T-Tubule Depolarization-induced SR Ca^{2+} Release Is Controlled by Dihydropyridine Receptor- and Ca^{2+}-dependent Mechanisms in Cell Homogenates from Rabbit Skeletal Muscle

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ABSTRACT   In vertebrate skeletal muscle, the voltage-dependent mechanism of rapid sarcoplasmic reticulum (SR) Ca^{2+} release, commonly referred to as excitation-contraction (EC) coupling, is believed to be mediated by physical interaction between the transverse (T)-tubule voltage-sensing dihydropyridine receptor (DHPR) and the SR ryanodine receptor (RyR)/Ca^{2+} release channel. In this study, differential T-tubule and SR membrane monovalent ion permeabilities were exploited with the use of an ion-replacement protocol to study T-tubule depolarization-induced SR $^{45}Ca^{2+}$ release from rabbit skeletal muscle whole-cell homogenates. Specificity of Ca^{2+} release was ascertained with the use of the DHPR antagonists D888, nifedipine and PN200-110. In the presence of the "slow" complexing Ca^{2+} buffer EGTA, homogenates exhibited T-tubule depolarization-induced Ca^{2+} release comprised of an initial rapid phase followed by a slower release phase. During the rapid phase, ~ 20% of the total sequestered Ca^{2+} (~ 30 nmol $^{45}Ca^{2+}$/mg protein), corresponding to 100% of the caffeine-sensitive Ca^{2+} pool, was released within 50 ms. Rapid release could be inhibited fourfold by D888. Addition to release media of the "fast" complexing Ca^{2+} buffer BAPTA, at concentrations ≥ 4 mM, nearly abolished rapid Ca^{2+} release, suggesting that most was Ca^{2+} dependent. Addition of millimolar concentrations of either Ca^{2+} or Mg^{2+} also greatly reduced rapid Ca^{2+} release. These results show that T-tubule depolarization-induced SR Ca^{2+} release from rabbit skeletal muscle homogenates is controlled by T-tubule membrane potential- and by Ca^{2+}-dependent mechanisms.

INTRODUCTION

In skeletal and cardiac muscle, excitation-contraction (EC) coupling refers to the process in which an action potential propagated along the surface membrane and its infoldings, the transverse (T)-tubules, triggers the rapid release of Ca^{2+} ions from an intracellular membrane compartment, the sarcoplasmic reticulum (SR). The resulting rise in myoplasmic Ca^{2+} allows contraction to occur. Coupling between T-tubule depolarization and SR Ca^{2+} release is believed to occur at junctional T-tubule SR regions, commonly referred to as triads, and is believed to be mediated by the T-tubule voltage-sensing/dihydropyridine receptor (DHPR) and SR ryanodine receptor (RyR)/Ca^{2+} release channel, respectively (for reviews, see Rios and Pizarro, 1991; Schneider, 1994). Based primarily on the observation that extracellular Ca^{2+} is required for EC coupling in cardiac (Beuckelmann and Wier, 1988; Fabiato, 1985) but not skeletal muscle (Armstrong, Bezanilla, and Horowicz, 1972; Brum, Stefani, and Rios, 1987; Miledi, Parker, and Zhu, 1984), different mechanisms of EC coupling have been proposed for these two tissues. In cardiac muscle, a small amount of extracellular Ca^{2+} entering the cell in response to surface membrane depolarization is believed to provide the initial trigger for SR Ca^{2+} release by activating the RyR/Ca^{2+} release channels. Extracellular Ca^{2+} entry is believed to be primarily mediated by the DHPR, which functions in this tissue as a voltage-sensitive Ca^{2+} channel. In contrast, in skeletal muscle the mechanical coupling model proposes that a T-tubule depolarization-induced conformational change in the DHPR is sensed by RyRs via a direct physical interaction between the two receptors, resulting in RyR/Ca^{2+} release channel opening (Rios and Pizarro, 1991; Schneider, 1994).

In addition to direct activation by DHPRs, the involvement of Ca^{2+} as an activator of SR Ca^{2+} release during EC coupling in skeletal muscle has been postulated. The most direct evidence for a role of Ca^{2+} in regulating SR Ca^{2+} release channel activity has been obtained from vesicle-ion flux and single-channel measurements. It has been shown that micromolar concentrations of Ca^{2+} activate whereas millimolar concentrations inhibit the Ca^{2+}-release channel (for review, see Meissner, 1994). However, because it is not known whether isolation of SR vesicles and Ca^{2+} release channels, leading to detachment of release channels from the T-tubule, affects channel behavior, the physiological significance of these studies has been questioned.

Unlike purified SR vesicle and RyR/Ca^{2+}-release channel preparations, intact triad complexes isolated from rabbit skeletal muscle have provided the opportunity to study regulation of RyR/Ca^{2+} release channels which have remained linked to the T-tubule membrane. T-tubule depolarization-induced SR Ca^{2+} release has been studied in vitro on a time scale of milliseconds (Ikemoto, Antoniu, and Meszaros, 1985) to seconds (Corbett, Bian, Wade, and Schneider, 1992). The question of whether or not the DHPR directly participates in mediating SR Ca^{2+} release was not addressed in these studies. Recently, an M_r 28,000 T-tubule membrane protein, rather than the DHPR, was implicated in mediating rapid SR Ca^{2+} release during EC coupling (Brandt, Caswell, Brunschwig, Kang, Antoniu, and Ikemoto, 1992).

Freeze-fracture morphological studies of toadfish swimbladder have suggested that only every other SR RyR is directly faced by a group of four T-tubule DHPRs (Block, Imagawa, Campbell, and Franzini-Armstrong, 1988). This observation led to specu-

lation that half the RyRs are linked to DHPRs and that during EC coupling only these DHPR-linked RyRs open in direct response to T-tubule depolarization. The resulting released Ca^{2+} then activates neighboring unlinked RyRs to amplify the Ca^{2+} release signal (Rios and Pizarro, 1991). Determination of DHPR/RyR ratios in rabbit and frog skeletal muscle have indicated that a significant population of RyRs may be unlinked to DHPRs in these species as well, consistent with the presence of two RyR activation mechanisms during EC coupling (Anderson, Cohn, and Meissner, 1994).

Evidence for a Ca^{2+} activation mechanism during EC coupling has been also obtained in studies with skinned, cut, and intact skeletal muscle fibers. Early reports of studies with skinned frog fiber preparations indicated that Ca^{2+} activation of SR Ca^{2+} release is significant only at nonphysiological levels of Ca^{2+} and Mg^{2+}, which raised doubts as to the physiological relevance of the Ca^{2+} activation mechanism (for review see Endo, 1977). Stephenson (1985), Volpe and Stephenson (1986), and Lamb and Stephenson (1990) showed the presence of a Ca^{2+}-sensitive component of depolarization-induced Ca^{2+} release in skinned frog fibers under more physiological conditions, but in these studies Ca^{2+} release was measured only on a slow time scale of seconds. An important role for Ca^{2+} in regulating SR Ca^{2+} release has been also obtained from studies in which microinjection into frog fibers of relatively high concentrations of fast complexing Ca^{2+} buffers such as BAPTA or fura-2 caused changes in the amount of Ca^{2+} released (Schneider, 1994).

Here we describe an in vitro assay we have developed for measuring T-tubule depolarization-induced SR Ca^{2+} release in cell homogenates from rabbit skeletal muscle. Our studies show that homogenates exhibit rapid T-tubule depolarization-induced SR Ca^{2+} release which is specifically inhibited by the DHPR antagonists D888, nifedipine and PN200-110, demonstrating that RyR/Ca^{2+} release channels have remained functionally coupled to DHPR/voltage sensors. Using these preparations and the "fast" Ca^{2+} buffer BAPTA, we find that depolarization-induced Ca^{2+} release is controlled by T-tubule depolarization-dependent and by Ca^{2+}-dependent mechanisms.

