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Enhancement of Ca^{2+} Current Does Not Regulate the Speed of Depolarization-induced Ca^{2+} Propagation Wave in Rat Atrial Myocytes

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Abstract – In atrial myocytes, lacking t-tubules, Ca^{2+} current (I_{Ca})-initiated Ca^{2+} release at the peripheral junctional sites propagates into the interior of the cell by diffusion of Ca^{2+}. We have previously reported that time of activation of the central sites is independent of I_{Ca}. In the present study we have probed the effects of Bay K 8644 on Ca^{2+} propagation wave to the center of the myocyte using rapid 2-D confocal Ca^{2+} imaging in the rat atrial myocytes. Enhancement of I_{Ca} by Bay K 8644 accelerated the rate of peripheral Ca^{2+} release, but did not affect the speed of propagation of central release. In contrast, enhancement of I_{Ca} by intracellular cAMP reduced the magnitude of peripheral and central Ca^{2+} transients, but significantly accelerated the speed of central Ca^{2+} release. Our data suggest that the speed of central Ca^{2+} propagation triggered by I_{Ca} is not regulated by the magnitude of either I_{Ca} or local cytosolic Ca^{2+} releases.

Key words □ Atrial Ca^{2+} signaling, Ca^{2+} propagation, Mitochondria, Ca^{2+} current

INTRODUCTION

Contraction of mammalian cardiac myocytes is controlled by a sequence of events (E-C coupling) that include Ca^{2+} current (I_{Ca})-gated opening of Ca^{2+} release channels (Ryanodine receptors, RyRs) and the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Beukelmann & Wier, 1988; Näbauer *et al*., 1989; Niggli & Lederer, 1990; Cleemann & Morad, 1991). In specialized junctions (dyads and peripheral couplings), RyRs are found in close proximity (Sun *et al*., 1995) to dihydropyridine (DHP)-sensitive Ca^{2+} channels, providing privileged cross-signaling between the DHP receptors (DHPRs) and RyRs (Sham *et al*., 1995; Adachi-Akahane *et al.*, 1996). In atrial myocytes lacking t-tubules, RyRs are also found in non-junctional or corbular SR throughout the central region of the cell without associated DHPRs (Sommer & Jennings, 1992; Carl *et al*., 1995; Hatem *et al*., 1997; Mackenzie *et al*., 2001). The mode of gating and the contribution of this set of RyRs to atrial functions remain uncertain.

Confocal Ca^{2+} imaging in field-stimulated and current-clamped atrial cells suggests that Ca^{2+} release initiated at the periphery propagates into the interior of the myocytes (Berlin, 1995; Lipp *et al*., 1996; Hüser *et al*., 1996; Mackenzie *et al*., 2001; Kockskämper *et al*., 2001; Tanaka *et al*., 2001; Blatter *et al*., 2003). Confocal imaging in voltage-clamped atrial myocytes has demonstrated that the amount of central Ca^{2+} release is dependent on the magnitude of Ca^{2+} influx through the Ca^{2+} channels (Woo *et al*., 2002; Sheehan & Blatter, 2003) as well as on Ca^{2+} diffusion (Woo *et al*., 2002). It is normally expected that Ca^{2+} released from RyRs would convey the signal from the peripheral junction to deeper cellular sites via Ca^{2+}-induced Ca^{2+} release in a regenerative manner. Inherent in this idea is that larger I_{Ca}-gated peripheral release would diffuse faster to activate RyRs in more central sites. Experimental evidence in both field-stimulated and voltage-clamped atrial myocytes, however, indicates that the magnitude of Ca^{2+} release is smaller in more central regions (Berlin, 1995; Hüser *et al*., 1996; Mackenzie *et al*., 2001; Woo *et al*., 2002; Sheehan & Blatter, 2003) and that the speed of Ca^{2+} propagation wave from the cell periphery to the center is nearly constant over a wide voltage range or at varying I_{Ca} magnitudes (Woo *et al*., 2002; Sheehan & Blatter, 2003).

In the present study we have examined if and how I_{Ca} controls the propagation speed of central Ca^{2+} release during activation of I_{Ca}. Using rapid 2-D confocal Ca^{2+} imaging we monitored spatiotemporal profiles of local peripheral and central Ca^{2+}

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transients on activation of I_{Ca} in voltage-clamped rat atrial myocytes, by examining the effects of Ca^{2+} channel modulators.

