Calcium-induced Calcium Release in Smooth Muscle
Loose Coupling between the Action Potential and Calcium Release

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Abstract Calcium-induced calcium release (CICR) has been observed in cardiac myocytes as elementary calcium release events (calcium sparks) associated with the opening of L-type Ca\(^{2+}\) channels. In heart cells, a tight coupling between the gating of single L-type Ca\(^{2+}\) channels and ryanodine receptors (RYRs) underlies calcium release. Here we demonstrate that L-type Ca\(^{2+}\) channels activate RYRs to produce CICR in smooth muscle cells in the form of Ca\(^{2+}\) sparks and propagating Ca\(^{2+}\) waves. However, unlike CICR in cardiac muscle, RYR channel opening is not tightly linked to the gating of L-type Ca\(^{2+}\) channels. L-type Ca\(^{2+}\) channels can open without triggering Ca\(^{2+}\) sparks and triggered Ca\(^{2+}\) sparks are often observed after channel closure. CICR is a function of the net flux of Ca\(^{2+}\) ions into the cytosol, rather than the single channel amplitude of L-type Ca\(^{2+}\) channels. Moreover, unlike CICR in striated muscle, calcium release is completely eliminated by cytosolic calcium buffering. Thus, L-type Ca\(^{2+}\) channels are loosely coupled to RYR through an increase in global [Ca\(^{2+}\)] due to an increase in the effective distance between L-type Ca\(^{2+}\) channels and RYR, resulting in an uncoupling of the obligate relationship that exists in striated muscle between the action potential and calcium release.

Key words: calcium-induced calcium release • smooth muscle • Ca\(^{2+}\) sparks • excitation-contraction coupling • action potential signaling

Introduction In striated muscle excitation-contraction (E-C\(^{1}\)) coupling is initiated by the gating of sarcolemmal L-type Ca\(^{2+}\) channels, which trigger the release of calcium from ryanodine receptors (RYRs) on the sarcoplasmic reticulum (Endo, 1977; Fabiato, 1983; Nabauer et al., 1989; Tanabe et al., 1990; McPherson and Campbell, 1993). While the mechanism of coupling between L-type Ca\(^{2+}\) channels and RYR is different in skeletal and cardiac myocytes, in both cell types local interactions between these proteins underlie calcium release. In skeletal myocytes, calcium entry is not required for calcium release (Armstrong et al., 1972), but gating of the L-type Ca\(^{2+}\) channel appears to be physically coupled to RYR opening (Tanabe et al., 1990; Nakai et al., 1998). Calcium entry through L-type Ca\(^{2+}\) channels triggers calcium-induced calcium release (CICR) in heart cells (Fabiato, 1985; Nabauer et al., 1989), resulting in localized calcium release, termed Ca\(^{2+}\) sparks (Cheng et al., 1993; Cannell et al., 1995; Lopez-Lopez et al., 1995). This coupling process involves a local increase in [Ca\(^{2+}\)] in the microdomain of the L-type Ca\(^{2+}\) channel, which is sensed by the RYR, resulting in RYR gating, and several lines of evidence indicate that the opening of a single L-type Ca\(^{2+}\) channel triggers a Ca\(^{2+}\) spark in an obligatory fashion (Niggli and Lederer, 1990; Cannell et al., 1995; Lopez-Lopez et al., 1995; Santana et al., 1996; Lipp and Niggli, 1996; Collier et al., 1999). This tight coupling between gating of the voltage-dependent sarcolemmal channel and the sarcoplasmic reticular release channel underlies the full mobilization of Ca\(^{2+}\) that occurs in cardiac myocytes with each action potential.

RYRs are widely expressed in nonsarcomeric (smooth) muscle, neurons, and nonexcitable cells, although their role in calcium release and cellular signaling is poorly understood. In smooth muscle, RYRs are expressed on the sarcoplasmic reticulum (Carrington et al., 1995; Lesh et al., 1998) and triggered Ca\(^{2+}\) release has been inferred from measurements of calcium-activated membrane currents and spatially averaged [Ca\(^{2+}\)] (Zholos et al., 1992; Ganitkevich and Isenberg, 1992, 1995), but little direct evidence of CICR exists and the function of RYRs in E-C coupling is poorly understood. Spontaneous Ca\(^{2+}\) sparks have been reported in smooth muscle (Nelson et al., 1995; Mironneau et al., 1996; Gordienko et al., 1998), and recent experiments combining confocal microscopy and patch-clamp techniques have demonstrated localized calcium release during depolarizing voltage-clamp steps (Arnaudeau et al., 1997; Imaizumi et al., 1998), further supporting the existence of CICR in smooth muscle. Here we show that the L-type Ca\(^{2+}\)
current \( (I_{Ca}) \) evokes CICR in single urinary bladder myocytes and establish the relationship between L-type Ca\(^{2+} \) channel opening and RYR-mediated calcium release in smooth muscle.

