downhill, \(\text{Ca}^{2+}\) release include RyRs, inositol (1,4,5)-trisphosphate receptors (InsP₃Rs), as well as an independent leak pathway. To determine the rate of passive \(\text{Ca}^{2+}\) release via all such pathways \((J_{\text{Release}})\) and its dependence on \(\text{Ca}^{2+}\) concentration, the following experiment was performed (Fig. 3). Cells were exposed to carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), and then were exposed rapidly to 100 \(\mu\text{M}\) t-BuBHQ to inhibit \(\text{SERCA}\)-mediated \(\text{Ca}^{2+}\) uptake. As before, this elicited a transient \([\text{Ca}^{2+}]\), rise (Fig. 3 A, left). Since the main, or only, \(\text{Ca}^{2+}\) fluxes responsible for the t-BuBHQ-induced \([\text{Ca}^{2+}]\), transient are passive \(\text{Ca}^{2+}\) release from the ER and \(\text{Ca}^{2+}\) extrusion across the plasma membrane, measurement of the total \(\text{Ca}^{2+}\) flux during the transient, along with a characterization of the rate of \(\text{Ca}^{2+}\) extrusion across the plasma membrane, permits calculation of the rate of passive \(\text{Ca}^{2+}\) release by subtraction at each point in time during the \([\text{Ca}^{2+}]\), transient. The rate of \(\text{Ca}^{2+}\) extrusion was determined from the recovery after a brief, high \(\text{K}^{+}\) depolarization elicited in the continued presence of t-BuBHQ and FCCP (Fig. 3 A, right). Under these conditions, \(\text{Ca}^{2+}\) extrusion is the primary, or only, mechanism of \(\text{Ca}^{2+}\) clearance.

Fig. 3 B shows the total cytoplasmic \(\text{Ca}^{2+}\) flux \((J)\) during the t-BuBHQ-induced \([\text{Ca}^{2+}]\), transient, determined by calculating the derivative of \([\text{Ca}^{2+}]\), at each point in time (inward fluxes are negative, outward fluxes are positive). \(J\), was initially negative and increased in magnitude to a peak before declining and changing sign after \([\text{Ca}^{2+}]\), attained its peak value, to become an outward flux. It then increased to maximum before finally declining to zero.

To determine \(J_{\text{Release}}\), it was necessary to dissect \(J\), into its component fluxes. The rate of \(\text{Ca}^{2+}\) extrusion across the plasma membrane \((J_{\text{pm}})\) was determined by measuring the total \(\text{Ca}^{2+}\) flux during the recovery after depolarization, and is plotted against \([\text{Ca}^{2+}]\), in Fig. 3 C; for comparison, \(J\), is also plotted throughout the t-BuBHQ-induced transient. In a previous study, it was shown that at each point in time, \(J_{\text{pm}}\) depends on the \([\text{Ca}^{2+}]\), level at that time (Colegrove et al., 2000a). This made it possible to determine the rate of \(\text{Ca}^{2+}\) extrusion at each time point during the t-BuBHQ-induced transient based on the magnitude of \(J_{\text{pm}}\) during the recovery after repolarization at the corresponding \([\text{Ca}^{2+}]\), level. The time course of \(J_{\text{pm}}\) determined in this way is shown in Fig. 3 B and parallels the \([\text{Ca}^{2+}]\), response. Subtracting \(J_{\text{pm}}\) from \(J\), at each point in time then gives the remaining \(\text{Ca}^{2+}\) flux (Fig. 3 B, shaded region). Since these measurements were performed in the presence of FCCP and a saturating concentration of t-BuBHQ, the remaining flux \((J_{\text{Release}})\) is expected to represent the rate of passive \(\text{Ca}^{2+}\) release from the ER (Fig. 3 B). If cytoplasmic and intraluminal buffers equilibrate rapidly with \(\text{Ca}^{2+}\) and the latter bind \(\text{Ca}^{2+}\) with low affinity, then at each instant in time, the produ-
uct $J_{\text{Release}}$, should be directly proportional to the net ER $Ca^{2+}$ flux, and, therefore, to the rate at which $[Ca^{2+}]_{\text{ER}}$ changes with time (see Appendix A). The properties of $J_{\text{Release}}$ are revealing. During the entire t-BuBHQ-induced $[Ca^{2+}]$, transient, $J_{\text{Release}}$ is negative, which is indicative of $Ca^{2+}$ release. During the rising phase, the magnitude of $J_{\text{Release}}$ increases to a peak. This increase occurs even though the driving force for passive $Ca^{2+}$ release should be falling (($[Ca^{2+}]$), is rising and the intraluminal $Ca^{2+}$ concentration is falling). As discussed in connection with Fig. 2, with our application protocol, t-BuBHQ appears to block SERCAs rapidly (within 200 ms) and completely, arguing that the initial increase in $J_{\text{Release}}$ observed in control cells does not simply reflect the time required for the inhibitor to act. This is supported by the finding that, in ryanodine-treated cells, the magnitude of $J_{\text{Release}}$ declines monotonically after t-BuBHQ application (see Fig. 4). This suggests that the pathway responsible for passive $Ca^{2+}$ release includes a $[Ca^{2+}]$, sensitive permeability.

Given measurements of $J_{\text{Release}}$, along with a description of the intraluminal $Ca^{2+}$ concentration, it is possible to characterize the $Ca^{2+}$ permeability of the ER and test the idea that it is sensitive to $[Ca^{2+}]$. When ER $Ca^{2+}$ uptake is inhibited, $J_{\text{Release}}$ gives the net ER $Ca^{2+}$ flux referred to the effective cytoplasmic volume, so multiplying by the ratio of effective cytoplasmic and intraluminal volumes ($v_{\text{ER}}/v_{\text{ER}}$) gives the rate at which $[Ca^{2+}]_{\text{ER}}$ changes with time. Integration of this quantity from the instant of t-BuBHQ application to the time $t$ thus provides a measure of the change in $[Ca^{2+}]_{\text{ER}}$ relative to its initial value just before the perturbation. Subtracting the integral during the entire t-BuBHQ-induced $[Ca^{2+}]$, transient gives the change in concentration relative to its final basal value. As shown in Appendix A, this latter quantity, which we call $\Delta [Ca^{2+}]_{\text{ER}}(t)$, is given by Eq. 2:

$$\Delta [Ca^{2+}]_{\text{ER}}(t) = \frac{v_i}{v_{\text{ER}}K_{\text{ER}}} \int_0^t J_{\text{Release}}K_i dt'$$

$$= \frac{v_i}{v_{\text{ER}}K_{\text{ER}}} \Delta [Ca^{2+}]_{\text{ER}}^{(i)}(t),$$

where minus the integral ($\Delta [Ca^{2+}]_{\text{ER}}^{(i)}(t)$) can be interpreted as the change in total cytoplasmic Ca concentration that would occur if from time $t$ onward, $J_{\text{Release}}$ were deposited into a closed compartment having the same volume as the cytoplasm. Accordingly, the initial value ($\Delta [Ca^{2+}]_{\text{ER}}^{(i)}(0)$) provides information about the resting $Ca^{2+}$ concentration within the ER. The relationship is (Eq. 3):

$$[Ca^{2+}]_{\text{ER}}(0) = [Ca^{2+}]_{\text{ER}}(\infty) + \frac{v_i}{v_{\text{ER}}K_{\text{ER}}} \Delta [Ca^{2+}]_{\text{ER}}^{(i)}(0).$$

A similar approach to assessing changes in ER $Ca^{2+}$ concentration in T cells was described previously by Bergling et al. (1998).

Before using $J_{\text{Release}}$ and $\Delta [Ca^{2+}]_{\text{ER}}(t)$ to characterize the $Ca^{2+}$ permeability of the ER, it is important to determine if these quantities have the expected properties. During the entire t-BuBHQ-induced $[Ca^{2+}]$, transient, the sign of $J_{\text{Release}}$ is indicative of net $Ca^{2+}$ release, and correspondingly $\Delta [Ca^{2+}]_{\text{ER}}(t)$ declines monotonically from the point of t-BuBHQ addition (Fig. 3 D). Analysis of sympathetic neurons gives an estimate of 10–20 for $v_i$ (unpublished data); although measurements of $K_{\text{ER}}$ have not been made in these cells, values have been reported in other cells: ~17 in AT20 cells (Wu et al., 2001) and ~20 in pancreatic acinar cells (Mogami et al., 1999). Based on these values, $\Delta [Ca^{2+}]_{\text{ER}}^{(i)}$ can be estimated to be ~0.5–1.2. Thus, the ~140-μM decline in $\Delta [Ca^{2+}]_{\text{ER}}$ shown in Fig. 3 C should correspond to a 70–168-μM decline in $[Ca^{2+}]_{\text{ER}}$. Since the basal value of $[Ca^{2+}]_{\text{ER}}$ after treatment with t-BuBHQ ($[Ca^{2+}]_{\text{ER}}(\infty)$) would be expected to approximate the resting cytoplasmic $Ca^{2+}$ concentration (~50–100 nM), using Eq. 3 we arrive at an estimated initial value of $[Ca^{2+}]_{\text{ER}}$ ~70.1–168.1 μM. This is consistent with the value obtained from the reduction in total ER $Ca^{2+}$ concentration induced by Tg, as determined from electron probe microanalysis in these cells (~20 mM; Hongpaisan et al., 2001) using the same values for $K_{\text{ER}}$: $\Delta [Ca^{2+}]_{\text{ER}}$ ~2 mM/20 = 100 μM. It should be noted that because slow net $Ca^{2+}$ release is increasingly difficult to resolve as $[Ca^{2+}]$, approaches its resting level, it is difficult to determine when $J_{\text{Release}}$ is truly zero and $Ca^{2+}$ within the ER is in equilibrium with the cytoplasmic compartment. Therefore, it is possible that the initial value of $\Delta [Ca^{2+}]_{\text{ER}}$ and hence our estimations of basal $[Ca^{2+}]_{\text{ER}}$ underestimate the actual values.