METHODS

Single cell isolation

Rat atrial myocytes were enzymatically isolated from male Wistar rats, (WKY, 200-300 g) as described previously (Woo *et al*., 2002). Briefly, rats were deeply anesthetized with sodium pentobarbital (150 mg kg^{-1}, i.p.). The chest cavity was opened and hearts were excised. This surgical procedure was carried out in accordance with institutional ethical guidelines. The excised hearts were retrogradely perfused at 7 ml min^{-1} through the aorta, first for 5 min with Ca^{2+}-free Tyrode solution composed of (in mM): 137 NaCl, 5.4 KCl, 10 HEPES, 1 $MgCl_2$, 10 Glucose, pH 7.3, at 37°C and then with Ca^{2+}-free Tyrode solution containing collagenase (1.4 mg ml^{-1}, type A, Roche) and protease (0.16 mg ml^{-1}, type XIV, Sigma) for 12 min, finally with Tyrode solution containing 0.2 mM $CaCl_2$ for 6 min. The atria of the digested heart were then cut into several sections and subjected to gentle agitation to dissociate the cells. The freshly dissociated cells were stored at room temperature in Tyrode solution containing 0.2 mM $CaCl_2$.

Whole-cell patch-clamp

Myocytes were whole-cell clamped (Hamill *et al*., 1981) with patch pipettes (tip resistance 2.5-3.5 MΩ) and dialyzed with a Cs^+-rich solution (see below) containing 0.5 mM fluo-3. Membrane currents were measured with a DAGAN (model 8900, Dagan Co., Minneapolis, MN) patch-clamp amplifier. Generation of voltage-clamp protocol and acquisition of data were carried out using pCLAMP software (version 5.5-1; Axon Instruments, Inc., Foster City, CA). The current signals were filtered at 10 KHz before digitalization and storage.

Two-dimensional confocal imaging and image analysis

Cells were loaded with the Ca^{2+} indicator dye fluo-3 via the patch pipette (see below) and were imaged using a Noran Odyssey XL rapid 2-D laser scanning confocal microscopy system (Noran Instruments, Madison, WI, USA) attached to a Zeiss Axiovert TV135 inverted microscope fitted with a × 40 water-immersion objective lens (Zeiss, 440052 C-Apochromat, NA 1.2) (Woo *et al*., 2002). The excitation wavelength of the argon ion laser was set to 488 nm (Omnichrome), and fluorescence emission (wavelengths greater than 510 nm) was detected by a high-efficiency photomultiplier tube (Hamamatsu, Middlesex, NJ, USA). The *y* direction (vertical direction in the figures) was scanned at 240 Hz (Figs. 1-2). The data were acquired by the Intervision program in a workstation computer (IRIX-operating system, Indy, Silicon Graphics) and were analyzed with the Intervision and PC computer program written in Visual Basic 6.0 (Microsoft) (Woo *et al*., 2002).

Fluo-3 fluorescence measurement was carried out following 6–7 min after rupture of the membrane with the patch pipette. After this period of dialysis, the intracellular fluo-3 concentration was typically at equilibrium throughout the atrial cells (data not shown). Approximately 3 min after rupture of membrane conditioning voltage pulses from −90 to −10 mV were applied at 0.1 Hz to maintain the Ca^{2+} load of the SR. To reduce photobleaching of the dyes and possible phototoxic effects to the cells, the laser was electronically shuttered and triggered to open by the command of patch-clamp program (pCLAMP) only during the data acquisition period. The average resting fluorescence intensity (F_o) was calculated from several frames measured immediately before depolarization. Tracings of local Ca^{2+} transients were shown as the average local fluorescence of each frame normalized relative to the average resting fluorescence (F/F_o). This type of normalization was used to permit comparison of results from different cells. To measure local Ca^{2+} transients, confocal images were arbitrarily divided into four domains from cell periphery to center as previously descried (Woo *et al*., 2002). The area up <2 μm immediately underneath the cell membrane was denoted as the peripheral region.

