Activation of calcium release assessed by calcium release-induced inactivation of calcium current in rat cardiac myocytes

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Zahradníková, Alexandra, Zuzana Kubalová, Jana Pavelková, Sándor Györke, and Ivan Zahradník. Activation of calcium release assessed by calcium release-induced inactivation of calcium current in rat cardiac myocytes. Am J Physiol Cell Physiol 286: C330-C341, 2004. First published October 1, 2003; 10.1152/ajpcell.00272.2003.—In mammalian cardiac myocytes, calcium released into the dyadic space rapidly inactivates calcium current (Ica). We used this Ca2+ release-dependent inactivation (RDI) of Ica as a local probe of sarcoplasmic reticulum Ca2+ release activation. In whole cell patch-clamped rat ventricular myocytes, Ca2+ entry induced by short pre-pulses from −50 mV to positive voltages caused suppression of peak Ica during a test pulse. The negative correlation between peak Ica suppression and Ica inactivation during the test pulse indicated that RDI evoked by the prepulse affected only calcium channels in those dyads in which calcium release was activated. Ca2+ ions injected during the prepulse and during the subsequent tail current suppressed peak Ica in the test pulse to a different extent. Quantitative analysis indicated that equal Ca2+ charge was 3.5 times less effective in inducing release when entering during the prepulse than when entering during the tail. Tail Ca2+ charge injected by the first voltage-dependent calcium channel (DHPR) openings was three times less effective than that injected by DHPR reopenings. These findings suggest that calcium release activation can be profoundly influenced by the recent history of L-type Ca2+ channel activity due to potentiation of ryanodine receptors (RyRs) by previous calcium influx. This conclusion was confirmed at the level of single RyRs in planar lipid bilayers: using flash photolysis of the calcium cage NP-EGTA to generate two sequential calcium stimuli, we showed that RyR activation in response to the second stimulus was four times higher than that in response to the first stimulus.

excitation-contraction coupling

Cardiac calcium channels inactivate by a calcium-dependent mechanism (40, 33), which is mediated by binding of Ca2+ ions to the two COOH-terminal binding sites on the calmodulin molecule prebound at the cytoplasmic side of the DHPR (41, 50). Therefore, in parallel with activation of calcium release, calcium current (Ica) becomes rapidly inactivated by the released calcium (1, 4, 16, 45, 46, 48, 58). Thus the calcium release-dependent inactivation (RDI) might be considered a convenient probe of calcium release because it senses the released Ca2+ ions directly in the dyadic junction (1, 14, 42, 45, 46). However, the difficulty in isolating RDI from the other two coexisting inactivation mechanisms of Ica inactivation, i.e., from the voltage-dependent and calcium current-dependant inactivation (17, 27, 32, 33, 40), has limited the use of RDI for quantitative assessment of calcium release mechanisms. Sham (46), using caffeine as an agent to deplete SR of calcium, visualized the calcium release-sensitive component of the calcium current by comparing calcium currents occurring in the presence of all three inactivation mechanisms and after RDI had been selectively removed by caffeine addition. By means of these intricate experiments, Sham (46) was able to show that calcium RDI results from local calcium release. For quantitative description of calcium release activation, however, this or analogous pharmacological approach would have serious limitations due to nonspecific effects of drugs and the complexity of experiments.

We have developed an alternative approach based on the known efficiency of tail calcium currents to induce Ca2+ release (5, 8, 10, 12, 14). We have varied the properties of the calcium current evoked by brief voltage pre-pulses and related them to the extent of RDI measured by the subsequent test pulse. This approach allowed us, for the first time, to induce RDI in the absence of other components of Ica inactivation, as well as to use it as a local, dyadic probe of calcium release activation. The relationship between Ca2+ influx and the ensuing RDI was analyzed on the basis of the law of mass action to show that the potency of a given calcium influx to induce calcium release, and hence RDI, was dependent on previous calcium influx. Increased probability of RyR activation by prior elevations of local Ca2+ was confirmed at the level of single RyRs in planar lipid bilayers by using brief, photolytically generated Ca2+ elevations under ionic conditions similar to those of the intracellular environment. Altogether, these findings suggest that the probability of calcium release activation by a single DHPR opening can be modulated by the recent history of calcium influx within the dyad.

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Part of this work has been published in abstract form (Ref. 34).

MATERIALS AND METHODS

Isolation of myocytes. The experiments were performed in accordance with the guidelines laid down by the Slovak Academy of Sciences animal welfare committee, as well as with the NIH Guide for the Care and Use of Laboratory Animals. Cardiac myocytes were enzymatically isolated from left ventricles of male Wistar rats (200–250 g) as previously described (58). The left ventricle and septum were dissected and further incubated up to 5 min in oxygenated running medium at room temperature for use within 1 h after isolation.

Calcium current measurements. The L-type ICa was recorded from single myocytes by using the standard whole cell patch-clamp technique (28). The external solution contained (in mM) 135 NaCl, 5.4 KCl, 5 MgCl2, 10 HEPES, 0.33 NaH2PO4, and 1 CaCl2, pH 7.3. The external solution was prepared by adding 6–10 mg of Collagenasa cruda (Sevapharma, Czech Republic) to 50 ml of the Ca2+-free Tyrode solution with free Ca2+ increased to 20 μM. The left ventricle and septum were dissected and further incubated up to 5 min in oxygenated running medium at room temperature for use within 1–4 h after isolation.

The external solution contained (in mM) 135 NaCl, 5.4 CsCl, 10 HEPES, 5 MgCl2, 0.33 NaH2PO4, and 1 CaCl2, pH 7.3. Patch pipettes (1–2 MΩ) were filled with the internal solution containing (in mM) 135 CsCH3SO3, 10 CsCl, 10 HEPES, 1 EGTA, 3 MgSO4, and 3 ATPNa2, pH 7.3. When necessary, sodium channels were inhibited with 20 μM tetrodotoxin (TTX) in the external solution and by a holding potential of −50 mV. In experiments involving hyperpolarizing stimuli, the external solution with Na+ ions replaced by Cs+ ions was used to minimize sodium channel currents on subsequent depolarization. Potassium currents were suppressed by replacement of K+ with Cs+ in all solutions. Calcium channels were kept in a phosphorylated state by adding 50 μM CAMP to the internal solution and 10 μM isobutylmethylxanthine (IBMX, a membrane-permeant phosphodiesterase inhibitor) to the external solution. Calcium currents could not be further stimulated by addition of 0.1 mM isoproterenol and were completely blocked by external Cd2+ or DHP antagonists. IBMX, ATP, and cAMP were from Sigma (St. Louis, MO). TTX was from Alomone Labs (Jerusalem, Israel), and ryanodine was from Calbiochem (La Jolla, CA). All other chemicals were of analytical grade. Whole cell currents measured with the Axopatch 200B (Axon Instruments, Union City, CA) patch-clamp amplifier were low-pass filtered at 2–5 kHz and digitized at 5–10 kHz by a Labmaster analog-to-digital board (Scientific Solutions) using pCLAMP (ver. 5.5.1, Axon Instruments) implemented on an IBM-AT type computer. Series resistance was electronically compensated by 50–85%. Capacitance charging current was canceled electronically. Where indicated, online subtraction procedure was used additionally to cancel the remaining linear currents. Experiments were carried out at room temperature.