**Materials and Methods**

**Cell Isolation**

Male New Zealand White rabbits were anesthetized (50 mg/kg ketamine, 5 mg/kg xylazine i.m.) and killed (100 mg/kg pentobarbital i.v.) in accordance with an approved laboratory animal protocol. The urinary bladder was removed, dissected in ice-cold oxygenated physiological salt solution, minced, and suspended in modified collagenase type II (Worthington Biochemical), 1 mg/ml protease type XIV, and 5 mg/ml bovine serum albumin (Sigma-Aldrich) at 37°C for 30–40 min. Digested tissue was triturated with a wide-bore Pasteur pipette and passed through a 125-μm nylon mesh; cells were concentrated by low speed centrifugation, washed with fresh medium, resuspended, and stored at 4°C.

**Electrophysiology**

Single myocytes were placed in a chamber mounted on an inverted Nikon TE300 microscope (Nikon) and whole-cell recordings made as previously described (Wang and Kotlikoff, 1997). In most experiments, pipettes were filled with (mM): 127 cesium glutamate, 10 HEPES (cesium-salt), 1 Mg-ATP, 5 Tris-creatine phosphate, and 0.1 fluo-4 (pentapotassium salt) at pH 7.2. Heparin (2–5 mg/ml) was added to the internal solution to increase the buffering capacity of the internal solution. Pipettes were 70V and 10 ms, respectively. Cells were incubated with 10 μM fluo-4 methoxymethyl ester (Molecular Probes, Inc.) and were pre-incubated with the drug for 1 h at room temperature and 10 μM ryanodine was added to the bath solution.

**Recording and Measurement of Fluorescence**

Fluo-4 fluorescence was excited with 488 nm light emitted from a Krypton/Argon laser and measured with a high speed laser scanning confocal head (Noran Oz), using a plan-apo, 60× water-immersion objective lens (1.2 NA; Nikon) and Intervision software on an Indy workstation (Silicon Graphics Inc.). x-y images were collected every 8.3 ms (256 × 240 pixels), and x-t images were obtained with line scans at 4.16 ms intervals for 2.13 s (512 × 480 pixels). Pixel size in the x axis was equal to 0.252 μm and in the y axis to 0.248 μm. To synchronize current and fluorescence measurements, a light emitting diode was placed in the path of the photomultiplier detector and switched on for 2 ms, 10 ms before the start of the voltage step. Images were analyzed using either Intervision software (Silicon Graphics Inc.) or a custom written analysis program using Interactive Data Language software (Research Systems Inc.).

**Results**

The L-type \( \text{Ca}^{2+} \) channel current triggers CICR

Depolarizing voltage-clamp steps activating \( I_{Ca} \) in single urinary bladder myocytes triggered one or several \( \text{Ca}^{2+} \) sparks and subsequent propagated \( \text{Ca}^{2+} \) waves (Fig. 1). Images acquired at 8.3 ms intervals showed that release began as elementary events at one or several foci, as
previously reported (Imaizumi et al., 1998), and progressively expanded to propagated Ca\textsuperscript{2+} waves. The velocity of propagation of the Ca\textsuperscript{2+} wave was 94 ± 15 μm/s (n = 4), similar to Ca\textsuperscript{2+} wave velocity described in cardiac myocytes (Wussling et al., 1997), but substantially faster than Ca\textsuperscript{2+} waves propagated by InsP\textsubscript{3} receptors in vascular cells (Bezprozvanny, 1994).