Solutions and statistics

The solution used for cellular equilibrium and formation of the gigaseal contained (in mM) 137 NaCl, 5.4 KCl, 2 $CaCl_2$, 10 HEPES, 1 $MgCl_2$, 10 Glucose, buffered to pH 7.4 with NaOH. Patch pipettes were filled with solutions containing (in mM) 110 CsOH, 110 Aspartic acid, 5 NaCl, 20 TEA-Cl, 20 HEPES, 5 Mg-ATP, 0.5 K_5fluo-3 (Molecular Probes Inc.), with the pH adjusted to 7.2 with CsOH. At 3 min following rupture of the membrane, myocytes were superfused with K^+-free Tyrode solution containing 30 μM tetrodotoxin (Sigma) to eliminate K^+ and Na^+ currents. Drugs were dissolved in the external experimental solutions, and applied rapidly using a concentration-clamp device (Cleemann & Morad, 1991). All the salts used to make Tyrode and internal solutions were purchased from Sigma. All experiments were carried out at room temperature (22–24°C). Numerical results are given as means±SEM (*n*=), where SEM is the standard error of the mean and *n* is the

number of cells. Statistical comparisons were carried out using Student's *t*-test. Differences were considered to be statistically significant to a level of $P<0.05$.

Precautions

Before a confocal plane was fixed for measurements, whole cell images were routinely monitored in vertical (z-) direction from top to bottom of a cell (average thickness: 11.1±0.72 μm, n=13) to determine the middle of the cell (defined as the image with sharp edges and most prominent nucleus). Currents were neither leak nor capacitance subtracted (to obtain high quality data and to control for myocyte viability), but were series resistance (1.5-3 times the pipette resistance) compensated. Since imaging of the myocytes with a two-dimensional imaging required 6-7 min of dye dialysis, only cells with low leak current and clear edges were included in the final analysis of the results.

RESULTS

We have previously shown that propagation speed of central Ca^{2+} release is independent of I_{Ca} and voltage (Woo *et al.*, 2002). To further examine this effect we tested if modulators of I_{Ca} also affect the speed and magnitude of central Ca^{2+} release. Figure 1 shows the effects of intracellular dialysis of cAMP (200 μM) on I_{Ca}-gated local Ca^{2+} releases in rat atrial myo-