Inactivation of calcium current was probed using a constant test pulse (70 ms, 0 mV) preceded by very brief (≤5 ms) test pulses varying in either prepulse potential or tail potential (i.e., the potential following the prepulse). Individual protocols are described in detail in RESULTS.

Bilayer experiments. Heavy SR microsomes were isolated from canine left ventricles by differential centrifugation as described previously (60). Single SR Ca2+ release channels were reconstituted by fusing heavy SR microsomes into planar lipid bilayers as described previously (24, 61). Single-channel currents through RyR2s were recorded in 400 mM CsCH3SO3, 10 mM Cs-HEPES, and 1 mM glutathione, pH 7.4. The cytoplasmic (cis) solution contained additionally 3 mM MgATP, and the luminal (trans) solution contained 1 mM CaCl2 (Orion). Single-channel currents were measured at +40 mV using Axopatch 200A (Axon Instruments), filtered at 5 kHz, digitized at 25 kHz, and acquired using Digidata 1200A and pCLAMP (ver. 7.0, Axon Instruments). All chemicals were from Sigma if not stated otherwise. Fast changes of the Ca2+ concentration in the microenvironment of the reconstituted channel were performed by flash photolysis of caged calcium as described previously (24, 26, 61), except that the Ca2+ cage compound NP-EGTA (Molecular Probes, Eugene, OR), which is highly selective for Ca2+ over Mg2+ (19), was used instead of DM-nitrophen. After channel incorporation, NP-EGTA (a final concentration of 3 mM) containing the appropriate amount of CaCl2 (Orion) to attain a free Ca2+ of 0.30 mM was added to the cytoplasmic (cis) side of the channel. Intense (2 mJ) and brief (9 ns) ultraviolet laser flashes produced by a pulsed, frequency-tripled Nd:YAG laser (Spectra-Physics, Mountain View, CA) were applied through a fused silica fiber optics (600-μm diameter) positioned in front of the bilayer (100-μm diameter) to illuminate the whole volume between the fiber optics and the bilayer. The concentration of steady-state free Ca2+ in the cis chamber was determined with a Ca2+-selective minielectrode (26). The local Ca2+ changes near the bilayer were calibrated by transforming the bilayer aperture into a Ca2+ electrode, using Ca2+ ionophore resin (ETR 129; Fluka, Switzerland) as described previously (26, 61). The amplitude and time course of free Ca2+ signals in response to the photolyzing laser pulses were computed in Mathematica (ver. 4.2, Wolfram Research, Champaign, IL) from the values of steady-state Ca2+ before and after the flash and from the concentration of total NP-EGTA (61), using published kinetic constants (18, 20). The calcium affinity of ATP was corrected for ionic strength and pH using the MaxChelator program (7, ver. 2.40; http://www.stanford.edu/~cpatton).

The fraction of the test pulse ICa, inactivated by the prepulse, Ipp, was estimated as

\[
F_{\text{ip}} = \frac{I_{\text{pp}} - I_p}{I_{\text{pp}}} \quad \text{(1)}
\]

where \(I_{\text{pp}}\) and \(I_p\) are the peak test calcium currents in the absence (control) and the presence of a prepulse, respectively.

The fraction of ICa inactivated during the test pulse, \(F_a\), was estimated as

\[
F_a = \frac{I_e - I_p}{I_p} \quad \text{(2)}
\]

where \(I_e\) is the amplitude of ICa at the end of the test pulse. Dependence of the Ca2+ influx on the prepulse voltage. The amount of Ca2+ ions, mc, entering via Ca2+-channels was calculated as \(Q_{Ca}(2F)\), where \(F\) is the Faraday constant. QCa was determined by integrating the inward ICa and separated into the prepulse and tail components. The amount of Ca2+ ions injected into the cell during the prepulse (\(mc,ip\)) was estimated using integration of the ICa during the depolarization. The amount of Ca2+ ions injected during the tail current (\(mc,ip\)) was estimated by integrating the tail calcium currents relative to the value observed 5 ms after hyperpolarization so that the exchange current did not contribute substantially to the measured \(mc,ip\).

The experimentally determined voltage dependence of \(mc,ip\) was fitted by the functions...
m_{Ca,p}(V_p) = m_{Ca}^\text{max} \cdot \frac{1}{1 + e^{-(V_p - V_{1/2,p})/k_{V,p}}}

(4)

where $m_{Ca,p}$ is the amount of Ca\(^{2+}\) ions entering the cell during the tail current at maximal activation of calcium channels, $V_{1/2}$ is the potential of half-maximal activation, and $k_V$ is the steepness of the voltage dependence of activation of $m_{Ca,p}$.

The voltage dependence of $m_{Ca,t}$ was fitted by the function

$m_{Ca,t}(V_p) = m_{Ca}^\text{max} \cdot \frac{1}{1 + e^{-(V_p - V_{1/2,t})/k_{V,t}}}

(3a)

and

$m_{Ca,t}(V_p) = 0$ for $V_p \approx V_t

(3b)

where $k_{V,t}$ is the proportionality factor, $V_t$ is the potential of maximal inactivation, and $S_t$ is the steepness of the voltage dependence of activation of $m_{Ca,t}$.

Calcium release and release-dependent inactivation of $I_{Ca}$

The relative potency of the calcium influx during the prepulse was estimated by fitting the fraction of inactivated calcium current by Eq. 8. A value of $\alpha = 1$ means that calcium ions injected by the prepulse and by the tail current have equal potency to induce inactivation of $I_{Ca}$, and values of $\alpha > 1$ and $\alpha < 1$ mean that calcium influx is more effective when occurring during the prepulse and during the tail, respectively. The apparent half-effective dose for tail calcium influx is therefore equal to $M_{50}$, and that for prepulse calcium influx is equal to $M_{50}/\alpha$.

For the purpose of visually comparing the measured data with the theoretical predictions, the values of $m_{Ca,p}$ and $m_{Ca,t}$ in the voltage range of $-50$ to $+60$ mV were calculated from the parameters obtained from the best fits of $m_{Ca,p}$ and $m_{Ca,t}$ by Eq. 3 and Eq. 4, respectively, and substituted into Eq. 8 to yield the theoretical values of $F_{pi}$. The theoretical dependence of $F_{pi}$ on $m_{Ca,p}$ only, or on $m_{Ca,t}$ only, was calculated using Eq. 8 by setting Ca\(^{2+}\) influx by the other pathway to zero.

