Fast release of $^{45}Ca^{2+}$ induced by inositol 1,4,5-trisphosphate and Ca^{2+} in the sarcoplasmic reticulum of rabbit skeletal muscle: evidence for two types of Ca^{2+} release channels

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ABSTRACT The kinetics of Ca^{2+} release induced by the second messenger D-myoinositol 1,4,5 trisphosphate (IP_3), by the hydrolysis-resistant analogue D-myoinositol 1,4,5 trisphosphorothioate (IPS_3), and by micromolar Ca^{2+} were resolved on a millisecond time scale in the junctional sarcoplasmic reticulum (SR) of rabbit skeletal muscle. The total Ca^{2+} mobilized by IP_3 and IPS_3 varied with concentration and with time of exposure. Approximately 5% of the $^{45}Ca^{2+}$ passively loaded into the SR was released by 2 μM IPS_3 in 150 ms, 10% was released by 10 μM IPS_3 in 100 ms, and 20% was released by 50 μM IPS_3 in 20 ms. Released $^{45}Ca^{2+}$ reached a limiting value of ~30% of the original load at a concentration of 10 μM IP_3 or 25–50 μM IPS_3. Ca^{2+}-induced Ca^{2+} release (CICR) was studied by elevating the extravesicular Ca^{2+} while maintaining a constant 5-mM intravesicular $^{45}Ca^{2+}$. An increase in extravesicular Ca^{2+} from 7 nM to 10 μM resulted in a release of 55 ± 7% of the passively loaded $^{45}Ca^{2+}$ in 150 ms. CICR was blocked by 5 mM Mg^{2+} or by 10 μM ruthenium red, but was not blocked by heparin at concentrations as high as 2.5 mg/ml. In contrast, the release produced by IPS_3 was not affected by Mg^{2+} or ruthenium red but was totally inhibited by heparin at concentrations of 2.5 mg/ml or lower. The release produced by 10 μM Ca^{2+} plus 25 μM IPS_3 was similar to that produced by 10 μM Ca^{2+} alone and suggested that IP_3-sensitive channels were present in SR vesicles also containing ruthenium red–sensitive Ca^{2+} release channels. The junctional SR of rabbit skeletal muscle may thus have two types of intracellular Ca^{2+} releasing channels displaying fast activation kinetics, namely, IP_3-sensitive and Ca^{2+}-sensitive channels.

INTRODUCTION

Receptor channels for the second messenger D-myoinositol 1,4,5 trisphosphate (IP_3) play a central role in the control of intracellular Ca^{2+} in neurons and secretory cells (Berridge and Gallione, 1988). However, their contribution to Ca^{2+} signals in muscle cells, particularly to the mechanism of excitation–contraction coupling in striated muscle, has remained elusive. The Ca^{2+} necessary to initiate muscle contraction is released from the sarcoplasmic reticulum (SR) in the muscle cell, essentially on the time scale of a single action potential lasting 10–200 ms. Several mechanisms by which the Ca^{2+} permeability of the SR could be increased in such a short time have been proposed (Schneider and Chandler, 1973; Endo, 1985; Fabiato, 1985), whereas others have been discarded (for review see Rios and Pizarro, 1991). Models involving protein–protein coupling between voltage sensors and Ca^{2+} release channels are particularly favored for skeletal muscle (Rios and Brum 1987; Tanabe et al., 1990), whereas Ca^{2+}-induced Ca^{2+} release (CICR) is strongly supported in heart (Beuckelmann and Wier, 1988; Niggli and Lederer 1990). A third model favored for smooth muscle (Somlyo et al., 1988) involves the action of IP_3 as a chemical messenger, which, once released from the plasma membrane in response to a stimulus, would bind to an IP_3 receptor in the SR to evoke release of the stored Ca^{2+}. This hypothesis has been extensively tested in skeletal muscle (Vergara et al., 1985; Volpe et al., 1985; Rojas et al., 1986; Donaldson et al., 1987; Valdivia et al., 1990) and cardiac muscle (Fabiato, 1986; Nosek et al., 1986; Kentish et al., 1990) but has been difficult to reproduce in all cases (Movsesian et al., 1985; Scherer and Ferguson, 1985; Lea et al., 1986; Mikos and Snow, 1987; Hanon et al., 1988).