Depolarizations activating smaller currents usually triggered a single Ca\textsuperscript{2+} spark and propagated Ca\textsuperscript{2+} wave, whereas larger currents initiated Ca\textsuperscript{2+} sparks from several sites that propagated and fused. The temporal relationship between I\textsubscript{Ca} and Ca\textsuperscript{2+} sparks varied with the magnitude of the current, but Ca\textsuperscript{2+} sparks always occurred with a delay after current activation. In some cases, Ca\textsuperscript{2+} sparks were observed only after I\textsubscript{Ca} was almost completely inactivated (Fig. 1 A). In separate experiments, Ca\textsuperscript{2+} sparks and Ca\textsuperscript{2+} waves were not altered by the dialysis of heparin (Fig. 1 C; n = 4), but were abolished by application of caffeine (10 mM; see Fig. 3; n = 9), incubation with ryanodine (10 μM; see Fig. 5; n = 11), or block of I\textsubscript{Ca} with CdCl\textsubscript{2} (500 μM; not shown; n = 9). The magnitude and kinetics of Ca\textsuperscript{2+} sparks triggered by I\textsubscript{Ca} was similar to previously reported values for spontaneous Ca\textsuperscript{2+} sparks in smooth muscle. The mean rise time of triggered release events was 26.6 ± 1.6 ms, peak F/F\textsubscript{0} = 1.9 ± 0.1, and the half time of decay of isolated (nonpropagated) Ca\textsuperscript{2+} sparks was 62 ± 16 ms (n = 5), which is similar to previous reports using similar methods (Perez et al., 1999). Thus, Ca\textsuperscript{2+} sparks and subsequent Ca\textsuperscript{2+} wave propagation triggered by the voltage-dependent calcium current is due to activation of RYRs by L-type Ca\textsuperscript{2+} channels.

Relationship between I\textsubscript{Ca} and CICR: Loose Coupling

The number of Ca\textsuperscript{2+} sparks triggered by I\textsubscript{Ca} and the latency between the onset of the current and the appearance of Ca\textsuperscript{2+} sparks is in sharp contrast to CICR ob-
served in heart cells, in which the latency of the release events (Wier et al., 1994; Cannell et al., 1995; Lopez-Lopez et al., 1995; Collier et al., 1999) closely follows the gating properties of individual L-type Ca\(^{2+}\) channels. The small number of Ca\(^{2+}\) spark sites evoked by \(I_{Ca}\) and the delay between L-type Ca\(^{2+}\) channel and RYR channel opening suggested a fundamentally different coupling process in smooth muscle. We hypothesized that, rather than sensing the local elevation of \([Ca^{2+}]_i\) in the vicinity of the Ca\(^{2+}\) channel, smooth muscle RYRs were not sensitive to the opening of individual channels, but required a global rise in \([Ca^{2+}]_i\).

To test this hypothesis, we first sought to determine whether activation of Ca\(^{2+}\) channels always lead to initiation of a Ca\(^{2+}\) spark.

As shown in Fig. 2, depolarizing voltage-clamp steps of short duration that initiated a calcium current, but little net calcium flux (\(J_{Ca^{2+}}\)), did not trigger Ca\(^{2+}\) sparks. When the duration of \(I_{Ca}\) was progressively increased, Ca\(^{2+}\) sparks were observed that occurred well after termination of the depolarizing step and did not propagate. Further lengthening the duration of \(I_{Ca}\) resulted in Ca\(^{2+}\) sparks that occurred closer to the period of current flow and finally in Ca\(^{2+}\) wave propagation. Activation of \(I_{Ca}\) without Ca\(^{2+}\) release does not occur in cardiac myocytes; rather, evidence suggests that the opening of a single L-type Ca\(^{2+}\) channel activates a Ca\(^{2+}\) spark (Santana et al., 1996; Collier et al., 1999). This observation and the demonstration of triggered Ca\(^{2+}\) sparks after current cessation indicate that L-type Ca\(^{2+}\) channels are loosely coupled to RYR channels. That is, L-type Ca\(^{2+}\) channels can open without initiating Ca\(^{2+}\) sparks in smooth muscle, and the probability of Ca\(^{2+}\) sparks occurring after activation of \(I_{Ca}\) is a function of current duration and magnitude (see below). It is unlikely that Ca\(^{2+}\) sparks during short \(I_{Ca}\) were missed and that late-occurring Ca\(^{2+}\) sparks were spontaneous events unrelated to \(I_{Ca}\) since: (a) measurements were made in line-scan mode using an extended slit width (z resolution at half max = 2.5 \(\mu\)M) to minimize the possibility of missed events; (b) spontaneous events were uncommon in the absence of \(I_{Ca}\) but were always observed if \(J_{Ca^{2+}}\) was sufficient; (c) the latency of late Ca\(^{2+}\) sparks decreased as \(J_{Ca^{2+}}\) increased (Fig. 2); and (d) propagated Ca\(^{2+}\) waves, which were never observed spontaneously, often occurred after the termination of \(I_{Ca}\).