Contribution of tail-current calcium channel reopenings to inactivation of $I_{Ca}$

Calcium ions triggering Ca\(^{2+}\) release enter the cell in quanta determined by the duration and amplitude of the single-channel openings. The single-channel amplitude of the calcium current, $i_{Ca}$, depends linearly on the membrane potential (23, 55). Duration of calcium channel openings has exponential distribution with mean open time independent of membrane potential (31, 43). Within the short time interval during which the tail calcium current flows, the channel may reopen only few times before it finally deactivates. Because of the exponential distribution of the mean open time, not every opening during the tail may be long enough to provide sufficient amount of Ca\(^{2+}\) ions to activate calcium release. However, if the activated channel reopens, its potency to induce calcium release might be substantially increased because of the pre-elevated free-Ca\(^{2+}\) concentration in the junctional space. We have attempted to quantify the contribution of calcium channel reopenings to induction of $I_{Ca}$ inactivation by analyzing the fraction of inactivated calcium current in experiments, in which a constant prepulse was followed by a variable tail potential.

The number of openings depends on the tail potential, $V_t$. At very negative voltages, only those channels that were open at the end of the prepulse will contribute to the tail current. Therefore, at very negative tail potentials, the amount of calcium ions entering the cell during the tail is equivalent to the amount entered during the first openings, $m_{Ca,t,1}$, which is

$m_{Ca,t,1} = N_o \cdot i_{Ca} \cdot t_o

where $N_o$ is the number of calcium channels open at the end of the prepulse, $i_{Ca}$ is the single-channel current amplitude at the tail potential, and $t_o$ is the mean open time of calcium channels. At less negative potentials, the channels that were open at the end of the prepulse have a chance to close and reopen before deactivating, and channels that were activated but not in the open state at the end of the prepulse may succeed to open before deactivating. All these openings will be called “reopenings,” and the average number of reopenings per channel open at the end of the prepulse we define as $n_l$. The amount of Ca\(^{2+}\) ions entering the cell during reopenings is

$m_{Ca,t} = m_{Ca,t,1} \cdot n_l

The total amount of ions, $m_{Ca,t}$, is thus decomposed into the contribution of $m_{Ca,t,1}$ and $m_{Ca,t}$.

$m_{Ca,t} = m_{Ca,t,1} + m_{Ca,t}

To analyze whether the first and subsequent reopenings during the tail differ in their ability to inactivate $I_{Ca}$, the quantities $m_{Ca,t,1}$ and $n_l$ had to be extracted from $m_{Ca,t}$. Because $t_o$ is voltage independent and $i_{Ca}(V_o)$ is linear (23, 55), the voltage dependence of $m_{Ca,t,1}(V_p)$ (see Eq. 9) is also linear and can be expressed as

$m_{Ca,t}(V_p) = k \cdot (V_p - V_t)$

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where $k$ is the slope of the voltage dependence and $V_i$ is the $I_{Ca}$ reversal potential. We set $V_i$ to +60 mV, a value typical for our cells, as determined from $I/V$ curves (data not shown). The voltage dependence of the number of reopenings, $n_r$, was described by the Boltzmann function

$$n_r(V) = \frac{n_{max}}{1 + e^{(V-V_{50})/S}}$$

(13)

where $n_{max}$ is the maximal number of reopenings, $V_{50}$ is the half-maximum potential, and $S$ is the steepness of the voltage dependence. The parameters characterizing the voltage dependence of $m_{Ca,i}$ and $n_r$ ($k$, $n_{max}$, $V_{50}$, and $S$) were estimated by substituting Eq. 12 and Eq. 13 into Eq. 11 and fitting the resulting voltage dependence of $m_{Ca,i}$. Then, in analogy to Eq. 7 and Eq. 8, it was possible to express $F_{pi}$ as a function of $m_{Ca,i}$, entering during the first openings of calcium channels, and of $m_{Ca,r}$, entered during the reopenings

$$F_{pi} = \frac{(\beta \cdot m_{Ca,i} + m_{Ca,r})}{(M_{50}) + (\beta \cdot m_{Ca,i} + m_{Ca,r})}$$

(14)

where $M_{50} = M_{50}/k_r$, $\beta = k_i/k_r$, and $k_i < 1$ and $k_r < 1$ are the efficiency coefficients for the first and the remaining reopenings, respectively. If $\beta = 1$, then the first openings and the reopenings of the calcium channels have the same potency to trigger RDI; if $\beta > 1$, then the first openings have larger potency to trigger RDI; and if $\beta < 1$, then the reopenings have larger potency to trigger RDI. The apparent half-effective dose for calcium influx during reopenings is therefore equal to $M_{50}$, and that for calcium influx during first openings is equal to $M_{50}/\beta$.

For the purpose of visually comparing the measured data with the theoretical predictions, the values of $m_{Ca,i}$ and $m_{Ca,r}$ obtained using the parameters $k$, $n_{max}$, $V_{50}$, and $S$ from the fit of the experimental data from Eqs. 11–13 were substituted into Eq. 14 to yield the theoretical values of $F_{pi}$. The theoretical dependence of $F_{pi}$ on $m_{Ca,i}$ only, or on $m_{Ca,r}$ only, was calculated using Eq. 14 by setting $Ca^{2+}$ influx by the other pathway to zero.

RESULTS

$I_{Ca}$ inactivation by short prepulses. The time course and the extent of $I_{Ca}$ inactivation substantially differed according to the state of the cardiac myocyte (Fig. 1). When the cell was depleted of releasable calcium by exposure to 1 mM caffeine (58), 20 µM ryanodine (4), or application of five 70-ms prepulses to 0 mV at a rate of 0.33 Hz (16), inactivation of the calcium current was much slower than activation (Fig. 1A). In the absence of pharmacological intervention and after a 10-s rest at −50 mV, calcium current inactivation was fast and well developed (Fig. 1C). Quantitative characterization of this fast inactivation would be difficult with standard two-pulse protocols because of the multiple inactivation stimuli evoked in parallel by the prepulse. These stimuli would include the voltage, the calcium influx, and the calcium release during and after the prepulse.

The slow inactivation that dominated $I_{Ca}$ decay in cells that were depleted of releasable calcium suggests that the effects of the mechanisms responsible for slow inactivation could be negligible if very brief prepulses were used. Indeed, as illustrated in Fig. 1, B and D, a brief prepulse to a positive potential induced pronounced suppression of the test $I_{Ca}$ only in cells showing the fast, calcium release-dependent component of inactivation. On average, $F_{pi}$ induced by a prepulse to 0 mV was 0.48 ± 0.05 in 6 cells with RDI and 0.06 ± 0.02 in 4 cells without RDI. Such a dramatic difference in the effect of the short prepulse could not have been due to a difference in peak calcium current density between these two groups, which was not found to be statistically significant ($P > 0.05$).

These experiments revealed that brief prepulses represent a potential tool for selective induction of the calcium release-dependent $I_{Ca}$ inactivation without the confounding contributions of the calcium current- and the voltage-dependent mechanisms inherent to inactivation evoked by prolonged prepulses.

We next explored the use of brief prepulses, using the calcium release-dependent inactivation as a probe of calcium release activation.