In favor of a role of IP_3 in excitation–contraction coupling of skeletal muscle are (*a*) the localized production of IP_3 in the transverse tubules (Hidalgo et al., 1986); (*b*) the presence of an active IP_3-degrading system in the same membrane (Sanchez et al., 1991); (*c*) the demonstration that IP_3 levels in skeletal muscle are actually increased by repetitive depolarization (Vergara et al., 1985, 1986; Lagos and Vergara, 1990); and (*d*) the recording of IP_3-sensitive channels in the SR of frog skeletal muscle (Suarez-Isla et al., 1988). However, against this mechanism are the observations that (*a*) heparin injected into frog muscle fibers, at concentrations that should presumably block the IP_3-sensitive release, did not block the excitation–contraction coupling (Pampe et al., 1988); and (*b*) the Ca^{2+} release in frog fibers activated by photolysis of caged IP_3 was notoriously slow (Walker et al., 1987). The latter result questioned whether IP_3 receptors could be kinetically

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competent to elevate cytosolic Ca^{2+} on a time frame compatible with the fast processes of skeletal muscle cells. Since the kinetics of activation of SR Ca^{2+} release by IP_3 are critical to evaluate the role of this second messenger in excitation–contraction coupling, in the present work we resorted to rapid filtration (Dupont, 1984). This technique permitted a direct exposure of the SR membrane of rabbit skeletal muscle to various activators and blockers of Ca^{2+} release. The kinetics of release evoked by IP_3 and IPS_3 were compared with those produced by Ca^{2+} in the micromolar range (Ikemoto et al., 1985; Meissner et al., 1986; Sumbilla and Inesi, 1987; Calviello and Chiesi, 1989) because CICR appears to be mediated by ryanodine receptor Ca^{2+} release channels (Smith et al., 1985, 1986, 1988; Lai et al., 1988). The release induced by IP_3 or D-myoinositol 1,4,5 trisphosphorothioate (IPS_3) was as fast as that induced by Ca^{2+} and was completed in 40–200 ms, depending on concentration. The results suggest that, in addition to ryanodine receptors, the junctional SR contains a fast-activated IP_3-sensitive Ca^{2+} channel that may contribute to Ca^{2+} release from the SR of skeletal muscle under various physiological conditions. The presence of two separate channels was independently supported by recordings in planar bilayers (Valdivia et al., 1991).

MATERIALS AND METHODS

Isolation of rabbit skeletal muscle junctional SR

Rabbit back and leg muscle was dissected, quickly frozen in liquid N_2, and stored at −80°C until use within six months. The procedure described by Meissner (1984) was implemented with the following modifications. Five portions of 50 g of thawed muscle were minced in a food processor and homogenized in a blender (Waring, New Hartford, CT) for 60 s in 250 ml of buffer A (0.3 M sucrose, 20 mM Hepes-Tris, pH 7.2). The following protease inhibitors were added to buffer A during homogenization: pepstatin A (1 μM), iodoacetamide (1 mM), PMSF (0.1 mM), leupeptin (1 μM), and benzamidine (1 mM). The homogenate was centrifuged for 30 min at 4,000 rpm (2,600 *g*) in a GSA rotor (Sorvall-Dupont, Willmington, DE). The supernatant was centrifuged for 30 min at 8,000 rpm (10,400 *g*) in a GSA rotor and the pellet was resuspended in 0.6 M KCl, 5 mM Na-Pipes (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)), pH 6.8. This material was incubated for 60 min at 0°C and centrifuged for 60 min in a rotor (Type 35, Beckman Instruments, Palo Alto, CA) at 32,000 rpm (80,000 *g*). The pellet was resuspended in 0.3 M sucrose, 0.4 M KCl, 5 mM Na-Pipes, pH 6.8. Samples were placed on top of a discontinuous sucrose gradient consisting of 9.5 ml each of 20, 30, and 40% (wt/wt) sucrose. Gradients were centrifuged in a rotor (SW 28, Beckman) for 16 h at 26,000 rpm (95,000 *g*). Heavy SR was recovered from the interphase between the 30 and 40% sucrose layers. Collected material was diluted three-fold with 0.4 M KCl, 5 mM Na-Pipes, pH 6.8, and centrifuged for 60 min at 32,000 rpm in a Type 35 rotor. The pellets were resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Na-Pipes, pH 6.8, frozen in liquid N_2 in 1-ml aliquots at a protein concentration of 10 mg/ml, and stored at −80°C.