Measurements of $J_{\text{Release}}$ and $\Delta [Ca^{2+}]_{\text{ER}}(t)$, along with the characterization of $K_{\text{ER}}$ from Fig. 1, can now be used to provide information about the $Ca^{2+}$ permeability of the ER ($P_{\text{ER}}$). $P_{\text{ER}}$ is defined operationally by Eq. 4:

$$J_{\text{Release}} = \frac{P_{\text{ER}}}{v_{\text{ER}}}$$

$$= \frac{P_{\text{ER}}}{v_{\text{ER}}} (\Delta [Ca^{2+}]_{\text{ER}} - [Ca^{2+}]_{\text{ER}})$$

$$\equiv P_{\text{ER}} ([Ca^{2+}]_{\text{ER}} - [Ca^{2+}]_{\text{ER}}),$$

where $P_{\text{ER}}$ is a lumped parameter giving the ratio of $P_{\text{ER}}$ to $v_{\text{ER}}$, and $[Ca^{2+}]_{\text{ER}}(t)$ is the free Ca concentration within the ER at time $t$. $P_{\text{ER}}$ would be expected to depend on the number, open probability, and unitary $Ca^{2+}$ permeability of ryanodine-sensitive $Ca^{2+}$ release channels expressed in these cells, as well as other channels that are permeable to $Ca^{2+}$ and influence the rate of passive $Ca^{2+}$ release. In particular, the $[Ca^{2+}]$, depen-
dence of $\tilde{P}_{ER}$ should provide information about how the activity of these channels varies with $[\text{Ca}^{2+}]_i$. In general, $\tilde{P}_{ER}$ could be influenced by $[\text{Ca}^{2+}]_i$, $[\text{Ca}^{2+}]_{ER}$, and could show explicit time dependence (e.g., as a result of desensitization). Finally, $\tilde{P}_{ER}$ would be expected to show a composite $[\text{Ca}^{2+}]_i$ dependence representing properties of both $P_{ER}$ and $\kappa_i$.

Given measurements of $J_{\text{Release}}$ and $([\text{Ca}^{2+}]_i - [\text{Ca}^{2+}]_{ER})$, $P_{ER}$ could be determined from the ratio $J_{\text{Release}}/([\text{Ca}^{2+}]_i - [\text{Ca}^{2+}]_{ER})$ at each point in time during the t-BuBHQ-induced $[\text{Ca}^{2+}]_i$ transient; measurements of $\kappa_i$ would then make possible to obtain information about $P_{ER}$. This approach requires information about $[\text{Ca}^{2+}]_{ER}(t)$. Although single cell measurements of $[\text{Ca}^{2+}]_{ER}(t)$ are not available, it is shown in APPENDIX A that $([\text{Ca}^{2+}]_i - [\text{Ca}^{2+}]_{ER})$ can be approximated by $-(v_i/v_{ER}\kappa_{ER})\Delta[\text{Ca}^{2+}]_{ER}$, making it possible to rewrite Eq. 4 as Eq. 5:

$$J_{\text{Release}}(t) = \frac{P_{ER}(v_i)\kappa_{ER}}{v_{ER}\kappa_{ER}} \Delta[\text{Ca}^{2+}]_{ER}(t),$$

and to obtain a quantity that is proportional to $P_{ER}$ from measured values,

$$P_{ER}(v_i/v_{ER}\kappa_{ER}) = \frac{J_{\text{Release}}(t)}{\Delta[\text{Ca}^{2+}]_{ER}(t)},$$

which is valid as long as $[\text{Ca}^{2+}]_{ER}$ is much larger than $[\text{Ca}^{2+}]_i$ (see APPENDIX A). Fig. 3 E shows $P_{ER}(v_i/v_{ER}\kappa_{ER})$ plotted against $[\text{Ca}^{2+}]_i$ during the t-BuBHQ-induced $[\text{Ca}^{2+}]_i$ transient, calculated according to Eq. 6. The dark noisy trace shows $P_{ER}(v_i/v_{ER}\kappa_{ER})$ during the rising phase of the transient, and the light trace represents the recovery phase. Three features of these measurements should be noted. First, $P_{ER}(v_i/v_{ER}\kappa_{ER})$ is not constant, but increases monotonically with $[\text{Ca}^{2+}]_i$ over the range $\sim$50–250 nM. Second, the $[\text{Ca}^{2+}]_i$ dependence of $P_{ER}(v_i/v_{ER}\kappa_{ER})$ during the onset and recovery (dark and light traces, respectively) is very similar. In other words, for a given value of $[\text{Ca}^{2+}]_i$, $P_{ER}(v_i/v_{ER}\kappa_{ER})$ has essentially the same magnitude during both the onset and the recovery. Since the intraluminal Ca$^{2+}$ concentration is expected to be very different during these phases of the t-BuBHQ-induced $[\text{Ca}^{2+}]_i$ transient, it appears that $P_{ER}(v_i/v_{ER}\kappa_{ER})$ depends much more strongly on $[\text{Ca}^{2+}]_i$ than on $[\text{Ca}^{2+}]_{ER}$. Moreover, if the underlying permeability undergoes desensitization, it must be very rapid, or very weak, compared with the $[\text{Ca}^{2+}]_i$-dependent changes observed in these experiments. Finally, extrapolation of $P_{ER}(v_i/v_{ER}\kappa_{ER})$ to $[\text{Ca}^{2+}] = 0$ gives an estimate of the basal Ca$^{2+}$ permeability of the ER (Fig. 3 E, dotted trace); however, since this value is based on extrapolation, there is uncertainty about its precise value. Importantly, these properties are shared by the macroscopic Ca$^{2+}$ permeability. Fig. 3 E (inset) shows $\tilde{P}_{ER}/v_{ER}\kappa_{ER}$ obtained after scaling $P_{ER}(v_i/v_{ER}\kappa_{ER})$ by $\kappa_i$, indicating that the macroscopic Ca$^{2+}$ permeability of the ER depends on $[\text{Ca}^{2+}]_i$. Results presented in the next section provide evidence that this $[\text{Ca}^{2+}]_i$-dependent permeability is dominated by ryanodine receptors.

**Changes in ER Ca$^{2+}$ Permeability Induced by Caffeine and Rynadone**

A number of observations indicate that sympathetic neurons express functional RyRs (Kuba and Nishi, 1976; Lipscombe et al., 1988; Friel and Tsien, 1992a,b; Hua et al., 1993; Akita and Kuba, 2000) that would contribute to the macroscopic Ca$^{2+}$ permeability of the ER, so we performed experiments to determine if $P_{ER}$ is sensitive to pharmacological modifiers of RyRs (Zucchi and Roncha-Testoni, 1997). Fig. 4 shows t-BuBHQ-induced $[\text{Ca}^{2+}]_i$ transients (Fig. 4 A) and the corresponding measurements of $J_{\text{Release}}$ (Fig. 4 B) and $\Delta[\text{Ca}^{2+}]_{ER}$ (Fig. 4 C) under control conditions, during continuous exposure to caffeine (after the caffeine-induced $[\text{Ca}^{2+}]_i$, transient) and after treatment with ryanodine, which rendered cells unresponsive to caffeine. Fig. 4 D compares the $[\text{Ca}^{2+}]_i$ dependence of $P_{ER}(v_i/v_{ER}\kappa_{ER})$ for each cell whose responses are illustrated in Fig. 4, A–C; population results are shown in Fig. 4 E. In the presence of caffeine, t-BuBHQ-induced $[\text{Ca}^{2+}]_i$, transients are faster, and $\Delta[\text{Ca}^{2+}]_{ER}$ declines more rapidly, compared with controls, reflecting an approximately three-fold increase in the peak magnitude of $J_{\text{Release}}$. In contrast, after treatment with ryanodine, the evoked $[\text{Ca}^{2+}]_i$ transients are slower, and $\Delta[\text{Ca}^{2+}]_{ER}$ declines more slowly than the control, reflecting a steady decline in $J_{\text{Release}}$ that contrasts markedly with the initial increase seen both in the control case and in the presence of caffeine. Given that the magnitude of $J_{\text{Release}}$ declines monotonically from the instant of t-BuBHQ application in ryanodine-treated cells, it is unlikely that the initial increase in $J_{\text{Release}}$ observed in control and caffeine-treated cells occurs simply because the inhibitor acts slowly, or because of $[\text{Ca}^{2+}]_i$-dependent changes in cytoplasmic Ca$^{2+}$ buffering strength. It also was found that the initial value of $\Delta[\text{Ca}^{2+}]_{ER}$ followed the order: control $(115 \pm 12 \mu M$, four cells) is greater than +caffeine $(38 \pm 4 \mu M$, four cells) is greater than +ryanodine $(20 \pm 3 \mu M$, three cells). Neither caffeine nor ryanodine had a systematic effect on $J_{pn}$ (unpublished data). These actions of caffeine and ryanodine on t-BuBHQ-induced $[\text{Ca}^{2+}]_i$, transients are consistent with their effects on $P_{ER}(v_i/v_{ER}\kappa_{ER})$ and $\tilde{P}_{ER}/v_{ER}\kappa_{ER}$ (Fig. 4 D). In the presence of caffeine and ryanodine, $\tilde{P}_{ER}/v_{ER}\kappa_{ER}$ shows higher values at resting $[\text{Ca}^{2+}]_i$, than the control, accounting for the lower basal values of $\Delta[\text{Ca}^{2+}]_{ER}$ (Fig. 4 C). In the presence of caffeine, $\tilde{P}_{ER}/v_{ER}\kappa_{ER}$ also increases more steeply with $[\text{Ca}^{2+}]_i$, than in the control and shows a supralinear $[\text{Ca}^{2+}]_i$ dependence, which presumably contributes to the accel-
Dissection of ER Ca Fluxes in Sympathetic Neurons