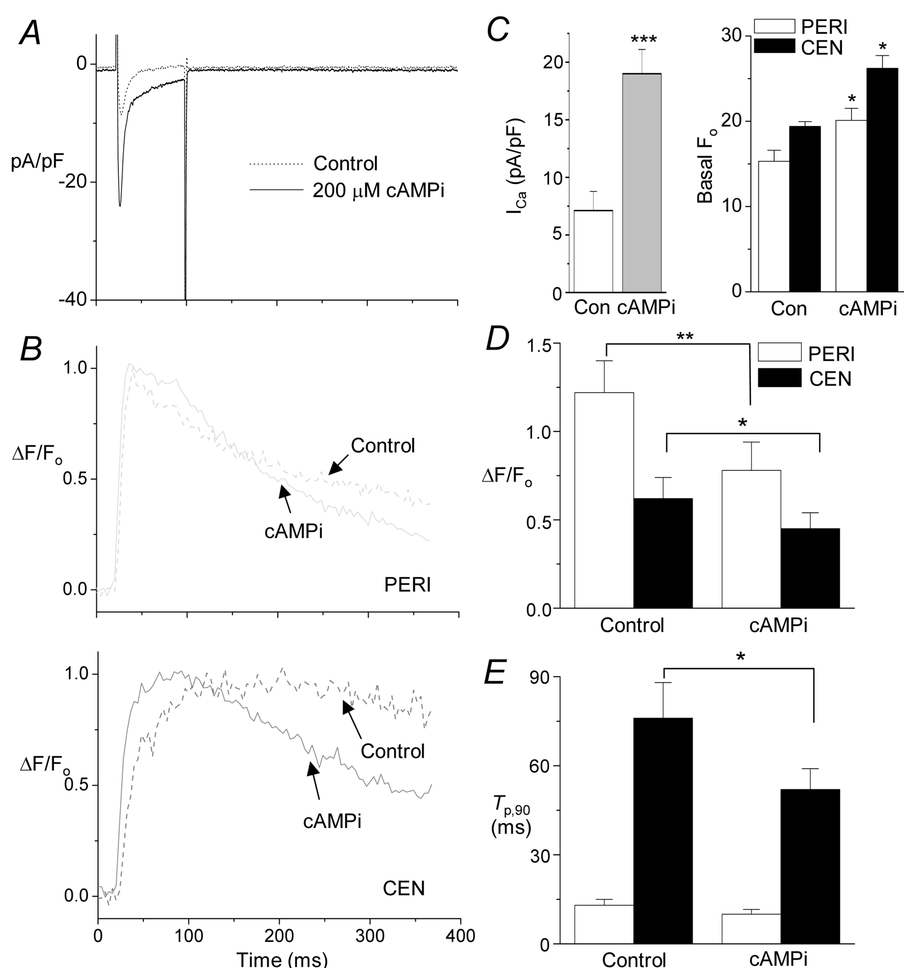

Fig. 1. Effect of intracellular cAMP on I_{Ca}-gated local Ca^{2+} releases. *A*, comparison of I_{Ca} measured in atrial cells dialyzed with 0 and 200 μM cAMP containing internal solution. *B*, peak-normalized peripheral (*PERI*; upper panel) and central (*CEN*; lower panel) Ca^{2+} transients measured in the presence and absence of internal cAMP (200 μM). Comparisons of means of magnitudes of I_{Ca} (*C*, *left* graph) and local Ca^{2+} transients (*D*) show that cAMP enhances I_{Ca} but decreases peripheral (*PERI*) and central (*CEN*) Ca^{2+} transients (Control, *n*=22; cAMP, *n*=9). *Right* graph in the panel *C* shows mean changes in the basal F_o by cAMP. *E*, comparison of mean $T_{p,90}$ of peripheral and central Ca^{2+} transients in the control cells (*n*=22) and in cells dialyzed with cAMP (*n*=9).
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. corresponding control.

cytes. Although I_{Ca} was enhanced by ~2.5 fold compared to control myocytes in 6-7 min cAMP-dialyzed myocytes (Fig. 1*A* & 1*C*), I_{Ca}-triggered Ca^{2+} transients in the cell periphery and center, were surprisingly smaller than in control myocytes (Fig. 1*D*). Note that basal Ca^{2+} fluorescence signal (F_o) was slightly higher both in the periphery and center of the cAMP-dialyzed myocytes (Fig. 1*C*, right panel; periphery: $P<0.05$; center: $P<0.05$; control, $n=22$; cAMP, $n=9$). Interestingly, the time-to-90% of peak ($T_{p,90}$) of the central Ca^{2+} transients, but not the peripheral Ca^{2+} transients, was significantly reduced by the cAMP ($n=9$, Fig. 1*B* & 1*E*) consistent with significant acceleration of the propagation speed of central Ca^{2+} release (distance/[$T_{p,90}$ of central transient $-$ $T_{p,90}$ of peripheral transient]; Woo *et al.*, 2002) by cAMP (control, 152 ± 14 μm s^{-1}, $n=20$; cAMP,