Passive loading of $^{45}Ca^{2+}$ into SR vesicles

Heavy SR stored frozen at −80°C was used in all experiments. To remove the sucrose used for storage, a thawed aliquot of SR was diluted in five volumes of 150 mM KCl, 50 mM Mes-Tris, pH 7.5, and kept on ice for 10 min. Membranes were pelleted by centrifugation for 10 min in a benchtop microfuge (Eppendorf-Brinkmann Instruments, Westbury, NY) at 12,000 rpm and resuspended at a protein concentration of 2.5 mg/ml in 150 mM KCl, 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes)-Tris, pH 7.5. Loading of $^{45}Ca^{2+}$ into SR vesicles was achieved passively in the presence of 5 mM $^{45}Ca^{2+}$. SR was incubated in 5 mM $^{45}CaCl_2$ (~7,500 cpm/nmol), 150 mM KCl, 50 mM Mes-Tris, pH 7.5, at room temperature for 2 to 3 h. The total $^{45}Ca^{2+}$ loaded averaged 14.2 ± 3 nmol/mg protein (n = 22). Upper and lower values of $^{45}Ca^{2+}$ passively loaded in this manner were 8 and 21 nmol/mg, respectively. Previously reported $^{45}Ca^{2+}$ loading capacity of rabbit skeletal muscle heavy SR varied between 30 (Kim et al., 1984; Calviello and Chiesi, 1989) and 100 nmol/mg protein (Miyamoto and Racker, 1984; Meissner et al., 1986). The lower values encountered in our preparation were explained by the fact that SR was isolated from muscle stored frozen in liquid N_2 instead of fresh tissue. When the same procedure was used to isolate SR from fresh tissue, the passive loading capacity was 58 ± 11 nmol/mg protein (n = 7). Control experiments showed that both preparations released the same percentage of the loaded $^{45}Ca^{2+}$. Percent releases in release medium and nonrelease medium described below were 51 ± 9 (n = 4) and 11 ± 8 (n = 6) in SR prepared from fresh tissue and 55 ± 7 (n = 4) and 9 ± 3 (n = 5) in SR prepared from frozen tissue. The difference in loading capacity was explained by a difference in the vesicular internal volume (McKinley and Meissner, 1978), which, in SR isolated from tissue stored frozen, was 2 ± 0.8 μl/mg protein whereas, in the SR isolated from fresh tissue, was 9 ± 2 μl/mg protein. Hence, the SR vesicles prepared from frozen tissue had the same Ca^{2+}-release properties as those prepared from fresh tissue but appeared to be smaller in size.

Measurements of $^{45}Ca^{2+}$ release by rapid filtration

The rapid filtration instrument consisted of a motor-driven syringe fitted to a holder for a 25-mm-diam filter (Biologic Instruments, Echirolles, France). SR loaded with $^{45}Ca^{2+}$ were placed on the surface of a standard nitrocellulose filter of 0.8 μm pore size (Millipore Corp., Danvers, MA), and the release medium containing the agonist was placed in the filtration syringe. Upon magnetic sealing of the holder and the syringe, the release medium in the syringe was forced through the filter for a specified time between 20 and 200 ms. A specified filtration time was executed by a microprocessor-controlled adjustment of the filtration volume at a constant filtration rate of 4 ml/s. Control filtrations showed that 10 ms was the upper limit for the time required to exchange the solution soaked into a filter. Before rapid filtration, the 5 mM $^{45}CaCl_2$ used for passive loading was lowered to ~7 nM by dilution 20 μl of the $^{45}Ca^{2+}$-loaded SR (50 μg protein) into 1 ml of 5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM Mes-Tris, pH 7.5. The diluted SR was immediately spread on the surface of a wet filter (Millipore) held in the filtration holder by a mild vacuum and the rapid filtration was then executed. The filtration syringe was filled with one of two solutions designated nonrelease (5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM Mes-Tris, pH 7.5) or release medium (6.25 mM EGTA, 6.23 mM $CaCl_2$, 150 mM KCl, 50 mM Mes-Tris, pH 7.5) or with the indicated concentration of IP_3, IPS_3, ruthenium red, or heparin prepared in release or nonrelease medium. The chosen pH, 7.5, maximized Ca^{2+} release channel activity (Ma et al., 1988). The calculated free Ca^{2+} of the nonrelease medium was 0.2 nM (assuming