erated $[\text{Ca}^{2+}]_i$ rise (Fig. 4, A and B). After ryanodine treatment, the $[\text{Ca}^{2+}]_i$ dependence of $P_{\text{ER}}(v/v_{\text{ER}})$ could be accounted for simply by the $[\text{Ca}^{2+}]_i$ dependence of $\kappa$, indicating that, under these conditions, $P_{\text{ER}}(v/v_{\text{ER}})$ does not vary with $[\text{Ca}^{2+}]_i$, accounting for the monotonic decline in the magnitude of $J_{\text{Release}}$ (Fig. 4 B) and the slower $[\text{Ca}^{2+}]_i$ transient (Fig. 4 A).

Each effect is consistent with known properties of RyRs and their modification by caffeine and ryanodine (Rousseau et al., 1987, 1988). Overall, the results illustrate how a caffeine- and ryanodine-sensitive Ca$^{2+}$ permeability representing the activity of a population of RyRs contributes to passive Ca$^{2+}$ release from the ER. Importantly, the properties of $P_{\text{ER}}$ in ryanodine-treated cells sug-
suggests that RyRs are responsible for most of the \([\text{Ca}^{2+}]_{\text{i}}\) dependence of ER \(\text{Ca}^{2+}\) permeability when \([\text{Ca}^{2+}]_{\text{i}} < 250\) nM and InsP3-generating agonists are not present.

Reconstruction of \(t\)-BuBHQ-induced \(\text{Ca}^{2+}\) Responses and the Underlying \(\text{Ca}^{2+}\) Fluxes

To determine if the rate descriptions presented above are sufficient to account for the dynamics of cytoplasmic and intraluminal \(\text{Ca}^{2+}\) concentration during \(t\)-BuBHQ-induced \([\text{Ca}^{2+}]_{\text{i}}\) transients, simulations were performed using the experimentally determined descriptions of \(\text{JSERCA}, \text{J}_{\text{pm}}, \text{P}_{\text{ER(vi/vER)}}\), and \(\kappa_i\) approximating \(V_{\text{ERK}_{\text{ER}}} \) by unity. In displaying the results, simulated \(\text{Ca}^{2+}\) concentrations are designated by \(c_i\) and \(c_{\text{ER}}\) to distinguish them from measured quantities. To facilitate comparison with responses from cells in Fig. 4, simulations were performed using the transport descriptions and initial \([\text{Ca}^{2+}]_{\text{i}}\) values obtained from those same cells. It was found that the simulations reproduce the experimental observations quite well, including the relative values of basal \(c_{\text{ER}}\) and the time courses of \(c_i, J_{\text{Release}},\) and \(c_{\text{ER}}\) (Fig. 5). This leads to three conclusions. First, \(J_{\text{pm}}, \text{JSERCA},\) and \(P_{\text{ER}}\) describe the main \(\text{Ca}^{2+}\) transport pathways responsible for cytoplasmic and intraluminal \(\text{Ca}^{2+}\) dynamics after rapid SERCA inhibition. Second, the equations used to describe these pathways do not ignore variables that are important for the dynamics of \([\text{Ca}^{2+}]_{\text{i}}\) and \(\Delta[\text{Ca}^{2+}]_{\text{ER}}\). Third,
the functional dependence of the fluxes on [Ca\(^{2+}\)], and [Ca\(^{2+}\)]\(_{\text{ER}}\) is sufficiently accurate to reproduce the salient features of the time courses of [Ca\(^{2+}\)]\(_{i}\) and \(\Delta[\text{Ca}^{2+}]\)\(_{\text{ER}}\). Thus, when mitochondrial Ca\(^{2+}\) transport is suppressed, the initial distribution of intracellular Ca\(^{2+}\), as well as the dynamics of Ca\(^{2+}\) after inhibition of SERCAs, can be explained in terms of the properties of \(J_{\text{pm}}, J_{\text{SERCA}}, P_{\text{ER}}, \) and \(k_{i}\).

**The Properties of \(J_{\text{pm}}, J_{\text{SERCA}}, P_{\text{ER}(\text{vi/vER})}, \) and \(k_{i}\) Account for Ca\(^{2+}\) Dynamics during Depolarization-evoked Ca\(^{2+}\) Entry**

We have shown that when [Ca\(^{2+}\)]\(_{i}\) is low (less than or equal to \(\sim 550 \text{nM}\)) during weak depolarization, the ER normally accumulates Ca\(^{2+}\), and that after treatment with ryanodine, the same stimuli lead to enhanced ER Ca\(^{2+}\) accumulation (Albrecht et al., 2001). We asked if the properties of \(J_{\text{pm}}, J_{\text{SERCA}}, P_{\text{ER}(\text{vi/vER})}, \) and \(k_{i}\) described above account for these observations. Fig. 6 shows simulations of depolarization-induced changes in [Ca\(^{2+}\)]\(_{i}\) and [Ca\(^{2+}\)]\(_{\text{ER}}\) using a measured Ca\(^{2+}\) current (\(I_{\text{Ca}}\)) as the basis for calculating the rate of stimulated Ca\(^{2+}\) entry (Fig. 6 A). Mitochondrial Ca\(^{2+}\) uptake was taken into consideration as described in Colegrove et al. (2000b) to facilitate comparison with measurements performed under voltage clamp in the absence of FCCP (Albrecht et al., 2001). Using parameters for \(P_{\text{ER}(\text{vi/vER})}\) obtained under control conditions, Ca\(^{2+}\) entry produces a \(c_{i}\) elevation that is accompanied by weak ER Ca\(^{2+}\) accumulation; consequently, the [Ca\(^{2+}\)]\(_{i}\) response is slightly accelerated when uptake is inhibited with Tg, as observed experimentally (Albrecht et al., 2001). Using \(P_{\text{ER}(\text{vi/vER})}\) parameters taken from ryanodine-treated cells, Ca\(^{2+}\) entry elicits a slower rise in \(c_{i}\) but a more robust increase in \(c_{\text{ER}}\), also as observed experimentally (Albrecht et al., 2001). Finally, using \(P_{\text{ER}(\text{vi/vER})}\) parameters from caffeine-treated cells, [Ca\(^{2+}\)]\(_{i}\) rises more rapidly in response to the same stimuli in concert with net Ca\(^{2+}\) release. Net Ca\(^{2+}\) release occurs in this case because \(J_{\text{Release}}\) increases more steeply with \(c_{i}\) than does \(J_{\text{SERCA}}\) over this \(c_{i}\) range. Resting \(c_{\text{ER}}\) is lower in this and the +Ryan case because basal \(P_{\text{ER}(\text{vi/vER})}\) is higher than it is under control conditions. These results validate the quantitative model presented in our previous study (Albrecht et al., 2001).

Comparing ER Ca\(^{2+}\) uptake and release rates shows how activation of the CICR pathway influences net ER Ca\(^{2+}\) transport and \(c_{i}\) and \(c_{\text{ER}}\) dynamics during such weak stimuli (Fig. 7). Before stimulation, ER Ca\(^{2+}\) uptake and release rates are in balance, accounting for the steady resting value of \(c_{\text{ER}}\). The resting rate of release is not modified by ryanodine because while \(P_{\text{ER}}\) is increased, the driving force is reduced, such that their product is unchanged. Under control conditions (Fig. 7 B), stimulation leads to Ca\(^{2+}\) accumulation by the ER (Fig. 7 B, dark trace) because \(J_{\text{SERCA}}\) increases more rap-