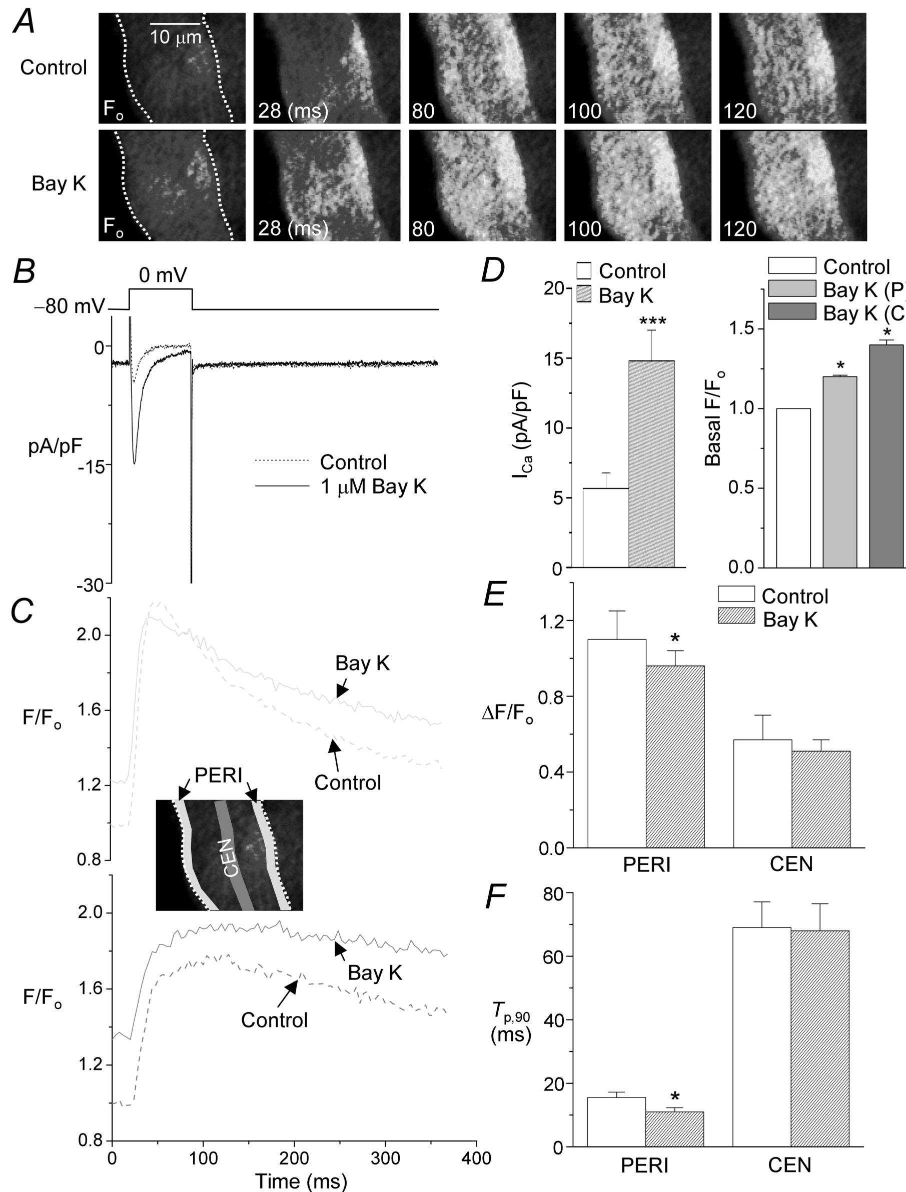

Fig. 2. Effect of Bay K 8644 on the I_{Ca}-gated local Ca^{2+} release in atrial myocytes. *A*, series of confocal Ca^{2+} images recorded during I_{Ca} in fluo-3 dialyzed rat atrial myocyte in the absence (control) and presence of 1 μM Bay K 8644 (Bay K). I_{Ca} was activated by depolarization from −80 to 0 mV (*B*). *C*, peripheral (*upper* traces) and central (*lower* traces) Ca^{2+} transients were measured from the local domains (see *inset*; periphery, *PERI*: center, *CEN*) of confocal images shown in the panel *A*. Comparison of means of peak I_{Ca} (*D, left*) and local Ca^{2+} transients (*E*) shows that Bay K enhances I_{Ca} but decreases peripheral Ca^{2+} releases. *Right* graph in the panel *D* shows mean changes in the basal F/F_o by Bay K. *F*, comparison of mean time-to-90% of peak ($T_{p,90}$) of peripheral and central Ca^{2+} transients in the control condition and after application of Bay K. $^{*}P<0.05$, $^{***}P<0.001$ vs. control ($n=5$).

228±21 μm s^{-1}, n=9, P<0.001). cAMP also accelerated the decay of Ca^{2+} transients both in the periphery (*decay time constant* [τ, ms]: control, 150±7.1, n=20; cAMP, 131±3.3, n=9, P<0.05) and center of myocyte (*slope* of decline [$-10^{-3}\cdot(F/F_o)$ ms^{-3}]: control, 1.2±0.3, n=22 *vs*. cAMP, 2.2±0.5, n=9, P<0.01; Fig. 1*B*), consistent with the enhanced activity of the SR Ca^{2+} pump by protein kinase A-dependent phosphorylation (Sham *et al*., 1991; Colyer, 1993).