METHODS

Materials

$^{45}Ca^{2+}$ was purchased from ICN Biomedicals (Irvine, California). S-Devapamil[(−)-D888)] and PN200-110 were gifts from Drs. Grunhagen and Unger, Knoll AG (Ludwigshafen, Germany) and Sandoz Research Institute (East Hanover, NJ), respectively, and nifedipine was from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

Preparation of Muscle Homogenates and Membrane Fractions

Whole-muscle homogenates were prepared by homogenizing rabbit leg and back muscle in a Waring blender (two times 30 s, high setting) in 8 vol of 0.1 M NaCl, 5 mM Tris maleate, pH 6.8, 2 mM EDTA, 0.2 mM EGTA, and various protease inhibitors (0.2 mM PMSF, 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidine). A crude microsomal fraction was obtained from the homogenate as a 2,500–30,000 g pellet. After resuspension for 1 h in 50 ml buffer A (100 mM NaCl, 10 mM K PIPES, pH 6.8, 100 μM EGTA, 75 μM Ca^{2+}, 1 μM leupeptin, and 0.2 mM PMSF), the crude microsomal fraction was repelleted by centrifu-

gation at 100,000 g for 30 min, resuspended in buffer A, quick-frozen, and stored at $-75°C$. Membrane fractions isolated from homogenates, prepared as described above, were shown previously to contain [^{3}H]PN200-110 and [^{3}H]ryanodine-binding activities that comigrated to the lower region of 20–45% sucrose density gradients, suggesting the presence of triad complexes in these preparations (Anderson et al., 1994).

$^{45}Ca^{2+}$ Release Assay

Unless otherwise indicated, homogenates or crude membrane fractions were incubated in 4 vol of 100 mM NaCl, and 10 mM Na PIPES, pH 7.0, in the presence and absence of DHPR antagonists for 20 min at 23°C and then diluted with 10 vol of T-tubule polarizing SR $^{45}Ca^{2+}$ uptake medium containing 150 mM K gluconate, 10 mM K PIPES, pH 7.0, 50 nM valinomycin, 5 mM Na$_4$ATP, 5 mM MgCl$_2$, 50 μM EGTA, and 20 μM $^{45}Ca^{2+}$ (free Ca^{2+} 475 nM) in the presence and absence of DHPR antagonists. After incubation for 1 min at 37°C, samples (~5–10 μg protein) were placed on a 0.65-μm filter (type DA, Millipore Corp., Bedford, MA) under vacuum and prerinsed with 3 ml of T-tubule polarizing—$^{45}Ca^{2+}$ uptake medium containing 50 μM EGTA, but no Ca^{2+}. The membranes on the filters were then washed for 50–1,000 ms using a Biologic Rapid Filtration apparatus (Meylan, France) with either T-tubule polarizing Ca^{2+} release media (50 nM valimomycin and 150 mM K gluconate), also referred to as control media, or T-tubule depolarizing Ca^{2+} release media (100 nM valinomycin and either 150 mM Tris Cl or 150 mM Na gluconate). Ca^{2+} release media flow rates during rapid filtration were 3, 3, and 1 ml/s resulting in total wash volumes of 0.15, 0.6, and 1 ml, for the 50-, 200-, and 1,000-ms time points, respectively. Unless otherwise indicated, T-tubule depolarizing and control media contained 5 mM Na$_4$ATP, 5 mM MgCl$_2$, 10 mM PIPES, pH 7.0, and varying concentrations of Ca^{2+}, ruthenium red (RR), and EGTA or BAPTA (Na$^+$ salts) as indicated in the figure legends. The $^{45}Ca^{2+}$ radioactivity remaining with the samples on the filters was quantitated by liquid scintillation counting. The statistical significance of effects of different release media on $^{45}Ca^{2+}$ efflux was analyzed by paired t test. If $P < 0.05$, the difference was considered to be significant.

Determination of Free Ca^{2+} Concentrations

The free Ca^{2+} concentration of $^{45}Ca^{2+}$ release and uptake solutions was determined with a Ca^{2+}-selective electrode (World Precision Instruments, Inc., Sarasota, FL). Free Ca^{2+} concentrations of <1 μM were achieved by including in solutions the appropriate amounts of EGTA or BAPTA, and Ca^{2+} as determined using the stability constants and computer program published by Schoenmakers, Visser, Flik, and Theuvene (1992).

Determination of $^{45}Ca^{2+}$ Sequestered by Homogenates

Total Ca^{2+} present in homogenates was determined as follows. Homogenate protein was precipitated with 5% trichloroacetic acid and removed by centrifugation. After neutralization, the Ca^{2+} content of the supernatant was determined photometrically using the Ca^{2+} dye, antipyrylazo. The Ca^{2+} concentration of the homogenates was found to be ~0.7 mM, which corresponds to 6 μmol Ca^{2+} per g wet muscle or 325 nmol Ca^{2+} per mg protein. Next, homogenates were diluted with 4 vol of 100 mM NaCl, 10 mM Na PIPES, pH 7.0 buffer followed by dilution with 10 vol of $^{45}Ca^{2+}$ uptake medium which contained no ATP. After a 2-h incubation at 23°C, which has been previously shown to result in equilibration of $^{45}Ca^{2+}$ across SR vesicle membranes (Meissner, 1984), active $^{45}Ca^{2+}$ uptake for 1 min at 37°C was initiated by addition of ATP to the uptake medium. After placing the sample on a filter and rinsing with Ca^{2+}-release channel inhibitory medium containing 5 mM Mg^{2+}, 5 mM EGTA, and 20 μM RR, the total $^{45}Ca^{2+}$/mg protein sequestered was determined by liquid scintillation counting. This

amounted to ~30 nmol/mg, ~95% of which could be released within 1 min of exposure to release media containing the Ca^{2+} ionophore, A23187 (10 μg/ml).

Determination of Protein Concentration

Protein was determined by the Lowry method using bovine serum albumin as a standard.

RESULTS

The initial goal of this work was to use isolated membrane fractions enriched in T-tubule–SR junctional complexes (triads) for studying the mechanism of T-tubule mediated SR Ca^{2+} release. Although triad-enriched membrane preparations, obtained from sucrose density gradients as previously described (Anderson et al., 1994), were found to exhibit SR Ca^{2+} release upon exposure to T-tubule depolarization media, in our hands, the Ca^{2+} release observed was slow and only partially inhibited by DHPR antagonists (data not shown). Because whole-cell homogenates, prepared as described in Methods, were found to exhibit significant rapid T-tubule depolarization-induced SR Ca^{2+} release which could be effectively inhibited by DHPR antagonists, these preparations were used in most of our studies.

Rationale of Ca^{2+} Release Assay

Our procedure for inducing T-tubule membrane potential-dependent SR Ca^{2+} release is based on the different monovalent ion permeabilities of T-tubule and SR membranes. Except during depolarization when Na^+ and K^+ channels are activated, the T-tubule membrane is largely impermeable to ions (for review, see Sabbadini and Dahms, 1989). In contrast, the SR membrane is highly permeable to monovalent ions due to the presence of SR monovalent ion channels which are open under the conditions used in this study (for review, see Meissner, 1983). We have exploited these differences in membrane permeability by exposing membranes to ionic conditions that create membrane potential changes across the T-tubule, but not the SR, membrane (Fig. 1). Samples equilibrated in 100 mM NaCl (equilibration in 150 mM Na gluconate was found to produce essentially identical results) in the presence and absence of a DHPR antagonist, were diluted into T-tubule polarizing—$^{45}Ca^{2+}$ uptake medium which contained 150 mM K gluconate, 5 mM MgATP, 475 nM free $^{45}Ca^{2+}$, and the K^+-selective ionophore, valinomycin. In this medium, along with active SR $^{45}Ca^{2+}$ uptake via the SR Ca^{2+} pump, T-tubule membrane polarization comparable to that in resting muscle cells was expected to occur primarily as a result of valinomycin-mediated movement of K^+ down the concentration gradient into the T-tubule lumen (Dunn, 1989). Two other processes that may have contributed to T-tubule membrane polarization were the outward movement of Cl^- from the T-tubule lumen, and cycling of the electrogenic T-tubule membrane Na^+, K^+ ATPase. Unless otherwise indicated, T-tubule membrane depolarization was achieved by rapidly exposing the polarized membranes on a filter under vacuum to Na gluconate release media which contained valinomycin. Because of the low K^+ concentration, these media were expected to cause rapid collapse of the T-tubule membrane potential (depolarization), as a result of reversed K^+ movement out of the T-tubule lumen. Polarized membranes were exposed in parallel to K gluconate
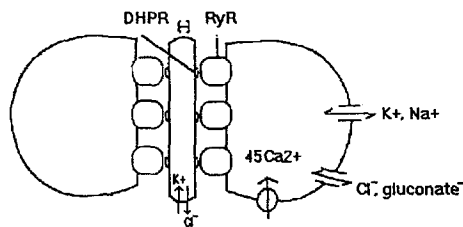
control (polarizing) release media. Because of the high K^+ concentration, these media were expected to leave the T-tubule membrane potential intact. Depolarization-induced Ca^{2+} release is defined as $\%^{45}Ca^{2+}$ release that could be inhibited by the DHPR antagonist, D888, in Na gluconate depolarizing media.
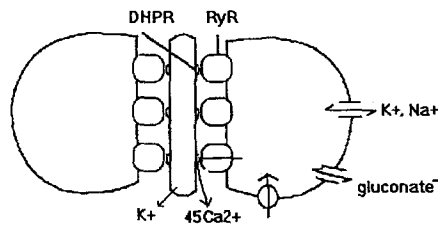
Ca²⁺ Release from Whole-Cell Homogenates