a contaminant $CaCl_2$ of 3.5 μM) and that of the release medium was 10 μM. Free Ca^{2+} was calculated by a computer program that used the stability constants of Fabiato (1988). A Ca^{2+} electrode (Orion Research Inc., Cambridge, MA) was used to verify the free Ca^{2+} of the release medium and to determine the contaminant free Ca^{2+} of solutions without added $CaCl_2$, $MgCl_2$, or EGTA, which was 1–3.5 μM. After rapid filtration, filters were rinsed under a mild vacuum with 3 ml of 150 mM KCl, 50 mM Mes-Tris, pH 7.5, 6 mM $MgCl_2$, 10 μM ruthenium red, and 20 μM quercetin (rinse medium). Filters were counted for $^{45}Ca^{2+}$ content in 5 ml of scintillation fluid.

$^{45}Ca^{2+}$ release controls and protocols

The following controls and protocols (1–6) ensured that the reported efflux of $^{45}Ca^{2+}$ represented a net transfer of Ca^{2+} across the SR membrane. (1) CICR was induced by a decrease, not an increase, in driving force for Ca^{2+} efflux. This was achieved by lowering the extravesicular free Ca^{2+} to 7 nM before the rapid filtration step. Consequently, the release induced by rapid filtration with 10 μM free Ca^{2+} occurred in spite of a net decrease in driving force for Ca^{2+} efflux out of the SR, from 5 mM lumenal Ca^{2+}, 7 nM extravesicular Ca^{2+} to 5 mM lumenal Ca^{2+}, 10 μM extravesicular Ca^{2+}. (2) Efflux induced by release medium (6.25 mM EGTA, 6.23 mM $CaCl_2$, 150 mM KCl, 50 mM Mes-Tris, pH 7.5) was not mimicked by Na_2EGTA in the absence of added CA^{2+} (6.25 mM EGTA, 150 mM KCl, 50 mM Mes-Tris, pH 7.5) or by micromolar Ca^{2+} in the presence of millimolar $MgCl_2$ (6.25 mM EGTA, 6.23 mM $CaCl_2$; 10 mM $MgCl_2$, 150 mM KCl, 50 mM Mes-Tris, pH 7.5). Thus, it was unlikely that the released $^{45}Ca^{2+}$ represented chelation by free EGTA of $^{45}Ca^{2+}$ bound to the SR surface. If the release actually represented removal of $^{45}Ca^{2+}$ electrostatically bound to the SR surface, Mg^{2+} should have increased this "removal" because of the collapse of the SR negative surface potential produced by the millimolar concentration of this divalent. (3) The nonreleasable $^{45}Ca^{2+}$ pool was evaluated in each experiment by a 50-fold dilution of $^{45}Ca^{2+}$-loaded SR into nonrelease medium containing 10 μM Ca^{2+} ionophore A23187. The nonreleasable pool was ≤ 7% of the total $^{45}Ca^{2+}$ present at $t = 0$. (4) Background $^{45}Ca^{2+}$ was evaluated in each experiment and was subtracted from each filtration time. To measure $^{45}Ca^{2+}$ background, a duplicate sample of $^{45}Ca^{2+}$-loaded SR was diluted 50-fold into nonrelease medium containing 10 μM Ca^{2+} ionophore A23187 and incubated at room temperature for 10 min. Samples were afterward subjected to rapid filtration in nonrelease medium for 200 ms and rinsed in 3 ml of the rinse medium described above. Thus, the background radioactivity subtracted from each data point comprised the nonreleasable $^{45}Ca^{2+}$ evaluated with A23187 and the nonspecific binding to filters. Background $^{45}Ca^{2+}$ was ≤1 nmol $^{45}Ca^{2+}$/filter per mg protein. (5) The total $^{45}Ca^{2+}$ present at $t = 0$ was evaluated in each experiment and was defined as the background-subtracted $^{45}Ca^{2+}$ that remained in the SR after a nominal 2-ms rapid filtration in nonrelease medium. (6) All filtrations were made in duplicate and averaged. Reported standard deviations were calculated from three or more filtrations from separate experiments. $^{45}Ca^{2+}$ efflux curves in release and nonrelease media were routinely included in parallel with each experiment as controls.

Chemicals and abbreviations

Na_2ATP was purchased from Sigma Chemical Co. (St. Louis, MO). Heparin prepared from porcine intestinal mucosa was purchased from ICN Biochemicals (Cleveland, OH). Ruthenium red (ruthenium III chloride oxide) was purchased from Alpha Products (Andover, MA). Sucrose, salts, and buffers were reagent grade. D-IP_3 and D-IPS_3 were synthesized as described (Cooke et al., 1987) and purified by ion-exchange chromatography on DEAE Sephadex A-25.