![Figure 6. Simulated responses to depolarization-evoked Ca\(^{2+}\) entry and their sensitivity to modifications of ER Ca\(^{2+}\) transport. Simulated changes in \(c_{i}\) (B) and \(c_{\text{ER}}\) (C) resulting from stimulated Ca\(^{2+}\) entry (A). For each simulation, \(J_{\text{SERCA}}\) was described as in Fig. 2 C, except for the case (+Tg) where \(V_{\text{max,SERCA}} = 0\), and \(J_{\text{pm}}\) was described by the smooth curve from the control cell in Fig. 3 C. \(P_{\text{ER}(\text{vi/vER})}\) was represented by the smooth curves in Fig. 4 D. In the case of +Tg, \(P_{\text{ER}(\text{vi/vER})}\) was described as in the control cell. \(I_{\text{Ca}}\) is based on a measured Ca\(^{2+}\) current elicited by a 40-s depolarization to \(-35 \text{ mV}\), scaled in amplitude, and extrapolated in time, with tail current omitted. \(c_{i} = 2 \text{ mM}\) and \(v_{\text{ER}} = 1\). Mitochondrial Ca\(^{2+}\) uptake was described as in Colegrove et al. (2000b) without release to facilitate comparison with experiments performed under voltage clamp in cells with low intracellular Na\(^{+}\), which inhibits release. Arrows in C indicate the 45- and 120-s time points at which measurements of total ER Ca concentration are available during steady depolarization in the absence and presence of ryanodine (Albrecht et al., 2001).](image-url)
idly than $J_{\text{Release}}$ increases because of its intrinsic $c_i$ dependence, whereas $J_{\text{Release}}$ rises in magnitude because of two factors: an increase in driving force (since $e_{\text{ER}}$ rises more rapidly than $c_i$) and an increase in $P_{\text{ER}}$. The $c_i$-dependent increase in $P_{\text{ER}}$ accelerates the increase in $J_{\text{Release}}$, reducing the imbalance between $J_{\text{SERCA}}$ and $J_{\text{Release}}$, ultimately causing the ER to be a less powerful Ca$^{2+}$ buffer. After inhibiting the $c_i$-dependent activation of $P_{\text{ER}}$ (Fig. 7 C), the magnitude of $J_{\text{Release}}$ increases more slowly in response to an increase in driving force only, and this exaggerates the imbalance between $J_{\text{SERCA}}$ and $J_{\text{Release}}$, leading to enhanced Ca$^{2+}$ accumulation. As a result, the ER becomes a stronger buffer, as observed experimentally (Albrecht et al., 2001).

**Properties of $P_{\text{ER}(v)/v_{\text{ER}},\kappa_{\text{ER}}}$ Account for Caffeine-induced [Ca$^{2+}$], Oscillations**

Previous work has shown that when caffeine-treated sympathetic neurons are depolarized to stimulate Ca$^{2+}$ entry, [Ca$^{2+}$]$_i$ rises rapidly to produce a spike and then, in many cases, oscillates (Lipscombe et al., 1988; Nohmi et al., 1992; Friel and Tsien, 1992b). To determine if these [Ca$^{2+}$] oscillations can be accounted for by the interplay between $J_{\text{pm}}$, $J_{\text{SERCA}}$, and $P_{\text{ER}(v)/v_{\text{ER}},\kappa_{\text{ER}}}$, simulations were performed using descriptions of $J_{\text{pm}}$ and $J_{\text{SERCA}}$ from control cells and of $P_{\text{ER}(v)/v_{\text{ER}},\kappa_{\text{ER}}}$ from caffeine-treated cells. Although Ca$^{2+}$ entry led to a steady increase in $c_i$ when Ca$^{2+}$ uptake by the store was inhibited (Fig. 8, $P_{\text{max,RyR}}$ dotted traces), oscillations were observed when both uptake and release pathways were enabled. The oscillations were much like those observed experimentally when caffeine-treated sympathetic neurons are depolarized by exposure to high K$^+$ (Friel and Tsien, 1992b). Using descriptions of $P_{\text{ER}(v)/v_{\text{ER}},\kappa_{\text{ER}}}$ from control and caffeine-treated cells contributing to the collected results in Fig. 4 E indicates that depolarization-evoked Ca$^{2+}$ entry does not elicit $c_i$ oscillations when control $P_{\text{ER}(v)/v_{\text{ER}},\kappa_{\text{ER}}}$ descriptions are used (4/4 cells), whereas oscillations can be elicited when descriptions are taken from caffeine-treated cells (4/4 cells). Thus, the quantitative descriptions of ER Ca$^{2+}$ uptake and release pathways obtained using t-BuBHQ-induced perturbations account for the observation that membrane depolarization typically elicits [Ca$^{2+}$] oscillations in caffeine-treated cells, but not in untreated cells.

**Quantitative Basis for Multiple Modes of CICR in Sympathetic Neurons**

Measurements in sympathetic neurons implicate distinct modes of CICR that operate over different ranges of [Ca$^{2+}$]. (Albrecht et al., 2001, Hongpaisan et al., 2001). When [Ca$^{2+}$], is low, the ER accumulates Ca$^{2+}$ at a rate that is reduced by activation of the CICR pathway (Mode 1 CICR). At higher [Ca$^{2+}$] levels, the ER releases Ca$^{2+}$, either at a rate that is slower than Ca$^{2+}$ clearance by other pathways (Mode 2 CICR), or faster, such that release overwhelms Ca$^{2+}$ clearance, leading to regenerative release (Mode 3 CICR). Do the measurements of $J_{\text{pm}}, J_{\text{SERCA}},$ and $P_{\text{ER}(v)/v_{\text{ER}},\kappa_{\text{ER}}}$ described above help explain these modes of CICR? Although we did not carry out rate measurements at high [Ca$^{2+}$], it is
possible to investigate the qualitative properties of these modes using the measured rate descriptions extrapolated to higher \([\text{Ca}^{2+}]_i\). \(J_{\text{SERCA}}\) and \(P_{\text{ER}}(v_{\text{ER}}/v_\text{ER})\) were represented by continuously extending the functions describing their \([\text{Ca}^{2+}]_i\) dependence at lower \([\text{Ca}^{2+}]_i\) levels.

The resulting descriptions coincide with the measured values at low \(c_i\) and represent approximations at high \(c_i\). Fig. 9 shows how in the steady state, \(c_{\text{ER}}\) varies with \(c_i\). When \(c_i\) is below \(\sim 200\) nM, an increase in \(c_i\) leads to net \(\text{Ca}^{2+}\) uptake and a rise in \(c_{\text{ER}}\). Increasing \(c_i\) further over the physiological range leads to net \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release and a decline in \(c_{\text{ER}}\). Further (nonphysiological) increases in \(c_i\) lead to \(\text{Ca}^{2+}\) accumulation and a rise in \(c_{\text{ER}}\). Thus, there are two major effects of the \(\text{Ca}^{2+}\) sensitive permeability on the relationship between \(c_i\) and \(c_{\text{ER}}\) under steady-state conditions. First, it leads to the definition of three distinct \(c_i\) ranges in which the ER plays qualitatively different roles in \(\text{Ca}^{2+}\) regulation: low \(c_i\) (ER is a \(\text{Ca}^{2+}\) sink), intermediate \(c_i\) (ER is a \(\text{Ca}^{2+}\) source), and high \(c_i\) (ER is a \(\text{Ca}^{2+}\) sink). Second, it would act as a safety valve to stabilize \(c_{\text{ER}}\) in the face of prolonged \([\text{Ca}^{2+}]_i\) elevations that would otherwise lead to large increases in intraluminal \(\text{Ca}^{2+}\) levels. With CICR in place, \(c_{\text{ER}}\) increases by less than a factor of two for a 10,000-fold change in \(c_i\) above resting levels.

To illustrate how the \(\text{Ca}^{2+}\)-sensitive permeability is expected to influence evoked \(\text{Ca}^{2+}\) signals, Fig. 10 (B and C) shows instantaneous flux/\(c_i\) relations like those described in Albrecht et al. (2001), calculated using the same parameter values as in Fig. 9 (with CICR). Fig. 9 A shows the \(c_i\) dependence of \(P_{\text{ER}}\) and \(P_{\text{ER}}/v_i\), whereas...
Fig. 9 B shows how $J_{ER}$ and its components $J_{SERCA}$ and $J_{Release}$ would be expected to change if $c_i$ was suddenly raised without perturbing $c_{ER}$ from its resting value, assuming a normal resting $[Ca^{2+}]_i$ level (50 nM). When $c_i$ is increased up to ~350 nM, the outward flux $J_{SERCA}$ is larger in magnitude than the inward flux $J_{Release}$, so that the ER accumulates $Ca^{2+}$. However, $Ca^{2+}$ accumulation becomes gradually slower because of progressive $Ca^{2+}$-dependent activation of $P_{ER}$, which we called Mode 1 CICR. When $c_i$ exceeds the threshold for net CICR, $J_{Release}$ is larger in magnitude than $J_{SERCA}$, causing $J_{ER}$ to be an inward flux, leading to net $Ca^{2+}$ release (Mode 2).