Ca^{2+} release from whole-cell homogenates was measured as described in Methods, using a phenylalkylamine (desmethoxyverapamil, D888) as a specific DHPR antagonist. Because D888 appears to bind most effectively to DHPRs when the T-tubule membrane is not polarized (Erdmann and Luttgau, 1989), homogenates were

FIGURE 1. Outline of $^{45}Ca^{2+}$ release assay. See text

initially incubated in the presence and absence of D888 before dilution into T-tubule–polarizing–$^{45}Ca^{2+}$ uptake medium. The effect of T-tubule membrane potential and D888 on SR Ca^{2+} release was investigated by exposing samples for different lengths of time to various depolarizing and control (polarizing) release media.

The total amount of $^{45}Ca^{2+}$ that could be released from SR vesicles containing Ca^{2+} release channels was determined by exposing homogenates to release media which contained 1 mM EGTA and 20 mM caffeine. Activation by caffeine is a distinguishing characteristic of the RyR/Ca^{2+} release channel, believed to occur via direct interaction of caffeine with the channel and which bypasses release channel

control by T-tubule membrane voltage sensors. Both depolarizing and control, caffeine-containing release media (Fig. 2 *A, circles and squares,* respectively) resulted in Ca^{2+} release which was comprised of an early rapid phase with 17% ± 3% (n = 5) of the sequestered $^{45}Ca^{2+}$ being released in the first 50 ms, followed by a much slower phase with an additional 15% ± 4% (n = 5) Ca^{2+} released by the end of 1 s. Control release media containing no caffeine resulted in comparatively slow Ca^{2+} release rates (Fig. 2 *A, triangles*). Paired t tests showed no significant differences between caffeine-induced release under depolarizing or control conditions in the presence and absence of D888 at each time point shown (Fig. 2 *A, circles and squares,* P > 0.05), demonstrating that caffeine-activated release channels overide voltage sensor control, and are not inhibited by D888. Because the caffeine release media used in these experiments have been shown previously to empty heavy SR Ca^{2+} stores in <1 s (Rousseau et al., 1988), the ~17% Ca^{2+} release observed in these media in the first 50 ms was assumed to represent the caffeine-sensitive SR Ca^{2+} pool. Sequestered Ca^{2+} not rapidly released in the presence of caffeine was assumed to be located primarily in SR vesicles lacking RyR/Ca^{2+} release channels.

Background, nonspecific Ca^{2+} release was determined by including in depolarizing and control media 5 mM Mg^{2+}, 5 mM EGTA, and 20 μM ruthenium red (RR), which in combination strongly inhibit the RyR/Ca^{2+} release channel (Fig. 2 *B*). With this condition, almost identical, slow time courses of $^{45}Ca^{2+}$ release (P > 0.05) were observed in depolarizing and control media in the presence and absence of D888. As described in METHODS, the total Ca^{2+} sequestered by homogenate fractions was determined to be ~30 nmol $^{45}Ca^{2+}$/mg protein (data not shown).

It has been well established that in the presence of 5 mM MgATP (0.7 mM free Mg^{2+}) micromolar concentrations of Ca^{2+} strongly activate the Ca^{2+} release channels in SR vesicle preparations, indicating a direct action of these ligands on the release channel (Meissner, 1994). To isolate T-tubule membrane mediated from Ca^{2+}-activated Ca^{2+} release the addition to release media of 50 μM EGTA was made. This concentration, taking into account the presence of 5–10 μM contaminating Ca^{2+}, buffers the free Ca^{2+} concentration close to the resting muscle cell level of 100–200 nM (Baylor, Chandler, and Marshall, 1983). Control Ca^{2+} release media containing 50 μM EGTA caused slow release rates comparable to those observed in the presence of 1 mM EGTA (Fig. 2 *A*). When these media were made depolarizing by the addition of Na gluconate, homogenates exhibited initially rapid release followed by a slower release phase (Fig. 2 *C, open circles*). During the rapid phase 17% ± 2% (n = 4) of the total sequestered Ca^{2+}, corresponding to 100% ± 11% (n = 4) of the caffeine-sensitive Ca^{2+} pool, was released within the first 50 ms. This rapid release represents a sixfold increase compared to the control condition and could be inhibited fourfold by D888 (Fig. 2 *C, filled circles*). The slow Ca^{2+} release phase, amounting to ~10% additional release by the end of 1 s, was not significantly inhibited by D888 (P = 0.20) and was therefore assumed to represent Ca^{2+} release from SR uncoupled from T-tubule membranes. We also tested two dihydropyridines (PN200-110, nifedipine) known to inhibit charge movement and EC coupling (Rios and Pizarro, 1991). Both were found to inhibit depolarization-induced Ca^{2+} release (Table I). These results indicate that homogenates exhibit SR $^{45}Ca^{2+}$ release that has remained functionally coupled to T-tubule voltage sensors.
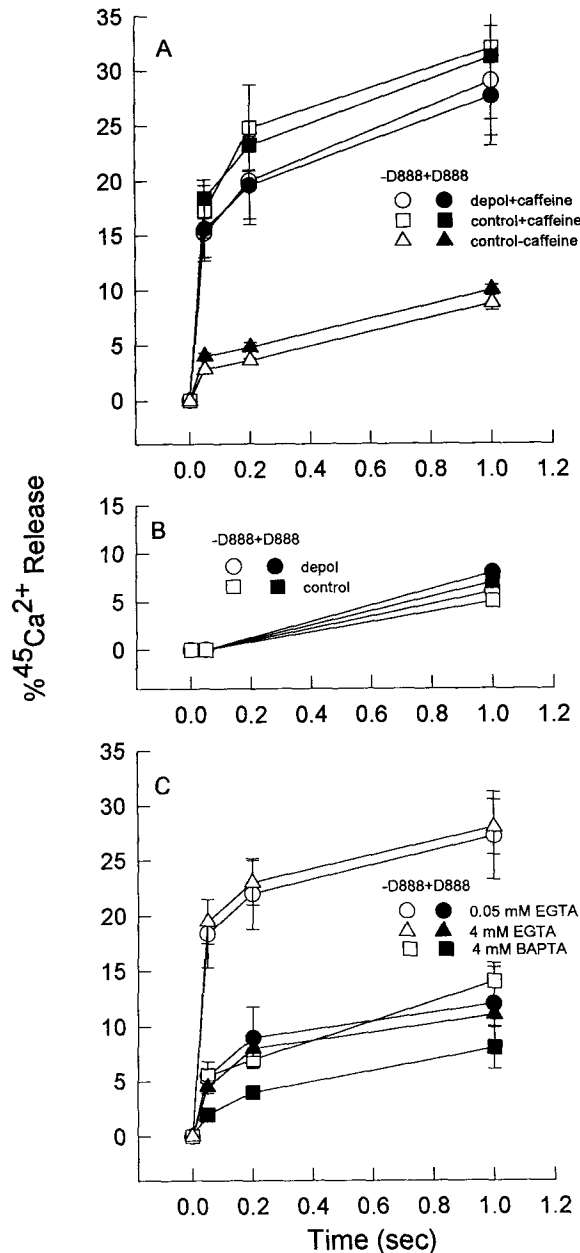
FIGURE 2. Muscle homogenates exhibit caffeine- and Na gluconate- T-tubule depolarization-induced SR Ca^{2+} release. (*A*) Caffeine-induced Ca^{2+} release is not inhibited by D888. $^{45}Ca^{2+}$ release was measured in the absence (*open symbols*) and presence (*filled symbols*) of 5 μM D888 in media containing 1 mM EGTA, 5 mM MgATP, and either (O, ●) 150 mM Na gluconate, 100 nM valinomycin, and 20 mM caffeine (depolarizing + caffeine), (□, ■) 150 mM K gluconate, 50 nM valinomycin, and 20 mM caffeine (control + caffeine), or (△, ▲) 150 mM K gluconate and 50 nM valinomycin (control minus caffeine). (*B*) $^{45}Ca^{2+}$ release was measured in the absence (*open symbols*) and presence (*filled symbols*) of 5 μM D888 in Ca^{2+} release channel inhibiting media containing 5 mM Mg^{2+}, 5 mM EGTA, 20 μM RR, and either (O, ●) 150 mM Na gluconate (depolarizing) or (□, ■) 150 mM K gluconate (control). (*C*) Na gluconate depolarization-induced Ca^{2+} release is inhibited by 5 μM D888 and by 4 mM BAPTA, but not by EGTA. $^{45}Ca^{2+}$ release was measured in the absence (*open symbols*) and presence (*filled symbols*) of 5 μM D888 in depolarizing media containing 150 mM Na gluconate, 5 mM MgATP, 100 nM valinomycin, and either (O, ●) 50 μM EGTA, (△, ▲) 4 mM EGTA, or (□, ■) 4 mM BAPTA. Data (mean ± SD) are the averages of four experiments carried out in triplicate.