Dependence of $I_{Ca}$ inactivation on the potential of the brief prepulse. To investigate the fast $I_{Ca}$ inactivation induced by brief depolarizations, we applied 5-ms prepulses from a holding potential of −50 mV to different potentials ranging between −40 and 0 mV (left) and +10 and +40 mV (right). The pulse protocol is shown at top. B: current traces recorded from the cell shown in A in response to a test pulse to 0 mV without a prepulse (I) and with a 5-ms prepulse to +30 mV separated by a 15-ms sojourn at the holding potential (2). Note that the application of the prepulse did not change the time course of $I_{Ca}$ substantially. C: a family of calcium currents recorded from a cell with the calcium release-dependent component of inactivation in response to the same voltage pulses as in A. D: currents recorded from the cell shown in C in response to voltage protocol as in B. Correspondingly, I and 2 denote traces with no prepulse and with prepulse separated by a 15-ms sojourn at the holding potential, respectively. Note the decrease of both the peak calcium current ($I_{Ca}$) and the inactivation rate in trace 2. Online subtraction of linear currents was used in all records.
partial inactivation has a dramatically decreased ability to trigger calcium release-dependent inactivation, even if it retains relatively large amplitude. In the case of the calcium current-dependent inactivation, the rate of fast inactivation was not decreased by ~50% preinactivation (52).

We compared the fraction, \( F_{\text{pi}} \), of the peak test \( I_{\text{Ca}} \) inactivated by the prepulse (Eq. 1) with the fraction, \( F_{\text{ui}} \), of the respective \( I_{\text{Ca}} \) that inactivated during the test pulse (Eq. 2). As shown in Fig. 2B, the larger was the fraction of the peak test calcium current inactivated by the prepulse; the smaller was the fraction of the test calcium current inactivated during the test pulse, resulting in a close negative correlation between the respective inactivation fractions for all prepulse potentials (\( R = -0.88, P < 0.0001 \)). In other words, the fraction of channels not subjected to calcium release-dependent inactivation by the prepulse underwent RDI during the test pulse. Such a relationship suggests that calcium channels undergoing inactivation by the prepulse and during the test pulse are recruited from the same pool of channels, namely, the pool of channels that face calcium ions released from the SR. This observation implies that prepulse-induced RDI affected only calcium channels in those dyads in which calcium release was activated by the prepulse.

\textbf{Prepulse- vs. tail-induced} \( I_{\text{Ca}} \), \textit{inactivation}. The prepulse as a trigger of calcium release can be thought of as a composite stimulus that can be divided into two parts, the calcium influx during the prepulse current and the Ca\(^{2+}\) influx during the tail current. We attempted to compare the potency of these two components in triggering calcium release and, thus, RDI. The amount of calcium flowing into the cell during the prepulse (\( m_{\text{Ca,p}} \)) and that during the tail current (\( m_{\text{Ca,t}} \)) was estimated by integrating the respective segments of the calcium currents. The amount of calcium ions injected into the cell during the tail current (Fig. 3A), \( F_{\text{p,t}} \), increased with the amplitude of the prepulse sigmoidally, whereas \( m_{\text{Ca,p}} \) had bell-shaped voltage dependence (Fig. 3A, \( \bullet \)). The voltage dependences of \( m_{\text{Ca,p}} \) and \( m_{\text{Ca,t}} \) were approximated by Eqs. 3 and 4, respectively. The resulting parameters of the best fits are given in Table 1, and the theoretical curves are overlaid with the data in Fig. 3A. It can be seen that \( m_{\text{Ca,p}} \) saturated at prepulses above \( +20 \) mV, in accordance with the voltage dependence of \( I_{\text{Ca}} \) activation (43).

The fraction, \( F_{\text{p,t}} \), of the peak test current \( I_{\text{Ca}} \) amplitude inactivated by the prepulse (Fig. 3B, \( \bullet \)) increased sigmoidally with the prepulse potential. It is obvious that the voltage dependence of \( m_{\text{Ca,t}} \) but not of \( m_{\text{Ca,p}} \) is similar to the voltage dependence of \( F_{\text{p,t}} \). Therefore, it can be speculated that the factual trigger of the calcium release-dependent inactivation of the test \( I_{\text{Ca}} \) might be constrained to the tail calcium current of the prepulse. Plotting the fraction of the test \( I_{\text{Ca}} \) inactivated by prepulses to different potentials against the amount of Ca\(^{2+}\) charge injected during the prepulse and tail currents (Fig. 3, C and D) revealed that although the relationship between \( F_{\text{p,t}} \) and \( m_{\text{Ca,p}} \) was quite complex (Fig. 3C), \( F_{\text{p,t}} \) increased sigmoidally with increasing \( m_{\text{Ca,t}} \) (Fig. 3D). This analysis suggests again that the tail current following the prepulse might be responsible for inactivation of the test \( I_{\text{Ca}} \). As in the absence of the calcium release-dependent inactivation the tail current did not induce significant inactivation of \( I_{\text{Ca}} \) (Fig. 1B), it appears that release of Ca\(^{2+}\) evoked by the tail current was the immediate cause of \( I_{\text{Ca}} \) inactivation. However, in the voltage range between \( -30 \) and \( +20 \) mV, the values of \( m_{\text{Ca,p}} \) and \( m_{\text{Ca,t}} \) are of comparable magnitude.

Fig. 2. Peak \( I_{\text{Ca}} \) inactivation by short prepulses of different amplitude. \( A \): current traces recorded from a typical cell in response to stimulation protocol (top) consisting of a 5-ms prepulse to a variable prepulse potential (\(-20 \) to \(+60 \) mV; indicated next to the traces), followed by a 10-ms interpulse interval at \(-50 \) mV and a 70-ms test pulse to \( 0 \) mV. Online subtraction of linear currents was used. \( B \): a scatter plot of the fraction of the test pulse peak calcium current inactivated by the prepulse (\( F_{\text{p,t}} \)) vs. the fraction of current inactivated during the test pulse (\( F_{\text{u,i}} \)). Solid squares, circles, and triangles; open squares, circles, and triangles; and crossed squares, circles, and triangles denote prepulse potentials of \(+60, +30, +20, +10, 0, -10; \) and \(-15, -20, \) and \(-50 \) mV, respectively. The line is the best linear fit for all data points.

Absence of prepulse, however, calcium currents with peak amplitude of about 40% of maximum peak \( I_{\text{Ca}} \) underwent fast and pronounced inactivation (see traces at \(-20 \) and \(+30 \) mV in Fig. 1C). This means that the calcium current remaining after
and prepulse Ca$^{2+}$, we assumed that the extent of calcium release, and consequently $m_{Ca,t}$ contribute differently to the effective calcium in $I_{Ca,p}$.