CICR Is a Function of the Magnitude of Ca\(^{2+}\) Influx, Not the Amplitude of \(I_{Ca}\)

As a further test of whether CICR in smooth muscle requires an increase in global \([Ca^{2+}]_i\), or results from the local response of RYR to the opening of single L-type Ca\(^{2+}\) channels, we designed experiments to maximize \(J_{Ca^{2+}}\) under conditions of low single-channel amplitude, and conversely to maximize the single channel current amplitude under conditions in which \(J_{Ca^{2+}}\) is low. As shown in Fig. 3 A, bladder myocytes were depolarized to
100 mV for 100 ms to open L-type Ca$^{2+}$ channels (without Ca$^{2+}$ ion permeation), and then to varied potentials to systematically alter the magnitude and duration of the Ca$^{2+}$ tail current. At more negative voltages (−70 mV) the magnitude of the instantaneous current (and the underlying single channel events) is relatively large (~0.3 pA; Rubart et al., 1996), but the current deactivation is rapid, resulting in little $I_{Ca}$. Ca$^{2+}$ sparks were never observed at clamp steps to −70 mV, indicating that brief channel openings of relatively large single channel amplitude do not trigger Ca$^{2+}$ sparks. Conversely, when cells were stepped to −10 mV, where the single channel current amplitude is approximately threefold lower, but $I_{Ca}$ is much larger due to a longer mean channel open time, CICR was routinely observed ($n = 6$). Thus, smooth muscle RYRs do not sense Ca$^{2+}$ in the microdomain of the L-type Ca$^{2+}$ channel, since large single channel events (which produce the highest local [Ca$^{2+}$]) do not evoke CICR in the absence of sufficient $I_{Ca}$. Rather, CICR occurs at low single channel amplitude if the net $I_{Ca}$ is sufficient. Moreover, the $I_{Ca}$ requirements for propagated Ca$^{2+}$ waves are incrementally greater than that required to achieve discrete Ca$^{2+}$ sparks. The estimated global [Ca$^{2+}$], achieved immediately before spark propagation was ~230 nM ($F/F_0 = 1.33 \pm 0.09$, $n = 8$). After depletion of sarcoplasmic reticulum (SR) Ca$^{2+}$ stores with caffeine (10 mM), tail current protocols did not result in Ca$^{2+}$ sparks or propagated Ca$^{2+}$ waves. As shown in Fig. 3 B, profiles from line-scan experiments obtained before and after caffeine exposure indicated that after SR depletion the tail protocol resulted in only a small rise in [Ca$^{2+}$], relative to that observed in control steps, despite the fact the equivalent $I_{Ca}$ obtained in both conditions (Fig. 3 C). In the experiment shown, the initial repolarization to −10 mV resulted in a rapid increase in local [Ca$^{2+}$], to greater than threefold baseline, whereas after caffeine application the increase was much smaller and slower.

The relationship between $I_{Ca}$ and Ca$^{2+}$ spark probability was examined quantitatively in voltage-clamp experiments. $I_{Ca}$ was calculated from the integrated $I_{Ca}$ in experiments such as that shown in Fig. 2, and the probability of a given $I_{Ca}$ evoking a Ca$^{2+}$ spark was determined. As shown in Fig. 4 A, the probability of an evoked Ca$^{2+}$ spark increased sharply with $I_{Ca}$. Fitting a generalized Boltzmann equation to the data, we determined that the flux at which the probability of evoking a Ca$^{2+}$ spark was 50% occurred with a $I_{Ca}$ of 4.0 fmol of Ca$^{2+}$. We also examined the latency to Ca$^{2+}$ spark in experiments at −30 and −10 mV (Fig. 4 B). Latencies were 32.0 ± 13.5 (n = 5) and 12.5 ± 2.7 (n = 10) in steps to −30 and −10 mV, respectively, significantly longer than observed in cardiac myocytes (~2 ms; Cannell et al., 1995). The voltage dependence of the latency is also consistent with a coupling mechanism related to net Ca$^{2+}$ flux. That is, since the integral of $I_{Ca}$ (and thus the flux of Ca$^{2+}$ ions) increases more rapidly with time at −10 mV than at −30 mV (due
mainly to the faster rate of current activation at $-10 \text{ mV}$, the same Ca$^{2+}$ flux is achieved in shorter time.