The $Ca^{2+}$ level at which this transition occurs, defined by a specific threshold (Fig. 10 B, arrow), depends on the quantitative relationship between the underlying fluxes and their $c_i$ dependence. Similarly, the interplay between $Ca^{2+}$ extrusion across the plasma membrane and net ER $Ca^{2+}$ transport defines the total cytoplasmic $Ca^{2+}$ flux ($J_{total}$; Fig. 10 C). When $c_i$ is low, $J_{pm}$ and $J_{ER}$ are both outwardly directed, so that if $c_i$ were increased rapidly to such a level and then allowed to relax, $c_i$ would decline at a rate that is jointly influenced by $Ca^{2+}$ extrusion and net uptake by the ER. If $c_i$ were increased to higher levels where $J_{ER}$ is negative, $c_i$ would also de-
cline, but at a rate that reflects the difference between Ca\(^{2+}\) extrusion and net release rates. Finally, if \(c_i\) is increased beyond \(\sim 935\) nM, the rate of net Ca\(^{2+}\) release exceeds the rate of Ca\(^{2+}\) clearance, leading regenerative release. Fig. 10 (right) illustrates simulated \(c_i\) and \(c_{ER}\) responses (Fig. 10, E and F) evoked by stimuli that raise \(c_i\) to levels below or above the threshold for net Ca\(^{2+}\) release, and above the threshold for regenerative net CICR. Simulations were performed using Ca\(^{2+}\) entry waveforms obtained from measured Ca\(^{2+}\) currents of 2 s duration (Fig. 10 D). As [Ca\(^{2+}\)] rises during the first (subthreshold) stimulus (curve 1), the ER accumulates Ca\(^{2+}\) (Fig. 10 F, inset). After the stimulus ends, \(c_{ER}\) continues to rise and \(c_i\) declines under the joint influence of Ca\(^{2+}\) extrusion across the plasma membrane and Ca\(^{2+}\) accumulation by the store. During the second stimulus, which exceeds the threshold for net CICR (curve 2), the ER is transformed into a source, with \(c_{ER}\) eventually declining below the basal level (Fig. 10 F, inset). When the stimulus ends, \(c_i\) declines initially at a rate that depends on Ca\(^{2+}\) extrusion and net Ca\(^{2+}\) release, but the recovery is eventually accelerated when the ER once again becomes a Ca\(^{2+}\) sink, causing an overshoot of the basal level before \(c_{ER}\) finally approaches the resting value. The third stimulus (curve 3) is sufficiently strong to bring \(c_i\) above the threshold for regenerative CICR, such that when the stimulus ends, \(c_i\) continues to rise under the influence of continued net Ca\(^{2+}\) release at a rate that exceeds the rate of Ca\(^{2+}\) extrusion. Thus, the extrapolated rate descriptions for \(J_{募集}, J_{SERCA}\), and \(P_{ER}\) provide an explanation for the observation that stimuli producing progressively larger [Ca\(^{2+}\)] elevations cause the ER to undergo a transition from sink to source (Hongpaisan et al., 2001).

**DISCUSSION**

The main goal of this study was to determine how Ca\(^{2+}\)-dependent activation of a ryanodine-sensitive CICR pathway contributes to [Ca\(^{2+}\)] responses evoked by weak depolarization. Our previous study showed that under these conditions of stimulation, the ER accumulates Ca\(^{2+}\), but we also presented indirect evidence that the rate of Ca\(^{2+}\) accumulation is attenuated by activation of the CICR pathway, which, in effect, makes the ER a weaker Ca\(^{2+}\) buffer. Since the rate of net ER Ca\(^{2+}\) transport depends on the relative rates of Ca\(^{2+}\) uptake and release via different transport pathways, we characterized these rates and their regulation by Ca\(^{2+}\) to test the hypothesis that the direction and rate of net ER Ca\(^{2+}\) transport depends on a slight imbalance between uptake and release rates of much larger magnitude, each showing a distinct functional dependence on [Ca\(^{2+}\)]. We found that Ca\(^{2+}\) uptake is regulated by SERCAs in a [Ca\(^{2+}\)]-dependent manner, and that passive Ca\(^{2+}\) release is regulated by a [Ca\(^{2+}\)]-sensitive permeability that is modified by caffeine and ryanodine in a way that indicates it is dominated by ryanodine-sensitive Ca\(^{2+}\) release channels. It was found that quantitative differences in the [Ca\(^{2+}\)] sensitivity of Ca\(^{2+}\) uptake and release rates account for Mode 1 CICR. Although these rates are in balance under resting conditions, small [Ca\(^{2+}\)] elevations stimulate Ca\(^{2+}\) uptake more strongly than Ca\(^{2+}\) release, accounting for Ca\(^{2+}\) accumulation. Moreover, increases in the rate of passive Ca\(^{2+}\) release by such small [Ca\(^{2+}\)] elevations, reflecting a [Ca\(^{2+}\)]-induced rise in ER Ca\(^{2+}\) permeability, reduces the imbalance between uptake and release rates, accounting for attenuated Ca\(^{2+}\) accumulation during stimulation. Taken together with the properties of Ca\(^{2+}\) extrusion across the plasma membrane, these same transport pathways account for caffeine-induced [Ca\(^{2+}\)] oscillations. Finally, extending the rate descriptions of Ca\(^{2+}\) uptake and release to higher [Ca\(^{2+}\)] levels provides an explanation for our finding that as depolarization-evoked [Ca\(^{2+}\)] elevations become larger, the ER becomes a less effective Ca\(^{2+}\) buffer, and at high [Ca\(^{2+}\)], becomes a Ca\(^{2+}\) source.

**Properties of the ER Ca\(^{2+}\) Uptake Pathway**

The impact of SERCAs (\(J_{SERCA}\)) on Ca\(^{2+}\) dynamics was assessed by measuring the t-BuBHQ-sensitive component of the total cytoplasmic Ca\(^{2+}\) flux (\(J\)) just after exposing cells to a high concentration of t-BuBHQ. As it was measured, the [Ca\(^{2+}\)] dependence of \(J_{SERCA}\) is expected to depend both on the rate of Ca\(^{2+}\) uptake via SERCAs at the instant of inhibition, and the properties of cytoplasmic Ca\(^{2+}\) buffering. The composite [Ca\(^{2+}\)] dependence of \(J_{SERCA}\) could be described by Eq. B8 quite well, and along with the other transport characterizations it made it possible to reconstruct the observed t-BuBHQ-induced [Ca\(^{2+}\)], transients. While the equations used to describe transport in this study were mechanistically motivated, they should be regarded as empirical descriptions of the transport rates and their [Ca\(^{2+}\)] dependence. Nevertheless, it is noteworthy that the apparent [Ca\(^{2+}\)] sensitivity of uptake (EC\(_{50, SERCA}\) < 100 nM) was higher than expected based on studies of SERCAs in isolation (Lytton et al., 1992). One possible explanation is that CICR raises local [Ca\(^{2+}\)], beyond the level detected by bulk [Ca\(^{2+}\)] measurements. Such an increase could partially saturate the uptake pathway, even though bulk [Ca\(^{2+}\)] is considerably lower. As shown by Albrect et al. (2001), ryanodine does not alter \(J_{SERCA}\) under resting conditions, arguing against such an effect when [Ca\(^{2+}\)] is at its resting level. However, with stronger activation of the CICR pathway at higher [Ca\(^{2+}\)], it is possible that increases in local [Ca\(^{2+}\)], near sites of ER Ca\(^{2+}\) uptake bring SERCAs close to saturation, so that at high [Ca\(^{2+}\)], the [Ca\(^{2+}\)] dependence of \(J_{SERCA}\) largely reflects the [Ca\(^{2+}\)] depen-
dence of \( k \). Assessment of this possibility will require additional information regarding the spatial distribution of Ca\(^{2+}\) near the ER and the specific SERCA isoforms that are expressed in sympathetic neurons.

**Characterization of the Ca\(^{2+}\) Permeability of the ER**

It has been difficult to characterize CICR in intact cells because the rate of passive Ca\(^{2+}\) release depends on two factors that are difficult to distinguish experimentally in intact cells: driving force and permeability. It is expected that Ca\(^{2+}\) release channel activity influences \( J_{\text{Release}} \) primarily through its effect on \( P_{\text{ER}} \), and secondarily through its effect on driving force; although the latter effect depends on other transport systems that influence ER Ca\(^{2+}\) loading, such as Ca\(^{2+}\) uptake and extrusion. We devised a way to distinguish between these contributions to \( J_{\text{Release}} \). Integration of \( J_{\text{Release}}K \) provided a measure of changes in intraluminal Ca\(^{2+}\) concentration, which in turn made it possible to estimate the driving force for Ca\(^{2+}\) release. Along with the definition of \( E_{\text{P.F.}} \) provided by Eq. 4, this made it possible to obtain a quantity that is expected to be proportional to the macroscopic permeability of the ER (\( E_{\text{P.F.}} \)) from the ratio of two measured quantities.

Based on this procedure, we characterized \( E_{\text{P.F.}} \) and its [Ca\(^{2+}\)] dependence, as well as its sensitivity to pharmacological agents known to modify RyR gating in vitro. We found that (1) \( E_{\text{P.F.}}/v_{\text{ERK}} \) increases with [Ca\(^{2+}\)] over the range studied (up to \( \sim 250 \) nM), which is consistent with regulation of RyR activity through low affinity Ca\(^{2+}\)-channel interactions with little cooperativity. (2) Caffeine increased, and ryanodine reduced, the [Ca\(^{2+}\)] sensitivity of \( E_{\text{P.F.}}/v_{\text{ERK}} \), arguing that its [Ca\(^{2+}\)] dependence is dominated by RyRs. The apparent loss of Ca\(^{2+}\) sensitivity after ryanodine treatment also provides a simple explanation for the previous finding that ryanodine enhances ER Ca\(^{2+}\) accumulation during depolarization. By preventing a Ca\(^{2+}\)-dependent increase in ER Ca\(^{2+}\) permeability, ryanodine also prevents an increase in the rate of Ca\(^{2+}\) release that normally occurs in response to evoked elevations in [Ca\(^{2+}\)]. This would exaggerate the imbalance between uptake and release rates under conditions where the ER is a Ca\(^{2+}\) sink, rendering the ER a more powerful Ca\(^{2+}\) buffer. (3) Ryanodine increased basal \( E_{\text{P.F.}}/v_{\text{ERK}} \), which is consistent with its effects on RyR open probability at the concentration used (1 \( \mu \)M). This result provides an explanation for our finding that ryanodine at the same concentration reduces basal intraluminal total Ca concentration (and presumably free Ca concentration) without altering the basal rate of Ca\(^{2+}\) uptake or release (Albrecht et al., 2001). (4) The [Ca\(^{2+}\)] dependence of \( E_{\text{P.F.}}/v_{\text{ERK}} \) was not detectably different during the rising and falling phases of the t-BuBHQ-induced [Ca\(^{2+}\)] transients.