$F_{pi}$ induced inactivation, $m_{Ca,t}$, amol 24.9

Table 1. Estimated values of parameters for dependence of $m_{Ca,p}$ on prepulse voltage (Eqs. 3 and 4)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$m_{Ca,p} = f(V_p)$ (Eq. 3)</th>
<th>$m_{Ca,t} = f(V_p)$ (Eq. 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$m_{Ca,t}$, amol</td>
<td>24.9 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>$k_{Ca}$, amol·m$^{-1}$·mV$^{-1}$</td>
<td>0.44 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>$V_{C}$, mV</td>
<td>67.03 ± 3.88</td>
<td></td>
</tr>
<tr>
<td>$V_{0.5}$, mV</td>
<td>−0.64 ± 1.58</td>
<td>−5.13 ± 1.72</td>
</tr>
<tr>
<td>$S$, mV</td>
<td>6.63 ± 0.78</td>
<td>8.62 ± 1.34</td>
</tr>
</tbody>
</table>

Values are means ± SE for $n = 8$ cells. The correlation coefficients for $m_{Ca,p} = f(V_p)$ and $m_{Ca,t} = f(V_p)$ were 0.988 and 0.997, respectively. See text for definitions.

Fig. 3. Dependence of Ca$^{2+}$ influx on prepulse potential. A: the dependence of prepulse and tail Ca$^{2+}$ influx (open and solid circles, respectively) on prepulse voltage ($V_p$). Solid lines represent the best fit to the prepulse and tail Ca$^{2+}$ influx by Eqs. 3 and 4, respectively, with the parameters given in Table 1. The dotted line represents the total calcium influx. B: the dependence of prepulse-induced inactivation, $F_{pi}$, on prepulse voltage ($V_p$). C: the relationship between the fraction of the test pulse calcium current inactivated by the prepulse ($F_{pi}$) and prepulse Ca$^{2+}$ influx ($m_{Ca,p}$). D: the relationship between the fraction of the test pulse calcium current inactivated by the prepulse ($F_{pi}$) and tail Ca$^{2+}$ influx ($m_{Ca,t}$). In B–D, the solid lines are the solution of Eq. 8 with parameters from Tables 1 and 2; the dotted and dashed lines are the solutions of Eq. 8 for $m_{Ca,t} = 0$, and $m_{Ca,p} = 0$, respectively. In all graphs, data are expressed as means ± SE ($n = 4$–6 from 8 different cells).

size (Fig. 3A). Therefore, we tested the hypothesis that $m_{Ca,p}$ contributes to prepulse-induced inactivation, albeit with a different potency (Eq. 6). As outlined in MATERIALS AND METHODS, we assumed that the extent of calcium release, and consequently the fraction $F_{pi}$ of $I_{Ca}$ inactivated by Ca$^{2+}$ release, are dose-dependently proportional to the effective calcium influx sensed by the RyRs and that the calcium influxes $m_{Ca,p}$ and $m_{Ca,t}$ contribute differently to the effective calcium influx. The potency of $m_{Ca,p}$ to induce a given $F_{pi}$ is then a constant fraction of the potency of $m_{Ca,t}$, i.e., the relative potency of $m_{Ca,p}$ with respect to $m_{Ca,t}$ can be expressed by the factor $\alpha$ (Eq. 8). Fitting Eq. 8 to the values of $F_{pi}$ provided the optimal value of $\alpha$ substantially less than 1 (Table 2). It means that the potency of $m_{Ca,p}$ is substantially less than that of $m_{Ca,t}$. Figure 3, B–D, show the theoretical relationships between $F_{pi}$, $m_{Ca,p}$, $m_{Ca,t}$, and membrane potential, calculated from the voltage dependencies of $m_{Ca,p}$ and $m_{Ca,t}$ (Eqs. 3 and 4, Table 1) and from the dependencies of $F_{pi}$ on $m_{Ca,p}$ and $m_{Ca,t}$ (Eq. 8, Table 2) overlaid on the experimental data measured at different prepulse potentials. It can be seen that the theoretical curves reproduce well the observed relationships.

Although it is not possible to expose the cells to the prepulse and tail Ca$^{2+}$ influx in separation from each other, the theoretical effects of these two modes of calcium influx on $F_{pi}$ can be calculated from their potencies (see dotted and dashed curves in Fig. 3, B–D). The difference between the effect of the prepulse and the tail Ca$^{2+}$ influx separated from each other is then even more pronounced than the difference in the potencies because in contrast to $m_{Ca,t}$, the value of $m_{Ca,p}$ is below the apparent half-effective dose at all potentials.

Dependence of $I_{Ca}$ inactivation on the tail potential. The above findings might be interpreted as if it were the increased amplitude of the single-channel current during the tail that increased the potency of calcium influx to evoke inactivation of $I_{Ca}$. However, variation in the tail potential invokes variation in the tail current kinetics as well. It follows from the recent understanding of calcium channel function that both the magnitude of the single-channel current and the rate of the current deactivation increase with hyperpolarizing membrane potential. Then, at less hyperpolarized tail voltages, the single-channel current amplitude is lower due to reduced driving force on calcium ions, but the current deactivates more slowly, due to increased probability of channel reopenings, than at more hyperpolarized voltages. From the point of release-dependent inactivation, we may speculate that if the amplitude of the single-channel openings is more important for triggering calcium release, RDI should increase with the tail hyperpolarization. Conversely, if reopenings are more important, then RDI should decrease with the tail hyperpolarization.

In this series of experiments, the extent of calcium channel activation at the end of the prepulse was kept constant by using constant prepulse duration, and Ca$^{2+}$ influx during the prepulse was minimized by depolarization to the reversal potential. A very short prepulse (3 ms, +60 mV) was followed by a 10-ms interval at a variable tail potential ($V_t$ = −120 to −40 mV), then by a 20-ms interpulse interval at the holding potential of −50 mV, and finally by the test pulse (70 ms, 0 mV). To avoid activation of sodium currents by repolarization steps from the very negative tail potentials to the holding potential and subsequent activation of the Na$^+$/Ca$^{2+}$ exchange, we used Na$^+$-
CALCIUM RELEASE AND RELEASE-DEPENDENT INACTIVATION OF $I_{\text{Ca}}$