Uncoupling of CICR by Chelation of Cytosolic Ca$^{2+}$

Loose coupling between L-type Ca$^{2+}$ channels and RYR could result from an increase in the effective distance between these proteins, or could indicate a decreased affinity of the ryanodine receptor for Ca$^{2+}$ ions. The spatial separation between a single L-type Ca$^{2+}$ channel and RYR in cardiac cells has been estimated to be $<100 \text{ nm}$, based on the fact that high concentrations of mobile Ca$^{2+}$ buffers such as EGTA do not disrupt CICR (Collier and Berlin, 1999), using models of radial diffusion of Ca$^{2+}$ in a concentric shell (Klingauf and Neher, 1997). To examine the effective distance between L-type Ca$^{2+}$ channels and RYR, we sought to determine whether CICR is disrupted in smooth muscle cells in the presence of high concentrations of EGTA, and compared this result with experiments in heart cells recorded under identical conditions. As shown in Fig. 5, CICR was completely eliminated in smooth muscle cells dialyzed with 17 mM EGTA and 3 mM Fluo 4 ([Ca$^{2+}]_i$, clamped at 100 nM; n = 6), whereas CICR was not affected in rat heart cells in equivalent protocols. Thus, Ca$^{2+}$ ions entering through L-type Ca$^{2+}$ channels at a distance $>100 \text{ nm}$ from RYRs are required for CICR in smooth muscle. We further investigated whether RYR are able to sense local Ca$^{2+}$ entry by performing experiments in which we clamped [Ca$^{2+}]_i$, at 250 and 500 nM, still maintaining 20 mM mobile Ca$^{2+}$ buffer. We reasoned that under conditions of increased global [Ca$^{2+}]_i$, CICR might be triggered by a small additional increase in Ca$^{2+}$ from near (<100 nm) L-type Ca$^{2+}$ channels. However, CICR was not triggered in these experiments, suggesting that the functional distance between the L-type Ca$^{2+}$ channel and RYR is substantially greater than in sarcomeric muscle, despite light microscopic evidence of a close association between calcium channels and RYRs in bladder smooth muscle (Carrrington et al., 1995).

Link between Action Potential Discharge and CICR

To determine the relationship between action potential discharge and CICR under relatively physiological conditions, we examined CICR in fluo-4AM–loaded myocytes stimulated at varying frequencies. Rapid acquisition of confocal images during depolarizing stimuli indicated that Ca$^{2+}$ release does not occur with each depolarization (Fig. 6). Rather, local Ca$^{2+}$ sparks and propagated Ca$^{2+}$ waves depend on action potential frequency, revealing complex signal integration at the level of calcium release. Thus, at low stimulation frequencies (0.5 Hz), nonpropagated Ca$^{2+}$ sparks were observed only after accumulation of sufficient depolarizing stimuli (n = 4), and CICR took the form of discrete Ca$^{2+}$ sparks. At higher frequency stimulation (10 Hz), similar to the frequency of spontaneous action potentials reported in guinea-pig bladder myocytes (Klockner and Isenberg, 1985), Ca$^{2+}$ sparks were propagated as Ca$^{2+}$ waves and were repeatedly triggered, often from the same Ca$^{2+}$ release site (n = 6). The frequency of initiation of the propagated Ca$^{2+}$ waves was substantially lower than the stimulation frequency, resulting in an effective low-pass filter of high-frequency electrical signals.
DISCUSSION