RESULTS

Kinetics of $^{45}Ca^{2+}$ release induced by IP_3, IPS_3, and Ca^{2+} in junctional SR

The kinetics of SR Ca^{2+} release were resolved by rapid filtration since this technique permitted a fast calibrated replacement of the extravesicular solution (Dupont, 1984). To eliminate the contribution of the Ca^{2+} pump to the release kinetics, all measurements were performed in passively loaded SR in solutions devoid of MgATP. We studied the release induced by IP_3 or IPS_3 side by side with CICR mainly since the latter represents a major efflux pathway for Ca^{2+} from the junctional SR and has been described extensively (Miyamoto and Racker, 1982; Ikemoto et al., 1985; Meissner et al., 1986; Sumbilla and Inesi, 1987; Calviello and Chiesi, 1989). The kinetics of $^{45}Ca^{2+}$ release produced by 25 μM IP_3 or 25 μM IPS_3 are shown in Fig. 1. At $t = 150$ ms, the $^{45}Ca^{2+}$ released by IP_3 and separately by IPS_3 was equivalent to 30 ± 8% ($n = 3$) and 33 ± 7% ($n = 3$) of the $^{45}Ca^{2+}$ loaded at $t = 0$, respectively. The $t_{1/2}$s established between $t = 0$ and $t = 150$ ms were 18 ± 8 and 20 ± 2 ms, and the initial rates averaged 118 and 117 nmol Ca^{2+}/mg^{-1} s^{-1}, respectively. These data established that at a concentration of 25 μM, the kinetics of efflux induced by IP_3 and IPS_3 were much faster than that described previously in nonmuscle cells (Meyer et al., 1988; Parker and Ivorra, 1990; Finch et al., 1991). The total $^{45}Ca^{2+}$ mobilized in these experiments agreed well with those described in actively loaded SR (Valdivia et al., 1990),

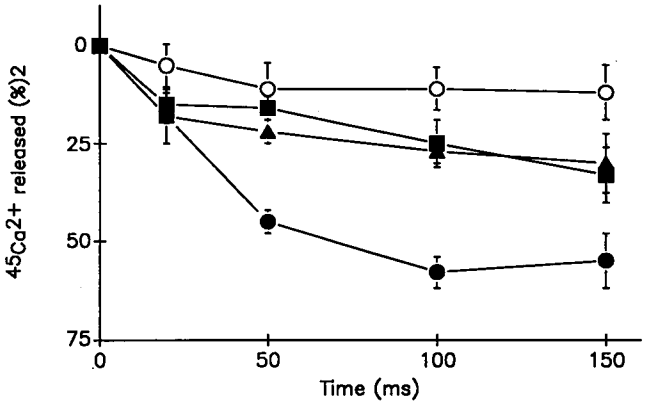

FIGURE 1 Time course of $^{45}Ca^{2+}$ release induced by 10 μM free Ca^{2+} and 25 μM IP_3 or IPS_3. Filtrations were carried out in nonrelease medium (5 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 50 mM Mes-Tris, pH 7.5; *open circles*); release medium (6.25 mM EGTA, 6.23 mM $CaCl_2$, 150 mM KCl, 50 mM Mes-Tris, pH 7.5; *filled circles*); nonrelease medium plus 25 μM IP_3 (*filled triangles*); or nonrelease medium plus 25 μM IPS_3 (*filled squares*). Mean ± SD of release at $t = 150$ ms are given in Table 1.

indicating that all the Ca^{2+} was mobilized from compartments that contained functional Ca^{2+} pumps. However, the rate of release in passively loaded SR was higher than that measured in actively loaded SR (not shown), which may be due to a larger Ca^{2+} gradient generated across the SR in the passive loading protocol. Because fast release was actually seen in the passive loading experiments, we concluded that IP_3 and the analogue increased the $^{45}Ca^{2+}$ efflux rate directly by increasing the SR Ca^{2+} permeability. Fig. 1 also shows that the CICR measured in the same SR preparation was equally fast. In release medium, the total $^{45}Ca^{2+}$ mobilized averaged 55 ± 7% and the $t_{1/2}$ was 31 ± 9 ms (n = 4), whereas in nonrelease medium, the total $^{45}Ca^{2+}$ mobilized averaged 9 ± 3%. Since the total $^{45}Ca^{2+}$ mobilized in nonrelease medium was small, the $t_{1/2}$ could not be measured accurately. On the basis of the data obtained between t = 0 and t = 50 ms, the initial rate of release induced by Ca^{2+} was ~126 nmol Ca^{2+}/mg^{-1} s^{-1}. Hence, the Ca^{2+} mobilized by CICR measured here at optimal Ca^{2+} (Ikemoto et al., 1985; Meissner et al., 1986; Calviello and Chiesi, 1989) was higher than that mobilized by a saturating concentration of IP_3 (see Fig. 4), although the initial rates of release were similar.