Figure 2 shows that ~2.5 fold enhancement of I_{Ca} by Bay K 8644 (Bay K, 1 μM), an agonist of the L-type Ca^{2+} channels (Hess *et al*., 1984) (Fig. 2*B* & 2*D*), had little or no effect on the amplitude of central Ca^{2+} transients even though it decreased the magnitude of peripheral Ca^{2+} transients ($\Delta F/F_o$) (Fig. 2*E*) (Fig. 2*E*). The decay of peripheral and central Ca^{2+} transients was delayed by Bay K (periphery: τ, control, 151±8.0 ms *vs*. Bay K, 189±10 ms, n=5, P<0.05; center: *slope* [$-10^{-3}\cdot(F/F_o)$ ms^{-1}], control, 1.2±0.2, *vs*. Bay K, 0.7±0.1, n=5, P<0.05; Fig. 2*B*). Bay K also increased basal Ca^{2+} levels (F/F_o) in the periphery and center of myocytes (Fig. 2*D*, right panel; P<0.05, n=5). The time-to-90% of peak ($T_{p,90}$) of peripheral, but not the central Ca^{2+} transients was significantly decreased by Bay K (Fig. 2*F*). Thus the speed of I_{Ca}-gated Ca^{2+} propagation from the periphery to the center appeared to be slower in the presence of Bay K (control, 169±15 μm s^{-1}; Bay K, 157±12 μm s^{-1}, P< 0.05, n=5). These results indicate that neither the magnitude of I_{Ca} nor the magnitude of I_{Ca}-gated peripheral or central Ca^{2+} release correlated directly with the propagation speed of central Ca^{2+} release.

DISCUSSION

The major finding of the present study is that increases in I_{Ca} do not appear to increase the velocity of a Ca^{2+} propagation wave into the center. This finding is consistent with our previous report that the delayed component of central Ca^{2+} releases was independent of the magnitude of I_{Ca} and membrane voltages (Woo *et al*., 2002). Although the time-to-peak of peripheral Ca^{2+} transients was shortened, large increases of I_{Ca} by Bay K affected neither the speed of Ca^{2+} propagation wave nor the magnitude of central Ca^{2+} release (Fig. 2*D* & 2*E*), resulting in a slower propagation of Ca^{2+} release wave. The observed rapid activation of peripheral sites in the presence of Bay K (Fig. 2*F*) results most likely from a rapid recruitment of peripheral release sites secondary to the enhanced Ca^{2+} channel conductance (Kass, 1987). Although the reason for the decreased peripheral Ca^{2+} release in the presence of Bay K is unclear, it is plausible that the drug induced decrease in the gain of Ca^{2+}-induced Ca^{2+} release, and the rise in resting cytosolic Ca^{2+} are responsible for this effect (Adachi-Akahane *et al*., 1999; Satoh *et al*., 1998).

It is well known that cAMP-dependent activation of protein kinase A phosphorylates phospholamban and increases the activity of SR Ca^{2+} pump (Sham *et al*., 1991; Colyer, 1993), consistent with the rapid decay of Ca^{2+} transients of myocytes dialyzed with cAMP (Fig. 1*B*). The smaller Ca^{2+} transients, observed in the cAMP-dialyzed myocytes, are however somewhat surprising, considering that cAMP or isoproterenol augments Ca^{2+} transients and enhances tension in ventricular and atrial muscle (Morad *et al*., 1981; Callewaert *et al*., 1988; Mackenzie *et al*., 2004). The decrease in the magnitude of Ca^{2+} transients in myocytes dialyzed with a high concentration (200 μM) of cAMP may result from enhanced SR Ca^{2+} leak secondary to dissociation of RyRs from FKBP12.6 (Marx *et al*., 2000). Although it is tempting to suggest that basal Ca^{2+} rise of Fig. 1*C* is consistent with such a phosphorylation-mediated Ca^{2+} leak from the SR, most of other drugs employed in this study also appeared to have significant effects on basal Ca^{2+} levels. Despite the reduced magnitude of Ca^{2+} transients in cAMP-dialyzed myocytes, the propagation speed of central Ca^{2+} release continued to be accelerated. Although the underlying mechanism for this effect of cAMP needs further investigation, it is likely that it is mediated by an acceleration of SR Ca^{2+} uptake (Fig. 1*B; see more rapid decay of* Ca^{2+} *transients*) leading to rapid refilling of luminal compartment of central SR. In addition, it appears that the rate of Ca^{2+} removal following Ca^{2+}-induced Ca^{2+} release, is closely related to the speed of activation of more centrally located release sites.

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