In Fig. 3 it is shown that partial replacement of Na gluconate by K gluconate in depolarizing Ca^{2+} release media reduces rapid ^{45}Ca^{2+} release. Half maximal release was observed in the presence of 75 mM K$^+$. Reduction of the K$^+$ concentration from 150 to 15 mM, 1.5, or nominally 0 mM resulted in release of the caffeine-sensitive SR Ca^{2+} pool in 50 ms. Applying the Nernst equation and assuming that valinomycin renders T-tubule membranes selectively permeable to K$^+$, we calculate that a membrane potential change of 60 mV is effective in releasing the caffeine-sensitive SR Ca^{2+} pool.

The importance of Ca^{2+} during depolarization-induced Ca^{2+} release was investigated by adding millimolar concentrations of Ca^{2+} chelators, either EGTA or BAPTA, to release media. When the free Ca^{2+} concentration in depolarizing media was lowered to $<10^{-9}$ M by the addition of 4 mM EGTA, rapid depolarization-induced Ca^{2+} release occurred which was similar in rate and extent to that seen in the presence of 50 μM EGTA. Rapid Ca^{2+} release could be largely inhibited by D888 (Fig. 2 *C, triangles*). If the free Ca^{2+} concentration in depolarizing media was lowered to $<10^{-9}$ M by the addition of 4 mM BAPTA, instead of 4 mM EGTA, depolariza-

TABLE I

Homogenates Exhibit T-tubule-mediated SR Ca^{2+} Release Which Can Be Inhibited by Dihydropyridines

Addition to depolarizing release medium	%^{45}Ca^{2+} released in 50 ms	P
No drug	15 ± 5.2	
5 μM D888	2.4 ± 1.1	0.040
5 μM PN200-110	2.7 ± 1.1	0.034
5 μM nifedipine	5.8 ± 2.3	0.038

^{45}Ca^{2+} release was measured in the absence of any drug or in the presence of either 5 μM D888, 5 μM PN200-110, or 5 μM nifedipine in standard T-tubule depolarization Ca^{2+} release medium. Data (mean ± SD) are from three experiments carried out in duplicate.

tion-induced Ca^{2+} release occurring in the first 50 ms was reduced from 19 ± 3% to 6 ± 2% ($n = 4$) of the total sequestered Ca^{2+} (Fig. 2 *C, squares*). This release was inhibited two- to threefold by D888 ($P = 0.04$).

Our rationale for comparing the effects of EGTA vs BAPTA on depolarization-induced Ca^{2+} release and our interpretation of the contrasting results obtained with these two Ca^{2+} buffers is as follows. Although EGTA and BAPTA have similar K_Ds for Ca^{2+}, BAPTA has a 1000-fold higher association rate constant (Stern, 1992). Modeling has indicated that due to this difference in association rate constants, millimolar concentrations of BAPTA will be more effective at suppressing a rise in the Ca^{2+} concentration of release media during SR Ca^{2+} release when compared with EGTA (Stern, 1992). The use of EGTA and BAPTA therefore potentially allows the distinction to be made between the importance of Ca^{2+} at the time of T-tubule depolarization versus the importance of a rapidly rising Ca^{2+} phase after depolarization. The marked decrease in rapid depolarization-induced Ca^{2+} release observed in the presence of 4 mM BAPTA, compared with 4 mM EGTA, suggests that initially

released Ca^{2+} amplifies SR Ca^{2+} release, presumably by binding to the Ca^{2+} activation sites of RyR/Ca^{2+} release channels.

In previously reported studies with triad-enriched vesicle fractions (Ikemoto et al., 1985) and skinned fibers (Stephenson, 1985; Lamb and Stephenson, 1990), T-tubule depolarization was induced by changing the major anion in the sample medium from a relatively impermeant one to chloride ion. We found also that exposure of homogenates to depolarizing media which contained Tris Cl instead of Na gluconate resulted in T-tubule depolarization-induced rapid SR Ca^{2+} release which was inhibited by D888 and by 4 mM BAPTA. These data are summarized in Table II.

Compared in Fig. 4 are the amounts of Ca^{2+} released from homogenates after exposure to depolarizing and control release media containing $< 10^{-8}$–10^{-4} M free Ca^{2+}. The indicated free Ca^{2+} concentrations were obtained by adding 1 mM BAPTA
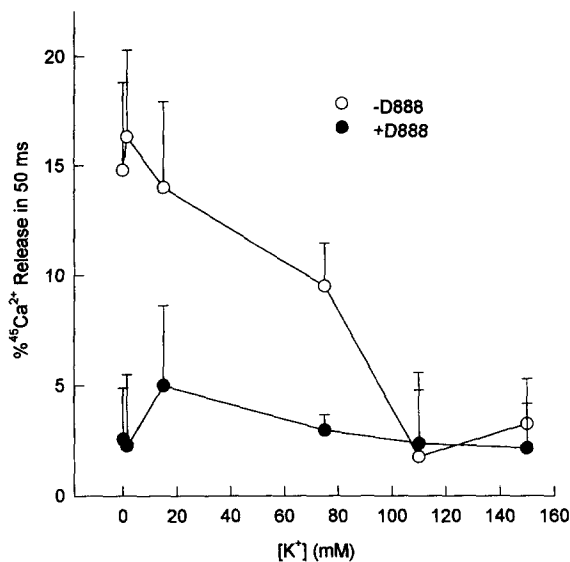

FIGURE 3. T-tubule depolarization-induced $^{45}Ca^{2+}$ release from homogenates is dependent on the K^+ concentration of depolarizing release media. $^{45}Ca^{2+}$ release was measured in the absence (○) and presence (●) of 5 μM D888 in Ca^{2+} release media containing 5 mM Na$_4$ATP, 5 mM MgCl$_2$, 50 μM EGTA (free Ca^{2+} 200 nM), 10 mM Na PIPES, pH 7.0, and varying concentrations of Na and K gluconate to result in the K^+ concentration indicated on the abcissa and a gluconate concentration which remained constant at 150 mM. Data (mean ± SD) are from three experiments carried out in duplicate.

and the appropriate amounts of Ca^{2+} to release media. The level of $^{45}Ca^{2+}$ release was dependent on free Ca^{2+} in both depolarizing and control release media, although the medium Ca^{2+} concentration yielding half-maximal activation was shifted from ~1.0 to ~0.07 μM, respectively. When the free Ca^{2+} concentration was 10–100 μM, the range reported from studies with purified vesicle and protein preparations to be optimal for direct Ca^{2+} activation of RyR/Ca^{2+} release channels (Meissner, 1994), the Ca^{2+} release induced by depolarizing and control media was not substantially different.