Fig. 4. Inactivation of $I_{\text{Ca}}$ by short prepulses with different potentials following the prepulse. $A$: a family of calcium currents in response to a 70-ms test pulse to 0 mV that was preceded by a 3-ms prepulse to +60 mV, followed by a 10-ms interval at a variable tail potential ($V_t = -120$ to $-40$ mV) and a second 20-ms interpulse interval at the holding potential of $-50$ mV. The pulse protocol is shown at top. In the external solution, Na$^+$ ions were replaced by Cs$^+$ to eliminate sodium currents. $B$: the dependence of prepulse-induced inactivation, $F_{pi}$, on the tail potential ($V_t$). $C$: the dependence of Ca$^{2+}$ influx during the tail, during the first opening, and during reopenings (half-solid, open, and solid circles, respectively) on the tail potential ($V_t$). Data were obtained by fitting the voltage dependence of $m_{Ca,1}$ as described in MATERIALS AND METHODS. The lines represent the theoretical values of $m_{Ca,1}$, $m_{Ca,s}$, and $m_{Ca,r}$, calculated using Eqs. 12 and 13 with the parameters given in Table 3. $D$: the relationship between Ca$^{2+}$ influx during the tail current ($m_{Ca,r}$) and the fraction of the test pulse calcium current inactivated by the prepulse ($F_{pi}$). Data are expressed as means ± SE ($n = 8$). $E$: the relationship between the fraction of the test pulse calcium current inactivated by the prepulse ($F_{pi}$) and the calcium influx during the first opening ($m_{Ca,1}$). $F$: the relationship between the fraction of the test pulse calcium current inactivated by the prepulse ($F_{pi}$) and the calcium influx during reopenings ($m_{Ca,s}$). In $C-F$, the solid lines are the solution of Eq. 14 corresponding to the tail potential range of $-120$ to $-40$ mV with parameters from Tables 3 and 4; the dotted and dashed lines are the solutions of Eq. 14 for $m_{Ca,1} = 0$, and $m_{Ca,s} = 0$, respectively. In all graphs, data are expressed as means ± SE ($n = 8$ cells).

free extracellular solutions. A typical experiment is presented in Fig. 4A. Suppression of the peak test $I_{\text{Ca}}$ and of the fast inactivation of calcium current during the test pulse increased with increasing (less negative) tail potentials (Fig. 4B) in parallel with increasing amount of calcium injected into the cell by the tail current (Fig. 4C, half-open symbols). As in the previous series of experiments, $F_{pi}$ had again a tendency to saturate at a level lower than unity (Fig. 4D). Incomplete suppression of the peak test $I_{\text{Ca}}$ suggests that there was a population of calcium channels that were not subjected to inactivation by Ca$^{2+}$ released from the SR. Similar to experiments with a variable prepulse amplitude, there was again a close negative correlation between $F_{pi}$ and $F_{t0}$ (data not shown), suggesting once more that the prepulse inactivates the same pool of channels as the test pulse.

In the range of tail potentials between $-100$ and $-40$ mV, $F_{pi}$ increased in a saturable manner with $m_{Ca,1}$ (Fig. 4D). Interestingly, however, although the tail-current Ca$^{2+}$ influxes at $-120$ mV and at $-100$ mV were not significantly different ($P > 0.05$), the former inactivated a smaller fraction of $I_{\text{Ca}}$ ($P < 0.005$), i.e., the potency of Ca$^{2+}$ influx was lower at $-120$ than at $-100$ mV despite presumably larger single-channel amplitude at $-120$ mV. The relationship between the extent of inactivation and the amount of Ca$^{2+}$ ions injected during the tail current suggests that it could be the number of calcium channel openings, rather than their single-channel amplitude, that determined the extent of the prepulse-induced inactivation. To verify this hypothesis, we attempted to compare the potency of Ca$^{2+}$ influx during the first opening and during the remaining openings in inducing inactivation of $I_{\text{Ca}}$, using again the mass action rationale for estimation of the Ca$^{2+}$ influx potency as that used already for comparison of the prepulse and the tail Ca$^{2+}$ influxes. We assumed that at a sufficiently negative tail potential, when the rate of deactivation is very fast, each channel that is activated at the end of the prepulse contributes to the total Ca$^{2+}$ charge only by one opening, the open time of which is independent of membrane potential (31, 43). The amount of Ca$^{2+}$ ions injected by the tail current was expressed as the sum of the calcium influx by the first openings $m_{Ca,1}$ and by the amount of Ca$^{2+}$ ions contributed by the tail-current reopenings $m_{Ca,s}$ (see MATERIALS AND METHODS). The parameters characterizing the voltage dependence of $m_{Ca,1}$ and $m_{Ca,s}$ (Fig. 4C), shown in Table 3, were estimated from the voltage dependence of $m_{Ca,1}$ using Eqs. 12
Table 3. Estimated values of parameters for voltage dependence of tail currents after brief pre-pulses (Eqs. 12 and 13)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k$, amol/mV</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>$V_0$, mV</td>
<td>+60*</td>
</tr>
<tr>
<td>$n_{max}$, amol</td>
<td>14.4 ± 2.6</td>
</tr>
<tr>
<td>$V_{50}$, mV</td>
<td>−45.7 ± 5.8 mV</td>
</tr>
<tr>
<td>$S$, mV</td>
<td>17.2 ± 1.0 mV</td>
</tr>
</tbody>
</table>

Values are means ± SE for $n = 8$ cells. *The reversal potential $V_R$ was set constant. The correlation coefficients of the fits for individual cells were 0.896 ± 0.0377. See text for definitions.

and 13 and then used in Eq. 14 to approximate the dependence of $F_{pi}$ on $m_{Ca,i}$ and $m_{Ca,r}$ (Fig. 4, E and F). Because the limited range of tail potentials did not allow $F_{pi}$ to vary in a sufficiently broad range, we have fixed the value of $n_{f}$, which is not expected to depend on the experimental protocol, to $n_{f} = 2$ as determined in the previous section (see Table 2). The value of $F_{max}$ (Table 4) was not significantly different from the value characterizing the dependence of $F_{pi}$ on prepulse amplitude (Table 2) as expected when the same population of calcium channels was addressed in the two types of experiments. However, the relative potency, $\beta$, of the first openings to induce inactivation of $I_{Ca}$ determined by fitting Eq. 14 to the observed values of $F_{pi}$ (Table 4) was significantly smaller than 1 ($P < 0.005$), suggesting that $F_{pi}$ was more under control of Ca$^{2+}$ influx during channel reopenings than during the first openings. The predominance of the lower-potency first openings (>95%, see Table 3) over reopenings at −120 mV explains the deviation from dose dependence between $m_{Ca,i}$ and $F_{pi}$ at this tail potential (Fig. 4C, arrow).

The theoretically expected effects of the pure Ca$^{2+}$ influx during the first openings and the reopenings, if they could occur independent of each other, on $F_{pi}$ were estimated from their potencies and from Eq. 14 (see dotted and dashed curves in Fig. 4, B, E, and F, and the corresponding legend). The difference in their effect arises from the fact that $m_{Ca,i}$, in contrast to $m_{Ca,r}$, is below its apparent half-effective influx ($M_{SO}/\beta$) at all studied potentials. The first opening and the reopenings contributed equally to the calcium influx at −60 mV, but due to their different potencies, the contribution of the two calcium influx modes to $F_{pi}$ would be equal at −90 mV. Therefore, at repolarization to the holding potential of −50 mV, the RDI of $I_{Ca}$ induced by the tail current following the prepulse was brought about mainly by the reopenings during the tail current.

In both types of experiments presented above (Figs. 3 and 4), Ca$^{2+}$ influx was more effective when occurring later. That is, the tail current was more effective than the prepulse current, and the reopenings were more effective than the first openings. The higher potency of Ca$^{2+}$ influx occurring shortly after previous Ca$^{2+}$ influx suggests that the potency of Ca$^{2+}$ influx to induce calcium release depends on the recent history of Ca$^{2+}$ influx rather than on the overall Ca influx.