In sarcomeric myocytes, tight coupling exists between gating of the L-type Ca\textsuperscript{2+} channel and RYR such that essentially every L-type Ca\textsuperscript{2+} channel gating event results in the opening of one or more RYR. This coupling derives either from a physical interaction between the proteins in skeletal myocytes (Tanabe et al., 1990; Nakai et al., 1998), or in cardiac myocytes from an interaction between the Ca\textsuperscript{2+} ions permeating the L-type Ca\textsuperscript{2+} channel and subsequently gating RYR. The latter coupling process appears to involve a local sensing of per-
meating Ca\textsuperscript{2+} ions within the microdomain of a single L-type Ca\textsuperscript{2+} channel, such that opening of a single L-type Ca\textsuperscript{2+} channel is sufficient to activate a Ca\textsuperscript{2+} spark, since: (a) the occurrence of Ca\textsuperscript{2+} sparks is stochastic with a voltage sensitivity equivalent to the gating behavior of the L-type Ca\textsuperscript{2+} channel (Cannell et al., 1995; Collier et al., 1999); (b) the latency to occurrence of a Ca\textsuperscript{2+} spark after depolarization is equivalent to the latency to opening of an individual L-type Ca\textsuperscript{2+} channel (Lopez-Lopez et al., 1995; Santana et al., 1996; Collier et al., 1999); and (c) the coupling process is not disrupted in the presence of high concentrations of mobile Ca\textsuperscript{2+} buffer (Collier and Berlin, 1999).

Despite the broad expression of L-type Ca\textsuperscript{2+} channels and RYR in many cell types, the existence and nature of CICR in nonsarcomeric cells, in which the distribution of L-type Ca\textsuperscript{2+} channels and RYR differs substantially from an orderly dyadic pattern, is not well established. In smooth muscle, evidence for CICR has been inferred from caffeine- and ryanodine-sensitive Ca\textsuperscript{2+} transients evoked upon I\textsubscript{Ca} activation (Zholos et al., 1992; Ganitkevich and Isenberg, 1992, 1995). More recently, confocal line-scan images acquired during flash photolysis of caged Ca\textsuperscript{2+} or peak I\textsubscript{Ca} activation gave rise to localized increases in Ca\textsuperscript{2+} (Arnaudeau et al., 1997) and 2-D confocal images acquired during step depolarizations demonstrated areas of increased fluorescence intensity or “hot spots” (Imaizumi et al., 1998). Direct examination of CICR and the mechanism underlying the coupling between L-type Ca\textsuperscript{2+} channels and RyR is lacking, however.

Using both 2-D and line-scan confocal modes, we examined CICR as a function of the amplitude and duration of I\textsubscript{Ca}, and provide direct visualization of CICR in x-y images obtained every 8.3 ms. A prominent feature of Ca\textsuperscript{2+} sparks activated by I\textsubscript{Ca} is the very low number of evoked Ca\textsuperscript{2+} sparks relative to that seen during depolarization of cardiac myocytes (Figs. 1 and 2). While the frequency of Ca\textsuperscript{2+} sparks may be a function of SR loading and modulatory factors (Porter et al., 1998), the number of sparks observed after activation of I\textsubscript{Ca} is dramatically lower than observed in cardiac cells and the ability to evoke Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} waves with caffeine application suggests that the low efficiency of CICR coupling cannot be explained by poorly loaded SR. Visualization of individual Ca\textsuperscript{2+} sparks in heart cells requires that the amplitude of I\textsubscript{Ca} be reduced (Cannell et al., 1995; Lopez-Lopez et al., 1995), while they were readily observed in bladder myocytes during voltage-clamp steps to activate I\textsubscript{Ca}. Moreover, in smooth muscle, individual Ca\textsuperscript{2+} sparks spread in the form of a propagated Ca\textsuperscript{2+} wave, whereas in cardiac myocytes depolarization appears to result in CICR from each dyad, with little required propagation. Our data indicate that both the initial Ca\textsuperscript{2+} spark and the subsequent propagation occurs through the gating of RyR, since both were eliminated in the presence of ryanodine, and neither were affected by dialysis with heparin (Fig. 1).