The depolarization-induced shift in Ca^{2+} sensitivity of Ca^{2+} release, shown in Fig. 4, can be interpreted in two ways. One possibility is that depolarization triggers a conformational change in the RyR/Ca^{2+} release channel, via mechanical coupling, which increases its sensitivity to activating Ca^{2+}. It is also possible that the Ca^{2+} buffer

TABLE II

Homogenates Exhibit T-tubule-mediated SR Ca²⁺ Release Induced by Cl⁻

Addition to Tris Cl release medium	%⁴⁵Ca²⁺ released in 50 ms		P
	−D888	+D888	
5 mM MgATP, 1 mM EGTA, 20 mM caffeine	17 ± 2	18 ± 2	0.4
5 mM Mg²⁺, 5 mM EGTA, 20 μM RR	0 ± 2	1 ± 2	0.8
1.5 mM Mg²⁺, 1.0 mM ATP, 50 μM EGTA	13 ± 1	4 ± 2	0.02
1.5 mM Mg²⁺, 1.0 mM ATP, 1 mM BAPTA	15 ± 2	5 ± 3	0.04
1.5 mM Mg²⁺, 1.0 mM ATP, 4 mM BAPTA	6 ± 1	3 ± 2	0.2

After ⁴⁵Ca²⁺ uptake and T-tubule polarization in the presence and absence of 5 μM D888 in standard uptake medium containing 150 mM K gluconate, homogenates were exposed to Ca²⁺ release media which contained 150 mM Tris Cl, pH 7.0, 100 nM valinomycin, and the indicated additions. Data (±SD) are averages of four experiments carried out in triplicate.

system used in this experiment (1 mM BAPTA) fails to sufficiently suppress the T-tubule depolarization-induced rise in Ca²⁺ concentration, such that Ca²⁺-induced amplification of Ca²⁺ release occurs. To distinguish between these two possibilities, depolarization-induced Ca²⁺ release was measured at a constant free Ca²⁺ concentration of 200 nM, but with varying levels of BAPTA. Table III shows that depolarization-induced Ca²⁺ release decreased greatly, from 20% of the total sequestered Ca²⁺ released in 50 ms to 2% released, as the uncomplexed BAPTA concentration was increased from 0.3 to 7.0 mM. In contrast, when 10 mM EGTA was added to release media no significant inhibition of T-tubule depolarization-induced rapid SR Ca²⁺ release was observed (Table III). It was therefore concluded that the shift in Ca²⁺ sensitivity of Ca²⁺ release resulted not simply from a change in the Ca²⁺ sensitivity of the release channel, but at least partly from Ca²⁺-induced amplification of Ca²⁺
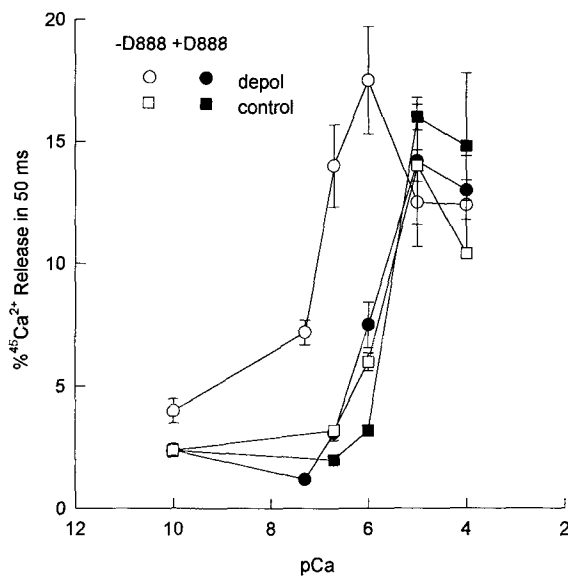

FIGURE 4. Ca²⁺ sensitivity of rapid SR Ca²⁺ release from homogenates under depolarizing and polarizing conditions. ⁴⁵Ca²⁺ release was measured in the absence (*open symbols*) and presence (*filled symbols*) of 5 μM D888 in media containing 5 mM MgATP and the indicated free Ca²⁺ concentration (buffered by 1 mM BAPTA), and either (○, ●) 150 mM Na gluconate (depolarizing) or (□, ■) 150 mM K gluconate (control). Data (mean ± SD) are averages of four experiments carried out in triplicate.

TABLE III

Rapid T-tubule Depolarization-induced SR Ca^{2+} Release Is Inhibited by Millimolar Concentrations of Either BAPTA, Ca^{2+}, or Mg^{2+}, and by Deletion of MgATP from the Release Medium

Addition to depolarizing medium	Free [Ca^{2+}]	Uncomplexed [Ca^{2+} buffer]	%45 Ca^{2+} released in 50 ms		P
			−D888	+D888	
	μM	mM			
1. 5 mM Mg^{2+}, 5 mM ATP, 50 μM EGTA	0.2	<0.03	20 ± 3	5 ± 2	0.007
2. 5 mM Mg^{2+}, 5 mM ATP, 1 mM BAPTA	0.2	0.3	20 ± 2	5 ± 0.5	0.005
3. 5 mM Mg^{2+}, 5 mM ATP, 4 mM BAPTA	0.2	2.0	11 ± 1.5	3 ± 1	0.009
4. 5 mM Mg^{2+}, 5 mM ATP, 10 mM BAPTA	0.2	7.0	2 ± 2	1 ± 2	0.6
5. 5 mM Mg^{2+}, 5 mM ATP, 10 mM EGTA	<0.001	10.0	30 ± 5	3 ± 2	0.03
6. 5 mM Mg^{2+}, 5 mM ATP, 1 mM Ca^{2+}	337	0	5 ± 4	3 ± 3	0.2
7. 6 mM Mg^{2+}, 5 mM ATP, 50 μM EGTA	0.2	<0.03	4 ± 3	2 ± 2	0.5
8. 0 mM Mg^{2+}, 0 mM ATP, 50 μM EGTA	0.2	<0.03	6 ± 5	1 ± 2	0.1

[45]Ca^{2+} release was measured in the absence and presence of 5 μM D888 in T-tubule depolarizing Ca^{2+} release media containing 150 mM Na gluconate, 50 nM valinomycin and the indicated additions. In the first four entries, the free Ca^{2+} concentration was kept constant at 200 nM while the Ca^{2+} buffering capacity of release media was varied. In the fifth entry, 10 mM EGTA was included which resulted in a nominal free Ca^{2+} concentration of <0.001 μM in the release medium. In the sixth entry, 1 mM Ca^{2+} was added in the absence of a specific Ca^{2+} chelator. Because of partial complexation by ATP, the free Ca^{2+} concentration in this case was 337 μM. In the seventh entry, the Mg^{2+} concentration was raised from 5 to 6 mM, which resulted in an increase of free Mg^{2+} concentration from 0.7 to 1.5 mM. In the eighth entry, the depolarizing medium contained no Mg^{2+} or ATP. Data (means ± SD) are from three experiments carried out in duplicate.
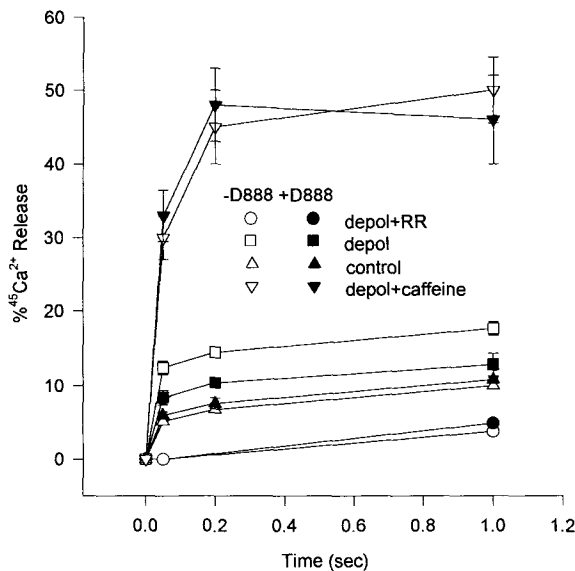