**Activation of RyRs by paired brief calcium elevations.** To directly examine whether the variable potency of a given amount of Ca$^{2+}$ ions to activate calcium release is apparent at the level of single RyR channels, we have fused cardiac SR microsomes into lipid bilayers and recorded the activity of single RyRs with Cs$^+$ as the charge carrier. Cytosolic concentrations of free Mg$^{2+}$ and total ATP in cardiac cells are in the range of 0.5–1.2 and 3–5 mM, respectively, and the free luminal Ca$^{2+}$ concentration is close to 1 mM (6). Therefore, to imitate the conditions present in the cell, the cytoplasmic solution contained 3 mM total ATP (free Mg$^{2+}$, ~0.6 mM) and the luminal solution contained 1 mM Ca$^{2+}$. To mimic two consecutive openings of a calcium channel, we applied two consecutive ultraviolet laser pulses at a 100-ms interval to generate rapid Ca$^{2+}$ changes by photolyzing the calcium cage compound NP-EGTA. The amount of Ca$^{2+}$ liberated by the two flashes was identical. Subtle differences between the shape of the first and the second stimulus were present because of the differences in the saturation of the Ca$^{2+}$ buffer, NP-EGTA, at the time of the stimulus. The first flash induced a rapid and brief (full duration at half amplitude, FDHA = 0.14 ms) Ca$^{2+}$ elevation from ~0.3 μM to ~65 μM, followed by a sustained Ca$^{2+}$ elevation to ~0.4 μM; the second flash produced a slightly longer Ca$^{2+}$ signal (FDHA = 0.19 ms) that again reached a peak amplitude of ~65 μM (Fig. 5A). The probability of RyR openings by the first Ca$^{2+}$ stimulus was low ($P = 0.03$; Fig. 5C), whereas the second Ca$^{2+}$ stimulus triggered many more openings ($P = 0.1$; Fig. 5, B and C). Based on results obtained in four individual experiments, the probability of RyR activation during the second stimulus was four times larger than that during the first Ca$^{2+}$ stimulus ($0.16 ± 0.03 vs. 0.04 ± 0.01, P < 0.05$). The changes in probability of RyR activation (300%) were much more pronounced that the changes in basal Ca$^{2+}$ concentration (~40%) and in the duration of the stimulus (~30%). These results support the notion that the probability of RyR activation by the same amount of liberated Ca$^{2+}$ steeply increases with the Ca$^{2+}$ preceding the calcium stimulus and/or with the duration of the stimulus.

**DISCUSSION**

Separation of RDI from other components of $I_{Ca}$ inactivation. In this study, we show that the RDI of the calcium current can be isolated from other inactivating mechanisms without pharmacological intervention. We also show that the extents of RDI evoked by two successive stimuli (i.e., prepulse-induced $F_{pi}$ and test-pulse induced $F_{pi}$) complement each other. These findings allowed us to use RDI as a local probe of the calcium release process. Our experiments revealed that the extent of calcium release activation depends on the recent history and mode of Ca$^{2+}$ influx rather than on the overall Ca$^{2+}$ influx, indicating that brief pre-elevations of dyadic Ca$^{2+}$ levels might potentiate the response of RyRs to the subsequent Ca$^{2+}$ influx.

Table 4. Estimated values of parameters for calcium dose dependence of the fraction $F_{pi}$ of $I_{Ca}$ inactivation by the first and remaining openings at different tail potentials (Eq. 14)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value ± SE</th>
</tr>
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<tbody>
<tr>
<td>$F_{max}$</td>
<td>0.60 ± 0.08</td>
</tr>
<tr>
<td>$\beta$</td>
<td>0.32 ± 0.15</td>
</tr>
<tr>
<td>$M_{SO}$, amol</td>
<td>3.04 ± 1.36</td>
</tr>
<tr>
<td>$n_{f}$</td>
<td>2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *The value of the parameter $n_{f}$ was set constant. The correlation coefficient for the fit was 0.96. See text for definitions.
We have directly demonstrated such potentiation at the level of single RyRs in planar lipid bilayers.

It is well known that the fast inactivation of \( I_{Ca} \) depends on the release of Ca\(^{2+}\) ions from the sarcoplasmic reticulum (1, 4, 45, 46, 48, 58). However, quantitative determinations of the extent of RDI have been limited due to lack of straightforward experimental approaches. RDI was isolated pharmacologically by comparing currents in the presence and absence of SR Ca\(^{2+}\) release (employing caffeine to deplete the SR of calcium) under conditions that supported also current- and voltage-dependent inactivation of \( I_{Ca} \) (46). However, besides depleting the SR of calcium, caffeine has several additional effects on the calcium current (58). In addition, the influence of RDI on the kinetics of current- and voltage-dependent inactivation mechanisms, which is manifested as a prominent increase in the rate of the slow calcium current-, and voltage-dependent inactivation observed in the absence of RDI (Fig. 1, see also Fig. 2 in Ref. 46) might present an additional limitation of the pharmacological approach. Employing brief prepulses as stimuli inducing RDI without contribution of other inactivation mechanisms (Fig. 1) enabled us to determine the extent of RDI directly.

**Relationships between RDI and calcium release activation.**

Our results and analysis indicate (Figs. 1 and 2) that the calcium release-dependent inactivation of \( I_{Ca} \) results from exposure of only a fraction of calcium channels to Ca\(^{2+}\) ions released from the nearby SR rather than from exposure of all the calcium channels to globally increased cytoplasmic calcium. This is inferred from the finding that the fraction of the test pulse calcium current that underwent fast inactivation in the absence of a prepulse could be inactivated by a brief prepulse (Fig. 2B), a stimulus specific for RDI. RDI affected \( \sim 75\% \) of the peak \( I_{Ca} \) amplitude (Figs. 2–4), suggesting that \( I_{Ca} \) activated only about 75% of release units under our conditions. Our estimates of excitation-contraction (E-C) coupling efficacy made by measuring RDI are consistent with those based on fluorescence imaging of local calcium signals (i.e., calcium “sparks” and “spikes”). Thus using rapid two-dimensional confocal imaging and fluo 3, Cleemann et al. (14) found that depolarization to 0 mV leads to activation of about 70% of release units in rat ventricular myocytes. By measuring spatially resolved fluorescence spikes with Oregon Green BAPTA-5N, Song et al. (51) estimated that 60–90% of all available dyads become activated during E-C coupling under fully phosphorylated conditions in the same voltage range. In addition, the dependencies of \( F_{pi} \) (this work) and calcium release (8) on the tail potential are very similar. Thus the principles of local calcium signaling that apply to regulation of SR Ca release (12, 25, 30, 38, 53, 56) also manifest themselves in features of RDI.