A second major feature of CICR in smooth muscle relates to the nature of the coupling between the channels. Rather than every opening of L-type Ca\textsuperscript{2+} channels activating a Ca\textsuperscript{2+} spark, Ca\textsuperscript{2+} spark activation in smooth muscle cells was only observed when I\textsubscript{Ca} was of sufficient magnitude or duration (Figs. 2 and 3). We term this relationship “loose coupling” since it differs dramatically from the obligate tight coupling that exists in heart cells. From experiments such as that shown in Figs. 2 and 3, it is clear that the opening of hundreds of L-type Ca\textsuperscript{2+} channels may not be sufficient to activate a Ca\textsuperscript{2+} spark if channel openings are not of sufficient duration. Experiments specifically designed to maximize single-channel amplitude and open-state probability, but minimize calcium flux, indicated that brief channel openings of maximal amplitude failed to activate Ca\textsuperscript{2+} sparks, whereas increasing the net Ca\textsuperscript{2+} flux at a lower single channel amplitude activated CICR. Thus, in smooth muscle, sufficient aggregate L-type Ca\textsuperscript{2+} channel activity is required to produce CICR in the form of discrete Ca\textsuperscript{2+} sparks, and further Ca\textsuperscript{2+} flux and increased global [Ca\textsuperscript{2+}], produces CICR in the form of propagated Ca\textsuperscript{2+} waves (Figs. 2 add 3). Taken together, these data indicate that RYRs appear to be coupled to L-type Ca\textsuperscript{2+} channels through a rise in global [Ca\textsuperscript{2+}], rather than local elevations near the channel. This finding was further supported by the disruption of coupling by high concentrations of mobile Ca\textsuperscript{2+} buffer, conditions that do not affect the coupling between L-type Ca\textsuperscript{2+} channels and RyR in cardiac myocytes (Fig. 5). While these data could be explained by an increase in the spacing distance between the sarcolemmal and sarcoplasmic reticulum Ca\textsuperscript{2+} channels (L-type and RYR), it is also possible that the relatively few sites at which Ca\textsuperscript{2+} sparks are repeatedly observed (Imaizumi et al., 1998; Gordienko et al., 1998) represent a concentration of RyR sufficient to generate a resolvable Ca\textsuperscript{2+} spark, and that close connections exist between L-type Ca\textsuperscript{2+} channels and individual RyR, as has been reported (Carrington et al., 1995), but that these do not occur in the density required to generate a resolvable Ca\textsuperscript{2+} spark.

What then is the likely physiological relevance of loose coupling? In skeletal and cardiac myocytes, each action potential results in a twitch response that derives from RyR-mediated calcium release, triggered by local signals in the microdomain of the L-type Ca\textsuperscript{2+} channels. Thus, every neural signal evoking a postsynaptic action potential is obligatorily linked to a mechanical response. Moreover, in addition to tight coupling, the signal gain is quite high, since each channel opening results in a Ca\textsuperscript{2+} spark (activation of several RyRs), the duration of which is longer than the L-type Ca\textsuperscript{2+} channel opening (Cannell et al., 1995). We show here that in
smooth muscle each action potential is not necessarily linked to CICR (Fig. 6), due to a coupling process that requires a sufficient rise in global \([\text{Ca}^{2+}]_i\). The uncoupling of \(\text{Ca}^{2+}\) release from the action potential introduces signals processing elements into the contractile response of smooth muscle. Features of "loose coupling" system are low gain (multiple L-type \(\text{Ca}^{2+}\) channels must open to produce \(\text{Ca}^{2+}\) sparks), discriminated responses (release takes the form of local \(\text{Ca}^{2+}\) sparks or globally propagated \(\text{Ca}^{2+}\) waves), and a marked lengthening of signal duration (\(\text{Ca}^{2+}\) waves last far longer than the action potential). Slight variations in this low gain, integrating system, such as a decrease in L-type \(\text{Ca}^{2+}\) channel density, likely underlies the fact that \(I_{\text{Ca}}\) does not appear to produce appreciable \(\text{Ca}^{2+}\) release in some smooth muscle cells, despite the presence of functional RyRs (Fleischmann et al., 1996; Kamishima and McCarron, 1996).

The dependence of \(\text{Ca}^{2+}\) release during E-C coupling on global increases in \([\text{Ca}^{2+}]_i\), contrasts with the local signaling that underlies relaxation. Features of "loose coupling" phenomenon are that this results in a functional uncoupling of a single action potential from \(\text{Ca}^{2+}\) release in smooth muscle cells. "Loose coupling" between L-type \(\text{Ca}^{2+}\) channels and RyR allows a functional uncoupling of the action potential and calcium release and provides a mechanism by which neural signals encoded at higher frequencies are transferred to slower mechanical responses.

We thank Drs. Clara Franzini-Armstrong, W.K. Chandler, and Joshua R. Berlin for helpful comments, and Mr. Mario Brenes for technical support.

Supported by National Institutes of Health (NIH) grants HL45239 and DK52620 (to M.I. Kotlikoff). M.L. Collier is a NIH postdoctoral fellow (T32-DK07708-06).

Submitted: 30 August 1999
Revised: 28 March 2000
Accepted: 28 March 2000

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