This leads to two conclusions about RyR regulation during these responses. First, \( E_{\text{P.F.}} \) is not very sensitive to reductions in intraluminal Ca\(^{2+}\) concentration that occur during these two phases of the transients. In other words, the major sites responsible for Ca\(^{2+}\)-dependent regulation of \( E_{\text{P.F.}} \) are directly accessible from the cytoplasmic solution. This agrees with the conclusions of Xu and Meissner (1998), who showed that intraluminal Ca levels influence gating of canine cardiac RyRs in a way that can be accounted for by Ca\(^{2+}\) release followed by interactions with cytoplasmic regulatory sites. Second, \( E_{\text{P.F.}} \) does not show appreciable intrinsic time dependence. This finding is consistent with the results of Schiefer et al. (1995) who showed there is little inactivation of canine cardiac RyRs when cis [Ca\(^{2+}\)] < 1 \( \mu \)M (our measurements of \( E_{\text{P.F.}} \) were made below 250 nM). Thus, the effects of caffeine and ryanodine on \( E_{\text{P.F.}} \) at low [Ca\(^{2+}\)], are in general agreement with in vitro studies of RyRs derived from mammalian cardiac cells.

**Assumptions Used in the Analysis**

An important step in characterizing \( E_{\text{P.F.}} \) was estimation of the driving force for passive Ca\(^{2+}\) release. Several approximations were made. First, it was assumed that the ER membrane potential is small enough that it has little or no effect on \( J_{\text{Release}} \). Second, it was assumed that intraluminal Ca\(^{2+}\) buffers bind Ca\(^{2+}\) rapidly and with low affinity. Third, it was assumed that \( \Delta [\text{Ca}^{2+}] \), is small enough compared with \( \Delta [\text{Ca}^{2+}]_{\text{ER}} \) (the differences between [Ca\(^{2+}\)] and [Ca\(^{2+}\)] \_er and their basal levels during t-BuBHQ-induced [Ca\(^{2+}\)] transients) that cytoplasmic Ca\(^{2+}\) has negligible effect on driving force (Appendix A).

Our results describe the quantitative properties of ER Ca\(^{2+}\) transport in intact cells under conditions where the spatial distribution of Ca\(^{2+}\) within cellular compartments is likely to be nearly uniform. How relevant are the rate descriptions obtained under these conditions to the case where Ca\(^{2+}\) is distributed nonuniformly (e.g., during depolarization-evoked Ca\(^{2+}\) entry)? In this case, [Ca\(^{2+}\)] \_er is highest near sites of Ca\(^{2+}\) entry and falls off with distance from the plasma membrane (Sala and Hernandez-Cruz, 1990; Hua et al., 1993). As long as the transport descriptions refer to populations of Ca\(^{2+}\) transporters that are distributed uniformly within the membranes delimiting compartments, and these membranes have simple geometry, the results are also expected to predict the spatial distribution of [Ca\(^{2+}\)] within compartments during periods of Ca\(^{2+}\) entry. Results that support this conclusion have been presented for the analogous problem of mitochondrial Ca\(^{2+}\) loading during depolarization (Pivovarova et al., 1999). Moreover, examination of the case where Ca\(^{2+}\) is spatially uniform provides an explanation for the basis of steady-state Ca\(^{2+}\) level within ER in terms of the quantitative properties of ER Ca\(^{2+}\) uptake and release rates.
and shows how \([\text{Ca}^{2+}]\), levels help determine which modes of CICR can be recruited in response to stimulation. Nevertheless, if the distribution of the ER or of transporters within ER or plasma membranes is non-uniform, for example, such that the density or intrinsic properties of ER transporters vary appreciably from one somatic region to another (e.g., peripheral versus central regions), or if proximity between intracellular membranes permits the development of \(\text{Ca}^{2+}\) microdomains (Rizzuto et al., 1998), an accurate description of \([\text{Ca}^{2+}]\) dynamics during stimulation would require detailed specification of the distribution of transporters and membrane geometry. An example where spatial nonuniformity may introduce an error in the \([\text{Ca}^{2+}]\), dependence of transport presented in this study was mentioned in connection with \(J_{\text{SERCA}}\).

Comparison with Previous Work

To our knowledge, this is the first description of ER \(\text{Ca}^{2+}\) uptake and release fluxes and \(\text{Ca}^{2+}\) permeability in intact cells. Our study builds on previous work in skeletal (Baylor et al., 1983; Kovacs et al., 1983; Melzer et al., 1987) and cardiac muscle (Sipido and Wier, 1991) describing CICR from the SR after membrane depolarization. As in these studies, we used the basic idea that at each instant in time, \([\text{Ca}^{2+}]\), changes at a rate that depends on the rates of passive \(\text{Ca}^{2+}\) release and removal, and on the properties of intracellular \(\text{Ca}^{2+}\) buffering. Thus, with information about the rate of \(\text{Ca}^{2+}\) removal and buffering, the rate of \(\text{Ca}^{2+}\) release can be calculated from the total \(\text{Ca}^{2+}\) flux. In these earlier studies, the rate of \(\text{Ca}^{2+}\) uptake was not measured, and was either included in an overall description of \(\text{Ca}^{2+}\) removal (Melzer et al., 1987) or was calculated (Sipido and Wier, 1991). In the present study, we used t-BuBHQ as a tool to measure both the rates of ER \(\text{Ca}^{2+}\) uptake and release and their \([\text{Ca}^{2+}]\) dependence. As a result, it was possible to describe the relationship between \(\text{Ca}^{2+}\) uptake and release fluxes during and after stimulation, which was essential for understanding the direction and rate of net ER \(\text{Ca}^{2+}\) transport during depolarization and how it is regulated by \([\text{Ca}^{2+}]\). With an understanding of the basis of net ER \(\text{Ca}^{2+}\) transport, it became possible to clarify how the interplay between net ER \(\text{Ca}^{2+}\) transport and \(\text{Ca}^{2+}\) extrusion across the plasma membrane sets the stage for multiple modes of CICR.

Caffeine-induced \(\text{Ca}^{2+}\) Oscillations

Previous studies have shown that when sympathetic neurons are exposed steadily to caffeine and then depolarized, \([\text{Ca}^{2+}]\), oscillates (Lipscombe et al., 1988; Friel and Tsien, 1992b; Kuba, 1994). Oscillations can be elicited by caffeine alone, but in our experiments, depolarization considerably increased the reliability with which they are evoked. In an earlier study (Friel, 1995), three independent components of the total \(\text{Ca}^{2+}\) flux underlying caffeine-induced \(\text{Ca}^{2+}\) oscillation were measured. These components represented caffeine-sensitive \(\text{Ca}^{2+}\) release, \(\text{Ca}^{2+}\) entry, and the remainder of the total cytoplasmic \(\text{Ca}^{2+}\) flux, interpreted as the sum of the rates of \(\text{Ca}^{2+}\) extrusion and \(\text{Ca}^{2+}\) uptake. A simple model was presented that accounted for small amplitude oscillations when \([\text{Ca}^{2+}]\), was low enough that mitochondrial \(\text{Ca}^{2+}\) transport was weak and linear approximations of the \([\text{Ca}^{2+}]\), dependence of \(\text{Ca}^{2+}\) extrusion and uptake are adequate. In this model, the rate of \(\text{Ca}^{2+}\) release was described as a product of a permeability factor and a driving force, as in the present study. The permeability was assumed to increase monotonically with \([\text{Ca}^{2+}]\), was insensitive to intraluminal \(\text{Ca}^{2+}\) concentration and did not show inactivation.

In the present study, this permeability was directly characterized and the third component of the total cytoplasmic \(\text{Ca}^{2+}\) flux was explicitly represented by the sum of two fluxes \(J_{\text{SERCA}}\) and \(J_{\text{pm}}\) that were measured in the absence of caffeine under conditions where \([\text{Ca}^{2+}]\), is not oscillating. It is remarkable that simulations performed using descriptions of \(J_{\text{pm}}, J_{\text{SERCA}}\), and \(k_i\) from control cells, and of \(P_{\text{ER}}\) from each of the four caffeine-treated cells, showed \([\text{Ca}^{2+}]\), oscillations in response to stimulated \(\text{Ca}^{2+}\) entry, whereas descriptions from the same number of control cells did not. It was possible to reproduce the basic properties of \([\text{Ca}^{2+}]\), oscillations evoked by stimulated \(\text{Ca}^{2+}\) entry, as well as the temporal properties of the three flux components measured in the earlier study (Fig. 7).

Importance of Studying \(\text{Ca}^{2+}\) Regulation by the Collection of Transporters Expressed together in Intact Cells

Studies from various in vitro preparations (e.g., vesicles, and isolated organelles) has provided information regarding the types of transporters that participate in \(\text{Ca}^{2+}\) signaling and their biophysical properties. However, since this information is derived from multiple tissues and species, it may not describe the particular collection of transporters that operate together in any one cell type. Such in vitro data have been invaluable in developing general concepts about \(\text{Ca}^{2+}\) regulation, but inferences drawn from them are limited. One reason is that the \(\text{Ca}^{2+}\) transport rates depend on \([\text{Ca}^{2+}]\), in a nonlinear manner. In a coupled system of nonlinear \(\text{Ca}^{2+}\) transporters, quantitative properties of the individual transport systems can influence qualitative properties of \(\text{Ca}^{2+}\) regulation. Therefore, to understand the \(\text{Ca}^{2+}\) signaling regimes that can exist in vivo, it is necessary to consider collections of transporters that are actually expressed together in living cells.