FIGURE 5. Crude membrane fractions exhibit T-tubule depolarization-induced SR Ca^{2+} release which is inhibited by D888. [45]Ca^{2+} release was measured in the absence (*open symbols*) and presence (*filled symbols*) of 5 μM D888 in depolarizing (150 Na gluconate) or control (150 mM K gluconate) media containing 5 mM MgATP and 50 μM EGTA (□, ■, △, ▲), or in media containing 5 mM MgATP, 1 mM EGTA and 20 mM caffeine (▽, ▼). [45]Ca^{2+} release was also measured in (○, ●) Ca^{2+} release channel inhibiting media containing 150 mM Na gluconate, 5 mM Mg^{2+}, 5 mM EGTA, and 20 μM RR. Data (mean ± SD) are the averages of three experiments carried out in triplicate.

release. The uncomplexed form of BAPTA was recently reported to directly inhibit IP_3 binding to the IP_3 receptor/Ca^{2+} channel (Richardson and Taylor, 1993). We found that caffeine-activated Ca^{2+} release was not inhibited by 10 mM uncomplexed BAPTA (data not shown), arguing against direct BAPTA inhibition of the release channel. However, we cannot rule out effects of BAPTA on the voltage sensor.

The effects of millimolar Ca^{2+} on rapid T-tubule depolarization-induced Ca^{2+} release, and of Mg^{2+} in the presence of EGTA and 200 nM free Ca^{2+}, were examined. As shown in Table III, 1 mM Ca^{2+} (free Ca^{2+}, 337 μM) or an increase in Mg^{2+} concentration from 5 to 6 mM (free Mg^{2+} from 0.7 to 1.5 mM) resulted in nearly complete inhibition of D888-sensitive Ca^{2+} release, suggesting that T-tubule depolarization-induced Ca^{2+} release is inhibited both by high concentrations of BAPTA and by millimolar concentrations of either Ca^{2+} or Mg^{2+}. Removal of MgATP from depolarizing Ca^{2+} release media similarly inhibited $^{45}Ca^{2+}$ release (Table III).

Ca^{2+} Release from Crude Membrane Fractions

Crude membrane fractions were also found to exhibit rapid T-tubule depolarization-induced Ca^{2+} release, with 13 ± 4% release observed in 50 ms (Fig. 5). However, this release represented only ~25% of the caffeine-sensitive pool and corresponded to only a 1.5-fold increase in release over the D888 control. The reduced T-tubule depolarization-induced Ca^{2+} release signal observed with crude membrane fractions, when compared with homogenates, indicates substantially less coupling in these preparations.

DISCUSSION

We describe in this report an in vitro assay for studying the mechanism of T-tubule depolarization-induced SR Ca^{2+} release from skeletal muscle whole-cell homogenates. Whole-cell homogenates from rabbit skeletal muscle sequestered ~30 nmol $^{45}Ca^{2+}$/mg protein, about one-fifth of which could be released in 50 ms by caffeine and was thus defined as the caffeine-sensitive SR Ca^{2+} pool. Upon exposure to T-tubule depolarizing conditions in the presence of EGTA, homogenates exhibited a rapid Ca^{2+} release phase followed by a slower release phase. During the rapid release phase, an amount corresponding to the caffeine-sensitive SR Ca^{2+} pool was released in 50 ms. The rapid release which represented a sixfold increase over the control could be largely inhibited by the DHPR antagonist, D888. This drug has been suggested to inhibit EC coupling by stabilizing the inactivated state of the voltage sensor (i.e., presumably the DHPR) (Erdman and Luttgau, 1989). Studies with D600, a highly related drug, have indicated that in voltage-clamped skeletal muscle fibers phenylalkylamines block SR Ca^{2+} release in a use-dependent manner without fully inhibiting charge movement (Pizarro, Brum, Fill, Fitts, Rodrigruez, Uribe, and Rios, 1988). A subsequent study suggested that D600 causes voltage-dependent inhibition of charge movements that control SR Ca^{2+} release (Feldmeyer, Melzer, and Pohl, 1990). In our studies, D888 appeared to specifically inhibit T-tubule depolarization-induced SR Ca^{2+} release, because direct activation of SR Ca^{2+} release by caffeine (Fig. 2 and Table II) or by medium Ca^{2+} (Fig. 4) was not significantly inhibited by the drug. Inhibition by D888 also showed that T-tubule depolarization-induced SR Ca^{2+}

release was not due to osmotic effects of depolarization media (Meissner, 1983). The slow Ca^{2+} release phase, which amounted to release of an additional 10–20% after 1 s of exposure, had a slope similar to that under control (nondepolarizing) conditions and was thus assumed to represent Ca^{2+} release not mediated by the DHPR. We conclude that homogenate preparations contain RyRs which have remained functionally coupled to DHPRs and should thus be useful for studying parameters believed to affect EC coupling.

We have used this preparation to investigate the importance of Ca^{2+} as an activator of SR Ca^{2+} release during EC coupling, by comparing depolarization-induced Ca^{2+} release in the absence and presence of millimolar concentrations of EGTA or BAPTA, which represent "slow" and "fast" complexing Ca^{2+} chelators, respectively. In the presence of 4 mM EGTA (nominal free Ca^{2+} concentration of $< 10^{-9}$ M), depolarization-induced Ca^{2+} release occurring in 50 ms was similar to that observed in 50 μM EGTA (nominal free Ca^{2+} concentration of 200 nM), and amounted to $\sim 20\%$ of the total sequestered Ca^{2+}. However, when a free Ca^{2+} concentration of 200 nM was established with 4 or 10 mM BAPTA, the depolarization-induced Ca^{2+} release occurring in 50 ms corresponded to only 11 and 2%, respectively (Table III). We conclude that, in addition to T-tubule depolarization, amplification of SR Ca^{2+} release by initially released Ca^{2+} appears to be important during EC coupling. Furthermore, our studies indicate that the Ca^{2+}-dependent component is large relative to the T-tubule–dependent component, although we cannot rule out the possibility that our preparations exhibit a low level of voltage-activated SR Ca^{2+} release because of partial loss of coupling or because of suboptimally activating T-tubule depolarization produced by depolarizing release media. We also found that rapid T-tubule depolarization–induced Ca^{2+} release was significantly inhibited by 1 mM Ca^{2+}, consistent with the presence of Ca^{2+}-dependent inactivation of SR Ca^{2+} release during EC coupling. Similar inhibition was observed when free Mg^{2+} was increased from 0.7 to 1.5 mM in release medium containing 50 μM EGTA. This observation may be of physiological significance, although the exact free Mg^{2+} concentration in resting muscle is not well known. The free Mg^{2+} concentration in resting muscle has been estimated to range from 0.5 to 1.0 mM, although concentrations as low as 0.2 mM and as high as 6 mM have been reported (Gupta and Moore, 1980; Baylor, Chandler, and Marshall, 1982). Deletion of MgATP from release media also inhibited rapid release (Table III). These results are consistent with studies with skinned fibers of toad (Lamb and Stephenson, 1991) and with isolated rabbit skeletal SR vesicles (Meissner, 1994) which have also shown a requirement of ATP for rapid SR Ca^{2+} release and inhibition by Ca^{2+} or Mg^{2+} when these exceeded millimolar concentrations. The effects of Mg^{2+} at concentrations < 0.7 mM were not tested in this study because it is well known that a decrease in free Mg^{2+} concentration in the presence of ATP directly activates the rabbit skeletal muscle SR Ca^{2+} release channel (Meissner, 1994).