A study of the concentration dependence of release induced by IPS_3 is presented in Fig. 2. In each curve, the loss of $^{45}Ca^{2+}$ measured in nonrelease medium in the absence of IPS_3 was subtracted from the release produced by IPS_3. In some but not all experiments, a concentration of 2 μM IPS_3 was sufficient to produce a small release, which occurred with a delay of ~100 ms. At higher concentrations, however, release could be observed at earlier times. At 10 μM IPS_3, a release of 10 ± 7% of the passively loaded $^{45}Ca^{2+}$ occurred with an average $t_{1/2}$ in the range of 70–100 ms. At 25 μM IPS_3, a release of 33 ± 6% of the original load occurred with an average $t_{1/2}$ of 20 to 50 ms, whereas at 50 μM, the $t_{1/2}$ was below 20 ms and the extent of release was 34 ± 6%. Thus the rate and the extent of the release varied with agonist concentration. Fig. 3 shows that the total $^{45}Ca^{2+}$ released at t = 100 ms (*circles*) or t = 150 ms (*squares*) reached a limit at ~30% of the passively loaded $^{45}Ca^{2+}$ at an IPS_3 concentration of 25 to 50 μM. When similar experiments were made using IP_3 (Fig. 4), there was a clear saturation of the releasable pool at a concentration of 10 μM IP_3. These results indicate that IP_3 has a higher affinity than IPS_3 for its receptor site, an observation in agreement with measurements in nonmuscle cells (Strupish et al., 1988). The saturation of the releasable pool at ~35% of the $^{45}Ca^{2+}$ loaded at t = 0 strongly suggests that only a fraction of the SR vesicles have the IP_3-sensitive release pathway. In addition, the putative IP_3-sensitive channel is unlikely to be the ryanodine receptor because millimolar Mg^{2+} was present in all of the solutions of these experiments.

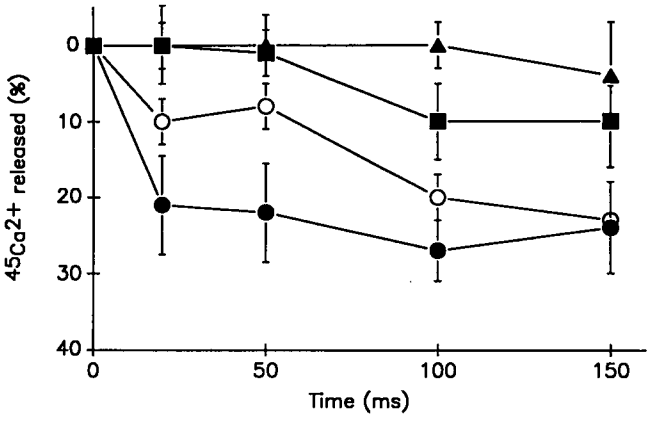

FIGURE 2 Concentration dependence of the rate of IPS_3-induced release. Time course of release was obtained in passively loaded SR. Measurements were carried out in nonrelease media containing 2 (*triangles*), 10 (*squares*), 25 (*open circles*), and 50 μM (*filled circles*) IPS_3. Each time point was measured in two to five separate experiments made in duplicate. The $^{45}Ca^{2+}$ released in the absence of IPS_3 was subtracted from each point. Release at t = 150 ms averaged 10 ± 4% in nonrelease media without IPS_3. Releases at 150 ms after subtraction were 4 ± 6% (*triangles*), 10 ± 7% (*squares*), 23 ± 7% (*open circles*), and 25% (*filled circles*).