Our results show that the relative rates of ER \(\text{Ca}^{2+}\) uptake and release are critical in determining whether depolarization-evoked \([\text{Ca}^{2+}]\), elevations lead to ER \(\text{Ca}^{2+}\)
accumulation or net Ca\(^{2+}\) release, and, therefore, whether intraluminal Ca\(^{2+}\) concentrations rise or fall in response to stimulation. The relative rates of transport are expected to depend on multiple factors, including transporter expression levels, sensitivity to Ca\(^{2+}\), and the state of modulation. Differences between the relative rates of Ca\(^{2+}\) uptake and release in vivo and those deduced from in vitro data could lead to completely different predictions regarding the direction in which ER Ca\(^{2+}\) concentrations change in response to particular stimuli. Certainly, from the standpoint of regulation of intraluminal Ca\(^{2+}\) sensors, this is an important difference.

**Implications for Studies of Ca\(^{2+}\) Signaling**

Our results provide a picture of CICR in intact cells in terms of the interplay between multiple transport systems that can lead to qualitatively different modes of Ca\(^{2+}\) dynamics in response to different patterns of stimulated Ca\(^{2+}\) entry. One approach illustrating this was in terms of instantaneous flux/\(c_i\) relations analogous to the momentary current voltage relations used previously to illustrate the basis for initial responses to depolarizing stimuli in excitable cells (Jack et al., 1983). We described how 

\[
\text{Ca}^{2+}\text{excitable cells (Jack et al., 1983). We described how the basis for initial responses to depolarizing stimuli in}
\]

tary current voltage relations used previously to illustrate

lizes intraluminal Ca\(^{2+}\) stimulation, the presence of a CICR pathway also stabilizes intraluminal Ca\(^{2+}\) concentrations over a wide

range of [Ca\(^{2+}\)]. This may be important in maintaining processes that are sensitive to intraluminal Ca\(^{2+}\) levels (Meldolesi and Pozzan, 1998; Corbett and Michalak, 2000) in the face of large swings in [Ca\(^{2+}\)], that might occur in response to excessive stimulation or injury.

**APPENDIX A**

**Assessment of ER Ca\(^{2+}\) Permeability in Intact Cells**

The purpose of this appendix is to derive an equation that relates the macroscopic Ca\(^{2+}\) permeability of the ER (\(P_{\text{ER}}\)) to measurements in intact cells presented in this study. Let \(J_{\text{ER}}\) be the rate of net Ca\(^{2+}\) transport between the ER and cytoplasm (e.g., in units of nmol/s). This flux would cause the total ER Ca concentration ([Ca\(_{\text{ER}}\)] to change at a rate given by Eq. A1:

\[
\frac{d[\text{Ca}^{2+}]_{\text{ER}}}{dt} = \frac{J_{\text{ER}}}{v_{\text{ER}}},
\]

where \(v_{\text{ER}}\) is the ER volume and outward fluxes from the cytoplasm are positive and inward fluxes are negative. For simplicity, it is assumed that Ca\(^{2+}\) is uniformly distributed within the ER and cytoplasm at all times, which is a reasonable approximation as long as transport between compartments is slow compared with diffusion within compartments. If binding to intraluminal Ca\(^{2+}\) buffers reaches equilibrium rapidly, then this flux would cause the intraluminal free Ca\(^{2+}\) concentration ([Ca\(_{\text{ER}}\)] to change at a rate of

\[
\frac{d[\text{Ca}^{2+}]_{\text{ER}}}{dt} = \frac{J_{\text{ER}}}{v_{\text{ER}}\kappa_{\text{ER}}},
\]

where \(\kappa_{\text{ER}}\) is the ratio of change in total ER Ca concentration to the accompanying change in free Ca concentration. In the absence of other forms of net cytoplasmic Ca\(^{2+}\) transport, the same flux would cause the cytoplasmic free Ca\(^{2+}\) concentration to change at a rate given by Eq. A3:

\[
\frac{d[\text{Ca}^{2+}]}{dt} = \frac{J_{\text{ER}}}{v_{i}\kappa_{i}},
\]

where \(J_{\text{ER}}\) can be interpreted as the net flux (e.g., in units nmol/liter s) of Ca\(^{2+}\) between the ER and cytoplasm per unit effective cytoplasmic volume (\(v_{i}\kappa_{i}\); Melzer et al., 1987). \(J_{\text{ER}}\) can be separated into two components:

\[
J_{\text{ER}} = J_{\text{SERCA}} + J_{\text{Release}},
\]

where \(J_{\text{SERCA}}\) represents Ca\(^{2+}\) uptake via SERCAs, and \(J_{\text{Release}}\) represents passive Ca\(^{2+}\) release. Results presented in Fig. 2 provide information about \(J_{\text{SERCA}}\), whereas Figs. 3 and 4 show measurements of \(J_{\text{Release}}\).
\(J_{\text{Release}}\) is expected to depend both on the \(Ca^{2+}\) permeability of ER and the driving force for \(Ca^{2+}\) movement across the ER membrane. If this driving force depends only on the difference between cytoplasmic and intraluminal \(Ca^{2+}\) concentrations, \(J_{\text{Release}}\) can be described at each point in time as follows:

\[
J_{\text{Release}} = \frac{\tilde{P}_{\text{ER}}}{\kappa_i} \left[ (\left[Ca^{2+}\right]_i - \left[Ca^{2+}\right]_{\text{ER}}) \right. \\
= \frac{\tilde{P}_{\text{ER}}}{\kappa_i} \left( [\text{Ca}^{2+}]_i - [\text{Ca}^{2+}]_{\text{ER}} \right) \ (A5)
\]

where \(\tilde{P}_{\text{ER}}\) is the permeability of the entire ER membrane (units cm\(^2\)s\(^{-1}\)) and \(P_{\text{ER}} = \frac{\tilde{P}_{\text{ER}}}{\kappa_i}\) (in units s\(^{-1}\)). \(\tilde{P}_{\text{ER}}\) is a macroscopic permeability that can be related to single-channel properties by \(\tilde{P}_{\text{ER}} = \sum_\nu N_\nu p_\nu \pi_\nu\), where \(N_\nu\) is the number of \(Ca^{2+}\)-permeable channels of type \(\nu\), \(p_\nu\) is their open probability, and \(\pi_\nu\) is the unitary \(Ca^{2+}\) permeability, if permeation through channels is the dominant form of passive ER \(Ca^{2+}\) transport. Note that if a channel contributing to \(P_{\text{ER}}\) has a \([Ca^{2+}]_i\)-dependent open probability (e.g., RyRs), then \(\tilde{P}_{\text{ER}}\) will depend on \([Ca^{2+}]_i\). Eq. A5 can be rewritten as follows:

\[
J_{\text{Release}}(t) = P_{\text{ER}} \{ ([Ca^{2+}]_i(t) - [Ca^{2+}]_{i,\text{basal}}) - \\
([Ca^{2+}]_{\text{ER}}(t) - [Ca^{2+}]_{\text{ER},\text{basal}}) + \\
([Ca^{2+}]_{i,\text{basal}} - [Ca^{2+}]_{\text{ER},\text{basal}}) \} \ (A6)
\]

where \(\Delta[Ca^{2+}]_i(t)\) and \(\Delta[Ca^{2+}]_{\text{ER}}(t)\) are the differences between \([Ca^{2+}]_i\) and \([Ca^{2+}]_{\text{ER}}\) at time \(t\) and their basal values after inhibition of SERCAs (e.g., with t-BuBHQ). If in the continuous presence of t-BuBHQ, \(Ca^{2+}\) becomes passively distributed between the ER and cytoplasm, then \([Ca^{2+}]_{\text{ER},\text{basal}} = [Ca^{2+}]_{i,\text{basal}}\) and Eq. A6 reduces to

\[
J_{\text{Release}}(t) = P_{\text{ER}}(\Delta[Ca^{2+}]_i(t) - \Delta[Ca^{2+}]_{\text{ER}}(t)). \ (A7)
\]

Now, if \(\Delta[Ca^{2+}]_i(t) \ll \Delta[Ca^{2+}]_{\text{ER}}(t)\), Eq. A7 can be approximated by

\[
J_{\text{Release}}(t) = -P_{\text{ER}}\Delta[Ca^{2+}]_{\text{ER}}(t). \ (A8)
\]

To obtain \(\Delta[Ca^{2+}]_{\text{ER}}(t)\), Eq. A2 can be integrated from the instant of SERCA inhibition to the time \(t\) and then offset by the integral over the entire t-BuBHQ-induced \([Ca^{2+}]_i\) transient:

\[
\Delta[Ca^{2+}]_{\text{ER}}(t) = \int_0^t \frac{d[Ca^{2+}]_{\text{ER}}}{dt} dt - \int_0^t \frac{d[Ca^{2+}]_{\text{ER}}}{dt} dt' \\
= -\int_t^\infty \frac{\tilde{J}_{\text{ER}}}{v_{\text{ER}} \kappa_{\text{ER}}} dt'
\]

(A9)
Therefore, if \( v_{ER} \kappa_{ER} \) is constant, \( P_{ER}(v_i/v_{ER} \kappa_{ER}) \) is expected to show a composite [\( \text{Ca}^{2+} \)] dependence reflecting both the properties of \( \kappa_i \) and \( P_{ER} \). In this study, we present measurements of \( P_{ER}(v_i/v_{ER} \kappa_{ER}) \) to avoid assumptions regarding \( (v_i/v_{ER} \kappa_{ER}) \). Multiplication by \( \kappa_i \) using results shown in Fig. 1 D then gives \( P_{ER}(v_i/v_{ER} \kappa_{ER}) \).