Triad-enriched membrane fractions isolated from rabbit skeletal muscle have been previously reported to exhibit T-tubule mediated SR Ca^{2+} release in vitro. T-tubule depolarization induced by rapid ion replacement was reported by Ikemoto et al. (1985) to result in three phases of Ca^{2+} release: (a) a lag phase; (b) the rapid release of a small amount of Ca^{2+} with a rate constant of $k = 100$ s^{-1}; and (c) the slow release

of a larger amount of Ca^{2+} with k ~ 1 s^{-1}. DHPR antagonists and antibodies have implicated the DHPR and the SR integral membrane protein, triadin, in mediating the slow release phase while a 28-kD protein, not part of the DHPR, was implicated in mediating the rapid release phase (Brandt et al., 1992). These results challenge the idea of DHPR-mediated control of rapid SR Ca^{2+} release during EC coupling. Corbett et al. (1992) have also described T-tubule depolarization–induced Ca^{2+} release from triads present in triad-enriched membrane fractions. However, in this study, Ca^{2+} release was measured only on a slow time scale of seconds and the question of participation of the DHPR in mediating SR Ca^{2+} release was not addressed. In neither of the above described in vitro studies was the role of Ca^{2+} in amplifying SR Ca^{2+} release during EC coupling specifically addressed.

Studies with muscle fibers have shown that the extent of SR Ca^{2+} release is dependent on the extent of T-tubule membrane depolarization, with maximal release occurring upon depolarization from ~ −90 to 0 mV (Miledi et al., 1984). In our studies, we could not directly correlate the extent of ^{45}Ca^{2+} release with the extent of T-tubule depolarization due to uncertainty of the T-tubule K$^+$, Na$^+$, and Cl$^-$ ion permeabilities and their lumenal concentrations before T-tubule depolarization. However, we were able to show that ^{45}Ca^{2+} release depended on the K$^+$ concentration in the depolarizing release medium. Using the Nernst equation, we calculated that a T-tubule membrane potential change of 60 mV is effective in releasing the caffeine-sensitive SR Ca^{2+} pool in 50 ms. Using triad-enriched vesicle fractions, Corbett et al. (1992) and Ikemoto, Antoniu, and Kang (1992) also found T-tubule depolarization-induced Ca^{2+} release to be a function of the degree of ionic replacement. Using the fluorescent potential-sensitive probe, WW781, and fluo-3, Ikemoto and Antoniu (1994) recently measured simultaneously membrane depolarization and Ca^{2+} release from a triad-enriched vesicle fraction and reported a voltage-dependent pattern of SR Ca^{2+} release which was similar to that of an intact fiber. Similar experiments using whole muscle homogenates are difficult to perform because of the low T-tubule content of mammalian skeletal muscle (<0.3% of cell volume and <5% of membrane content; Eisenberg, 1983), resulting in low signal-to-noise ratios.

The mechanism of T-tubule depolarization-induced Ca^{2+} release has also been investigated using ion replacement protocols with skinned amphibian skeletal muscle fibers. Some of the results obtained with this approach have been contradictory. Stephenson (1985) concluded that KCl or choline Cl replacement of K Mes produced T-tubule depolarization-induced ^{45}Ca^{2+} efflux comprised of a small Ca^{2+}-insensitive component, measured in the presence of EGTA, and a much larger Ca^{2+}-dependent component. Volpe and Stephenson (1986) reported that application of the Na$^+$(K$^+$)/H$^+$ exchanger, monensin, or the K$^+$/Na$^+$ channel former, gramicidin D, to Ca^{2+}-loaded fibers elicited T-tubule depolarization-induced ^{45}Ca^{2+} efflux which was entirely Ca^{2+} dependent. Lamb and Stephenson (1990) described D600-sensitive contractions, induced by replacement of K HDTA with either Na HDTA or choline Cl, which were considered to be largely Ca^{2+}-independent. In none of these studies were fast Ca^{2+} buffers used to address the importance of Ca^{2+} in amplifying Ca^{2+} release. The effect of Mg^{2+} on T-tubule depolarization–induced Ca^{2+} release has been also examined using skinned fibers. Millimolar concentrations were reported to inhibit both ^{45}Ca^{2+} efflux initiated by Cl$^-$ replacement of propionate$^-$ (Stephenson

and Podolsky, 1977) and D600-sensitive contractions initiated by Na^+ replacement of K^+ (Lamb and Stephenson, 1991). The above cited skinned fiber studies were carried out on a nonphysiologically slow time scale of seconds, making the physiological relevance of the results difficult to assess.

It is of interest to compare the results of our study showing an inhibition of depolarization-induced SR Ca^{2+} release by BAPTA with the changes in SR Ca^{2+} release produced by BAPTA and fura-2 in frog skeletal muscle fibers. In voltage-clamped intact and cut frog muscle fibers, depolarization of the surface membrane normally results in an intracellular Ca^{2+} transient comprised of an early peak, occurring in ≤ 50 ms, and a quasi-steady component which is maintained until repolarization of the surface membrane. The latter component is believed to be under the direct control of the T-tubule voltage sensor (Rios and Pizarro, 1991). The rising and falling phases of the early peak have been postulated to result from Ca^{2+} activation and Ca^{2+} inactivation of SR Ca^{2+} release, respectively (Baylor et al., 1983; Melzer, Rios, and Schneider, 1984; Simon, Klein, and Schneider, 1991). Studies with purified SR vesicle and RyR preparations confirmed that Ca^{2+} concentrations of 1–50 μM activate, whereas relatively high concentrations of Ca^{2+} (> 100 μM in the presence of physiological concentrations of Mg^{2+} and ATP) inhibit the SR Ca^{2+}-release channel (Meissner, 1994). The intracellular microinjection into intact frog muscle fibers of relatively high concentrations (~ 2–4 mM) of fast complexing Ca^{2+} buffers such as BAPTA or fura-2 was found by Jacquemond, Csernoch, Klein, and Schneider (1991) to cause elimination of the early peak component of depolarization-induced Ca^{2+} release while leaving the maintained quasi-steady level unaffected. These results suggested that the early peak component of Ca^{2+} release is activated by Ca^{2+}, because it was eliminated by fast calcium Ca^{2+} buffers. In contrast, Holling-worth, Harkins, Kurebayashi, Konishi, and Baylor (1992) found that the injection of fura-2 into intact fibers increased the amount of Ca^{2+} released during an action potential. Pape, Jong, Chandler, and Baylor (1993) and Jong, Pape, Chandler, and Baylor (1993) observed that the rate and amount of SR Ca^{2+} release in cut frog muscle fibers during action potential stimulation and depolarization was dependent on the concentration of the Ca^{2+} buffer present in their fiber preparations. Fura-2 concentrations of 0.5–3 mM increased both the amount of Ca^{2+} release and the rate of release, which was attributed to the removal by fura-2 of Ca^{2+}-induced inactivation of Ca^{2+} release. When the fura-2 concentration was increased to ~ 6–8 mM, SR Ca^{2+} release was reduced, which was considered to be possibly due to a reduction of Ca^{2+}-induced Ca^{2+} release or to a pharmacological effect not related to Ca^{2+} buffering. Our results showing an inhibition of T-tubule depolarization-induced SR Ca^{2+} release by high [BAPTA] and 1 mM Ca^{2+} provides direct evidence for the idea that the released Ca^{2+} activates and inhibits the SR Ca^{2+} release channel during T-tubule depolarization, depending on the Ca^{2+} concentration in the junctional gap.