Selective block of CICR or IP_3-sensitive release in junctional SR

To establish whether the release induced by IP_3 occurred by activation of ryanodine receptors, we investi-

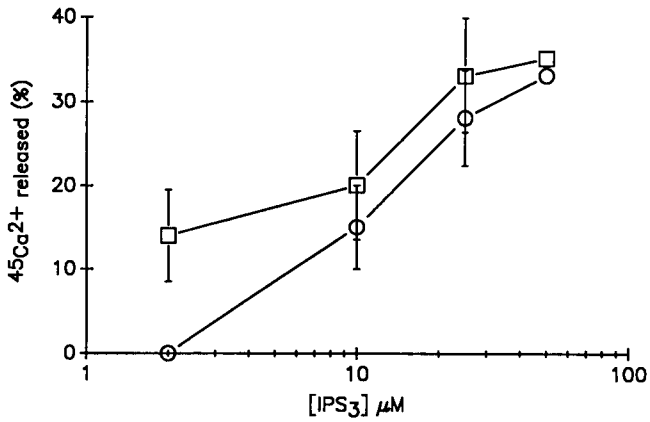

FIGURE 3 Saturation of the $^{45}Ca^{2+}$ mobilized by IPS_3. All filtrations were carried out in nonrelease medium containing the indicated micromolar concentration of IPS_3. Each time point ± SD corresponds to three to five separate experiments in duplicate. The highest IPS_3 concentration corresponds to a single experiment in duplicate. The total $^{45}Ca^{2+}$ released was measured at a fixed time at 100 (*circles*) or 150 ms (*squares*).

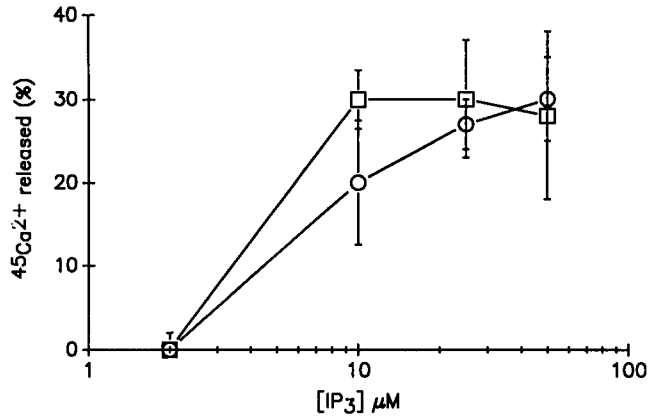

FIGURE 4 Saturation of the $^{45}Ca^{2+}$ mobilized by IP_3. All filtrations were carried out in nonrelease medium containing the indicated micromolar concentration of IP_3. Each mean ± SD corresponds to three separate experiments in duplicate. The total $^{45}Ca^{2+}$ released was measured at a fixed time of 100 (*circles*) or 150 ms (*squares*).

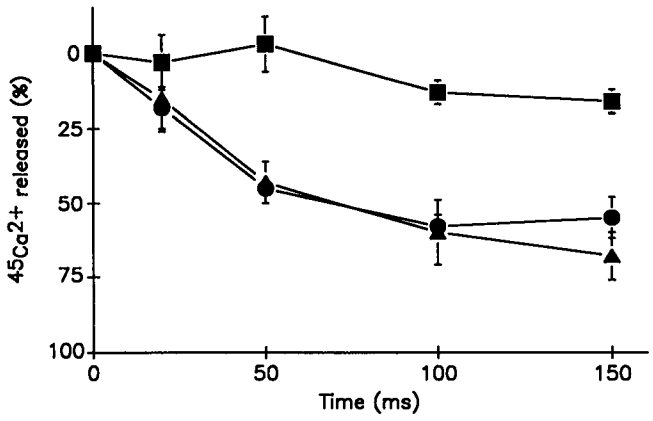

FIGURE 5 Effect of heparin and ruthenium red on CICR. Filtrations were carried out in passively loaded SR in release medium (*circles*), release medium plus 10 μM ruthenium red (*squares*), or release medium plus 2.5 mg/ml heparin (*triangles*).