If \( v_{ER} \kappa_{ER} \) is constant, this is proportional to the macroscopic \( \text{Ca}^{2+} \) permeability of the ER. Given measurements of \( \Delta[\text{Ca}^{2+}]_{ER}(t) \), estimates of \( \Delta[\text{Ca}^{2+}]_{ER}(t) \) are obtained after multiplying by \( (v_i/v_{ER} \kappa_{ER}) \).

The validity of Eq. A13 depends on the approximation \( \Delta[\text{Ca}^{2+}]_i(t) - \Delta[\text{Ca}^{2+}]_{ER}(t) = -\Delta[\text{Ca}^{2+}]_{ER}(t) \) used in obtaining Eq. A8 from Eq. A7. If during evoked t-BuBHQ transients \( \Delta[\text{Ca}^{2+}]_i(t) < 250 \text{ nM} \) and \( \Delta[\text{Ca}^{2+}]_{ER}(t) > 25 \text{ \mu M} \), the error in estimating \( P_{ER}(v_i/v_{ER} \kappa_{ER}) \) would be \( \sim 1\% \) and would be smaller when \( \Delta[\text{Ca}^{2+}]_{ER}(t) \) is higher. During the final phase of the recovery, \( -\Delta[\text{Ca}^{2+}]_{ER}(t) \) systematically overestimates \( \Delta[\text{Ca}^{2+}]_i(t) \) in magnitude, leading to an underestimate of \( P_{ER}(v_i/v_{ER} \kappa_{ER}) \). However, such an underestimate was difficult to resolve (Fig. 3 E, light trace) possibly because in this case \( P_{ER}(v_i/v_{ER} \kappa_{ER}) \) is determined from the ratio of two small and noisy numbers.

**APPENDIX B**

**Description of the Model**

The dynamics of the free \( \text{Ca}^{2+} \) concentration within the cytosol \( (c_i) \) and the ER \( (c_{ER}) \) were represented by the following differential equations (Eqs. B1 and B2):

\[
\frac{dc_i}{dt} = -J_i \tag{B1}
\]

\[
\frac{dc_{ER}}{dt} = J_{ER} \frac{v_i \kappa_i}{v_{ER} \kappa_{ER}}, \tag{B2}
\]

where the total cytoplasmic \( \text{Ca}^{2+} \) flux is given by Eq. B3

\[
J_i = J_{pm} + J_{ER}, \tag{B3}
\]

and the intercompartmental fluxes \( J_{pm} \) and \( J_{ER} \) depend on the relative rates of transport via different pathways as follows:

\[
J_{pm} = J_{extru} + J_{ICa}, \tag{B4}
\]

and

\[
J_{ER} = J_{SERCA} + J_{Release}, \tag{B5}
\]

where

\[
J_{extru} = \left\{ k_{\text{leak.pm}} (c_i - c_o) + \frac{V_{\text{max,extru}}}{1 + \left( \frac{EC_{50,\text{extru}}}{c_i} \right)^{n_{\text{extru}}}} \right\} \frac{1}{\kappa_i} \tag{B6}
\]

and

\[
J_{SERCA} = \frac{V_{\text{max,SERCA}}}{\kappa_i \left[ 1 + \left( \frac{EC_{50,\text{SERCA}}}{c_i} \right)^{n_{\text{SERCA}}} \right]}, \tag{B7}
\]

\[
J_{Release} = \tilde{P}_{ER} \frac{(c_i - c_{ER})}{v_i \kappa_i}, \tag{B9}
\]

and

\[
\frac{\tilde{P}_{ER}}{v_i} = \tilde{P}_{\text{basal}} + \frac{\tilde{P}_{\text{max,RYR}}}{\left[ 1 + \left( \frac{EC_{50,\text{RYR}}}{c_i} \right)^{n_{\text{RYR}}} \right]}, \tag{B10}
\]

where \( k_{\text{leak.pm}}, c_o, V_{\text{max,extru}}, EC_{50,\text{extru}}, n_{\text{extru}}, F, V_{\text{max,SERCA}}, EC_{50,\text{SERCA}}, n_{\text{SERCA}}, \tilde{P}_{\text{basal}}, \tilde{P}_{\text{max,RYR}}, EC_{50,\text{RYR}}, \) and \( n_{\text{RYR}} \) are constants. \( c_o \) is the extracellular \( \text{Ca}^{2+} \) concentration and \( F \) is the Faraday constant. Eqs. B4–B9 describe the rate of total \( \text{Ca}^{2+} \) transport by the respective pathways divided by the cytoplasmic volume \( (v_i) \) and the cytoplasmic buffering factor \( \kappa_i \). According to the sign convention used, fluxes that raise \( c_i \) are negative while fluxes that lower \( c_i \) are positive. \( J_{extru} \) is the sum of plasma membrane pump and leak fluxes. \( \tilde{P}_{ER}/v_i \), a lumped quantity representing the total ER permeability per unit cytoplasmic volume, consists of a constant basal component \( (\tilde{P}_{\text{basal}}) \) and a \( c_i \)-dependent component that increases saturaingly with \( c_i \) (half-maximal activation when \( c_i = EC_{50,\text{RYR}} \)) and approaches \( \tilde{P}_{\text{max,RYR}} \) when \( c_i \) is high.

To obtain an analytical description of ER \( \text{Ca}^{2+} \) permeability for use in simulations, measurements of \( P_{ER}(v_i/v_{ER} \kappa_{ER}) \) were described by Eq. B11:

\[
\frac{P_{ER}}{v_{ER} \kappa_{ER}} = \frac{1}{\kappa_i} \left[ \frac{\tilde{P}_{ER}}{v_i} \right] \left[ \frac{v_i}{v_{ER} \kappa_{ER}} \right] \tag{B11}
\]

where \( \kappa_i \) was treated as a known function of \( [\text{Ca}^{2+}]_i \) based on measurements shown in Fig. 1 D, \( (\tilde{P}_{ER}/v_i) \) was described by Eq. B10, \( v_{ER} \kappa_{ER}/v_i \) was estimated to be unity. The description of mitochondrial \( \text{Ca}^{2+} \) transport used in simulations (Figs. 6–8) is from Colegrove et al. (2000b).

In carrying out simulations, the resting value of \( c_i \) was determined as the solution of Eq. B6 when \( J_{pm} = 0 \). Alternatively, if simulations were to be compared at the same value of resting \( c_i \), (Fig. 5, A and B, insets), Eq. B6 was solved determine the value of \( k_{\text{leak.pm}} \) yielding that value of \( c_i \). The initial value of \( c_{ER} \) was determined as the particular value where \( \text{Ca}^{2+} \) uptake and release rates are equal at the prevailing basal \( c_i \) according to Eq. B12:

\[
c_{ER}(0) = c_i(0) + \frac{J_{SERCA}(c_i(0))}{P_{ER}(c_i(0))/v_i}. \tag{B12}
\]

Therefore, at rest \( c_{ER} \) is defined by the properties of \( J_{SERCA} \) and \( P_{ER}/v_i \) and basal \( c_i \).
The authors thank Drs. Hille Chiel and Steve Jones for their helpful comments on the manuscript.

This work was supported by a grant from the National Institutes of Health/National Institute of Neurological Disorders and Stroke (NS 35514) to D.D. Friel.

Submitted: 2 August 2001
Revised: 22 January 2002
Accepted: 25 January 2002

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The abscissa of Fig. 5 D was not labeled and did not correspond to the inset. The corrected figure appears below:

Figure 5. Reconstruction of t-BuBHQ-induced [Ca\textsuperscript{2+}]\textsubscript{i} transients and their modification by caffeine and ryanodine. Simulated effects of sudden inhibition of ER Ca\textsuperscript{2+} uptake on c\textsubscript{i} (\sim [Ca\textsuperscript{2+}], A), \textit{J}_{\text{Release}} (B), and intraluminal Ca\textsuperscript{2+} concentration, c\textsubscript{ER} (\sim \Delta [Ca\textsuperscript{2+}]\textsubscript{ER}(t)). Simulations were performed as described in APPENDIX B using experimentally determined descriptions of \kappa, from Fig. 1 D, \textit{J}_{\text{SERCA}} from Fig. 2 C, and \textit{P}\textsubscript{ER}(n/v_{\text{ER}}) from Fig. 4 D. \textit{J}\textsubscript{pore} was described by smooth curves obtained from analysis of individual cells from Fig. 4 as in Fig. 3 C, with initial values of c\textsubscript{i} based on estimates of resting [Ca\textsuperscript{2+}] in those same cells. Insets in A and B show simulations performed assuming a fixed initial value (50 nM) for c\textsubscript{i}. 

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The Journal of General Physiology. Volume 119, No. 3, March 2002. 211–233.