In the presence of high BAPTA or millimolar Ca^{2+} or Mg^{2+}, a small remaining release of Ca^{2+} was seen. Although this release was not statistically significant, it was consistent with the idea that the T-tubule coupled release component is relatively insensitive to these inhibitors (Rios and Pizarro, 1991). We did not observe a consistent potentiation of Ca^{2+} release by EGTA and BAPTA. One reason for our failure to detect an increase in Ca^{2+} release by Ca^{2+} buffers may have been the

limiting time resolution of our methodology. No additional Ca^{2+} release in the presence of millimolar concentrations of BAPTA or EGTA was expected since our caffeine controls indicated that essentially all the releasable Ca^{2+} was released in 50 ms in our control condition (i.e., in the presence of 50 μM EGTA).

Activation of the SR Ca^{2+} release channel by caffeine at high [BAPTA] argued against a direct effect of BAPTA on the channel, although we cannot rule out effects of BAPTA on the voltage sensor. Csernoch, Jacquemond, and Schneider (1993) observed that control injections with the poorly buffering BAPTA analogue anisidine did not produce a substantial suppression of the early peak component of depolarization-induced Ca^{2+} release, which also indicated that the effect of BAPTA is most likely related to Ca^{2+} buffering.

In this study, the earliest time point of measuring Ca^{2+} efflux was 50 ms. This time resolution is not adequate, however, for resolving the rising and falling phases of the initial Ca^{2+} peak which is observed in muscle fibers. This represents a major disadvantage of the approach which could possibly be overcome with the use of a Ca^{2+} indicator dye. The rapid mixing/Ca^{2+} dye method described by Ikemoto et al. (1985) allowed measurement of changes in Ca^{2+} concentration within 5 ms of stimulation. However, our approach offers two major advantages. First, upon exposure of vesicles to Ca^{2+} release medium, the released ^{45}Ca^{2+} is continuously removed by filtration, making it possible to directly measure Ca^{2+} release in the presence of MgATP without concern for Ca^{2+} reuptake. Second, with our approach there is no limitation on the concentration of Ca^{2+} and/or Ca^{2+} buffer which may be included in Ca^{2+} release media, making it therefore possible to evaluate the effects of Ca^{2+} on SR Ca^{2+} release during EC coupling.

Some caution is necessary when comparing mammalian and amphibian muscle preparations. Biochemical analysis, and cloning and sequencing of the complementary DNAs of the RyR have revealed that mammalian skeletal muscle expresses a single isoform (RyR1), whereas amphibian muscle expresses two RyR isoforms (termed α and β) (Olivares, Tanksley, Airey, Beck, Ouyang, Deerink, Ellisman, and Sutko, 1991; Murayama and Ogawa, 1992; Lai, Liu, Xu, El-Hashem, Kramarcy, Sealock, and Meissner, 1992). The α and β amphibian isoforms are present as discrete homooligomers, have immunological properties characteristic of the mammalian skeletal and cardiac RyR isoforms, respectively, and may differ in their Ca^{2+} sensitivity. There is morphological evidence to suggest that, at least in some skeletal muscle, only a subpopulation of RyRs are mechanically linked to a group of four DHPRs (for review, see Franzini-Armstrong and Jorgensen, 1994). This observation has raised the possibility that Ca^{2+} released by DHPR-linked Ca^{2+} release channels could serve to amplify SR Ca^{2+} release by opening Ca^{2+}-release channels not linked to DHPRs. [^{3}H]PN200-110 and [^{3}H]ryanodine binding measurements indicating a low DHPR/RyR of ~1 and 0.6 for rabbit and frog skeletal muscle, respectively, support the idea of the presence of DHPR-linked and -unlinked RyRs in mammalian and amphibian skeletal muscle (Anderson et al., 1994). Considering the presence of two RyR isoforms, six different forms of SR Ca^{2+} release must then be considered to possibly take place in amphibian skeletal muscle: voltage-dependent Ca^{2+} release by DHPR-linked RyR α and β isoforms, and Ca^{2+}-dependent Ca^{2+} release involving both DHPR-linked and -unlinked α and β isoforms. In mammalian skeletal muscle,

because of the presence of a single RyR isoform, the number of possible activation mechanisms is reduced by half.

Stern (1992) describes a method for determining the free Ca^{2+} concentration gradient near a Ca^{2+}-conducting channel. He derived a simple expression (Eq. 13 of his paper) which allows calculation of the Ca^{2+} concentration as a function of distance, if the initial rate of Ca^{2+} flux through the channel and the initial concentrations of total and uncomplexed Ca^{2+} and Ca^{2+} buffer are known. Therefore, the concentrations of Ca^{2+} and Ca^{2+} buffer which result in inhibition of depolarization-induced Ca^{2+} release can in theory be used to estimate the distance between the Ca^{2+}-release channel pore and Ca^{2+}-activation sites. We calculated this distance using the following constants for Ca^{2+} and BAPTA: $k_{on} = 1.7 \ 10^9 \ M^{-1} \ s^{-1}$; $K_d = 4 \ 10^{-7} \ M$, $D_{Ca} = 3 \ 10^{-6} \ cm^2 \ s^{-1}$, $D_{BAPTA} = D_{CaBAPTA} = 10^{-5} \ cm^2 \ s^{-1}$. The number of Ca^{2+}-release channels (~ 0.4 pmol/mg protein) was determined by measuring the B_{max} value of high affinity [^{3}H]ryanodine binding (Anderson et al., 1994). Using this value and the release of 6,000 pmol Ca^{2+}/mg protein at 50 ms, which is our earliest time point of measuring $^{45}Ca^{2+}$ release, we estimate that each Ca^{2+} release channel releases $\sim 15,000 \ Ca^{2+}$ which corresponds to a current of ~ 0.1 pA. This may represent an underestimate because of release of most of the 15,000 Ca^{2+} in < 50 ms. Assuming that the current falls within the range of 0.1–1.0 pA and that 1 μM free Ca^{2+} is sufficient to activate the release channel, then the near complete inhibition of Ca^{2+} release by 7 mM uncomplexed BAPTA gives a lower limit of 15 nm (for a current of 0.1 pA) and 25 nm (for a current of 1 pA) for the distance separating the Ca^{2+} channel pore and Ca^{2+}-activation site(s). The purified rabbit skeletal muscle RyR/Ca^{2+}-release channel complex has been interpreted to exhibit dimensions of 27 × 27 × 14 nm (Wagenknecht, Grassucci, Frank, Saito, Inui, and Fleischer, 1989). If Ca^{2+}-release channels are assumed to lie adjacent to one another in the SR membrane (Franzini-Armstrong and Jorgensen, 1994), 15–25 nm would appear to be a reasonable distance for Ca^{2+} leaving one channel to travel before reaching the activation site(s) of a nearby channel. Another possibility we cannot rule out, however, considering the large size of the Ca^{2+}-release channel complex, is that released Ca^{2+} amplifies SR Ca^{2+} release by interacting with activation sites located on the same channel.

In conclusion, the results reported here support the mechanical coupling mechanism of EC coupling in skeletal muscle, but also show that Ca^{2+}-dependent mechanisms play an important role in controlling skeletal muscle SR Ca^{2+} release. The mechanical coupling mechanism proposes that DHPR conformational changes induced by T-tubule depolarization trigger the opening of RyR/Ca^{2+} release channels that are physically linked to DHPRs. The involvement of additional proteins in mediating this interaction has been invoked (Brandt et al., 1992). As shown in this study with the use of the fast Ca^{2+} buffer BAPTA, the initially released Ca^{2+} amplifies SR Ca^{2+} release by binding to Ca^{2+} activation sites of the same channel or neighboring Ca^{2+} release channels which may or may not be linked to DHPRs. The data do not distinguish between these possibilities because the size of the Ca^{2+}-release channel complex is comparable to the distance between neighboring channels and it is not known in which way linkage to the DHPR alters regulation of the RyR by Ca^{2+}. Rapid release of Ca^{2+} is dependent on the presence of MgATP, whereas an

increase in the free Ca^{2+} or Mg^{2+} concentration to millimolar levels inhibits the release channels. In support of the mechanical coupling model, peptides derived from the DHPR were recently shown to activate the purified rabbit skeletal muscle SR Ca^{2+}-release channel (Lu, Xu, and Meissner, 1994).

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