The direct proportionality between the fraction of activated calcium release units and the extent of RDI, ensuing from the local control of RDI, enabled us to derive the relationships among calcium influx, activation of calcium release, and induction of RDI (Eqs. 8 and 14). Varying single-channel properties during the prepulse and tail current portions of the calcium release-inducing stimulus enabled us to show for the first time that different modes of calcium influx contributed differently to the effective calcium trigger signal. In the two-pulse experiments with \( \sim 50 \) mV tail potential, changing the amplitude of the prepulse varied the relative proportion of calcium ions entering the cell during the prepulse and during the tail current. Analysis of the relationship between \( I_{Ca} \) inactivation and Ca\(^{2+}\) influx (Fig. 3), based on the principle of mass action (Eq. 8), showed that the same amount of calcium influx was much more effective in triggering calcium release when entering during the tail \((m_{Ca,t})\) than when entering during the prepulse \((m_{Ca,p})\). Similar observations are usually interpreted as showing that the probability of triggering calcium release increases with the single-channel amplitude of the calcium current (12, 44, 51). It should be noted, however, that the dependence of the extent of RDI on the prepulse tail potential appeared to be at variance with such conclusion. When the DHPR channels were preactivated in the absence of Ca, \( I_{Ca} \) near the reversal potential, and their deactivation rate and single-channel amplitude were varied by changing the tail potential, the potency of calcium influx to activate calcium release (i.e., the extent of RDI observed for the same amount of calcium influx) appeared to increase with decreasing single-channel amplitude. This apparent paradox...
was resolved by realizing that tail calcium influx comprises of two phenomena with different voltage dependences: the single-channel current amplitude and the number of channel openings. Because of the much higher potency of calcium channel openings to activate calcium release, they contributed more substantially to the effective Ca$^{2+}$ influx than did the first openings at all tail potentials positive to $-90$ mV (compare the dashed and dotted curves in Fig. 4B). These observations strongly indicate that the probability that a solitary DHPR opening will activate its neighboring RyRs is less than one even at the most negative voltages ($-120$ mV).

The difference in efficiency between single and multiple openings can be understood on the grounds of our previous findings (59, 61) that significant levels of RyR activation may be reached only with sufficiently prolonged exposure of RyRs to elevated Ca$^{2+}$. The effective exposure time for any given concentration of calcium is determined by the kinetics of RyR activation. In addition, preceding influx of Ca$^{2+}$ that failed to activate release may result in Ca$^{2+}$ accumulation in the junctional space. The increased level of Ca$^{2+}$ may potentiate the effects of subsequent influx of Ca$^{2+}$ by partial occupation of the mobile and immobile calcium buffers (39) that reduces calcium-buffering capacity of the junctional space. In effect, less Ca$^{2+}$ from the subsequent Ca$^{2+}$ influx is consumed by calcium buffers, and a larger amount of free Ca$^{2+}$ is left available for activation of RyRs. Both these nonlinear processes are effective only locally and briefly due to the fast chemistry of Ca$^{2+}$ ions and the quantal character of calcium influx from the point source(s). Preceding elevated junctional Ca$^{2+}$ may also have direct potentiating effect on RyR activation by increasing the fraction of Ca$^{2+}$-bound RyR states at the expense of Mg$^{2+}$-bound states (36, 57). Local changes of dyadic Ca$^{2+}$ on a longer time scale were speculated to contribute to the increase of the frequency of spontaneous calcium sparks upon inhibition of the forward mode sodium-calcium exchange (22) and to potentiation of calcium current-induced calcium release by the calcium influx by the reverse-mode Na$^+$/Ca$^{2+}$ exchange (37).

In agreement with these notions, we have directly demonstrated in single-channel experiments that the probability of activation of RyRs by a given Ca$^{2+}$ influx increases with repeated stimulus application. The observed increase in open probability could have been due to changes in the Ca$^{2+}$ concentration preceding the stimulus and/or due to prolongation of the stimulus, both due to the partial saturation of the calcium buffer. Because of the long time constant of diffusion in the 0.1-mm$^3$ volume exposed to flash photolysis in our experimental setting (~10 s; Ref. 26), the changes in buffer saturation fully persisted during the 100-ms interval between the two stimuli. The volume of the dyadic gap is only 0.001 μm$^3$ and, therefore, the changes in buffer saturation due to local calcium influx are expected to persist only on the millisecond time scale (49).

**Implications for excitation-contraction coupling.** The voltage dependence of $P_{o,DHPR}$ on the calcium influx through an isolated DHPR opening enables direct estimation of the fidelity of coupling for a given number of DHPR channels in the dyad and known DHPR open probability ($P_{o,DHPR}$). Previous estimates, in addition to experimental measurements, required also specifying the amplitude of the DHPR single-channel current and DHPR open time, as well as of the amplitude and duration of the calcium influx during a single spark (62), parameters that can be inferred only indirectly. Assuming 20 DHPRs per calcium release unit (6, 21) and a maximum $P_{o,DHPR}$ of 5% (43), our data (Table 4) suggest a probability of Ca$^{2+}$ release activation by a solitary DHPR opening (coupling fidelity: Refs. 51, 55, 62) of 35% at $-120$ mV, 15% at $-40$ mV, 6% at 0 mV, and <3% at +20 mV. These values are in good accord with the previous estimates of coupling fidelity (15 and 2% at $-40$ and 0 mV; Ref. 62). Extrapolation to calcium currents in the presence of the calcium agonist FPL 64176 by assuming a 20-fold increase of $m_{Ca}$ per opening (43, 51) and $P_{o,DHPR} = 0.8$ (51) gives an estimate of coupling fidelity of $-0.65$ at 0 mV in agreement with direct measurements of coupling fidelity in the presence of FPL 64176 (55). The good correspondence between our estimates for two widely different conditions and the corresponding estimates by two different methods (51, 55) suggests that the relationship between Ca$^{2+}$ influx and calcium release activation for solitary DHPR openings (dotted line in Fig. 4E) might be quite general.

In summary, we have shown that tail calcium currents following repolarization from short depolarizing pulses to positive membrane potentials are better triggers of calcium release than calcium currents carrying a similar charge during the depolarizing pulses themselves. Moreover, the potency of calcium current to trigger calcium release is higher at less negative repolarization potentials. This higher potency might result from a combination of the potentiating effects of futile channel openings that failed to activate RyR channels but increased the basal Ca$^{2+}$ level during depolarization and of the high temporal synchronization of the calcium influx through preactivated calcium channels on repolarization.

The physiological consequences of these findings may become comprehensible when brief stimuli in our experiments are related to the shape of the action potential: the rising phase and the peak of the action potential resembles the brief depolarizing prepulse, whereas the early repolarization resembles the end of the prepulse. Our results then suggest that the efficiency of calcium influx to trigger calcium release should achieve its maximum during the early repolarization phase of the action potential. In this way, our findings explain why the peak of calcium release occurs within a few milliseconds after the peak of the action potential (9, 29, 42).

**ACKNOWLEDGMENTS**

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**GRANTS**

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