gated the effect of ruthenium red, which is a blocker of the Ca^{2+} release channel formed by the ryanodine receptor (Miyamoto and Racker, 1982; Smith et al., 1985, 1986, 1988; Meissner et al., 1986). We also studied the effect of heparin because this glycosaminoglycan inhibits IP_3-sensitive channels in a selective manner (Ehrlich and Watras, 1988). For these experiments we selected a concentration of 10 μM ruthenium red and 2.5 mg/ml heparin, since Ehrlich and Watras (1988) reported that up to 1 mg/ml heparin had no effect on the skeletal ryanodine receptor, whereas in the same study, 10 μM ruthenium red could block skeletal ryanodine receptors without affecting IP_3-sensitive channels in aortic SR. Fig. 5 shows the effect of both agents on the CICR measured in release medium. In control experiments (*circles*), the released $^{45}Ca^{2+}$ was 55 ± 7% and the $t_{1/2}$ was 31 ± 9 ms. Addition of heparin to the release medium did not produce a significant change in the total $^{45}Ca^{2+}$ mobilized at t = 150 ms (68 ± 8%) or in the $t_{1/2}$ (39 ± 5 ms). In contrast, a release medium containing 10 μM ruthenium red blocked release almost as effectively as the Mg^{2+}-containing nonrelease medium described earlier. Thus, the CICR established by changing the solution free Ca^{2+} was readily blocked by ruthenium red but was totally insensitive to 2.5 mg/ml heparin. The effects of 2.5 mg/ml heparin and 10 μM ruthenium red on the release activated by 25 μM IPS_3 in nonrelease medium are shown in Fig. 6. In control experiments (*circles*), IPS_3 mobilized 33 ± 7% of the loaded $^{45}Ca^{2+}$ and the $t_{1/2}$ was 20 ± 2 ms. Heparin tested in nonrelease medium plus 25 μM IPS_3 inhibited release as effectively as nonrelease medium without IPS_3 (5 ± 4%). On the other hand, 10 μM ruthenium red did not modify the release initiated by 25 μM IPS_3 in nonrelease medium, which at t = 150 ms was 31 ± 6%. These results clearly show that the chosen concentrations of heparin and ruthenium red were effective in separating the IP_3-sensitive and Ca^{2+}-sensitive release of Ca^{2+} in junctional SR. This rules out the possibility that the IP_3-induced release may have occurred by activation of ryanodine receptor Ca^{2+} release channels.

Colocalization of IP_3 and ryanodine receptors in junctional SR

Because two distinct release pathways were present in the preparation of junctional SR, we investigated whether

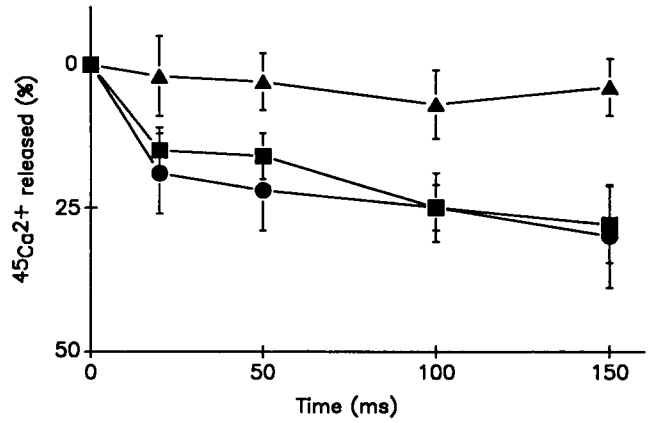

FIGURE 6 Effect of heparin and ruthenium red on the release induced by IPS_3. Filtrations were carried out in passively loaded SR in nonrelease medium plus 25 μM IPS_3 (*circles*), nonrelease medium plus 25 μM IPS_3 and 10 μM ruthenium red (*squares*), and nonrelease medium plus 25 μM IPS_3 and 2.5 mg/ml heparin (*triangles*).

Ca^{2+} and IPS_3 acted on the same or separate pools of stored Ca^{2+}. This was achieved by comparing the release evoked via CICR and IPS_3 to that produced when both triggering agents were combined. Fig. 7 shows averages of three experiments in which $^{45}Ca^{2+}$ was mobilized by 10 μM Ca^{2+}-containing release medium or by release medium plus 25 μM IPS_3. Nonrelease medium plus 2.5 mg/ml heparin served as control for both agonists. The Ca^{2+} mobilized at t = 150 ms via CICR (55 ± 7%) was not significantly different from that mobilized by CICR and IPS_3 combined (52 ± 11%). This result indicates that, after activation of CICR, the Ca^{2+} pool mobilized by IP_3 may have been depleted. Alternatively, it could be argued that micromolar Ca^{2+} inhibits the IPS_3-sensitive release so that, in the presence of both agents, only CICR is activated. However, micromolar Ca^{2+} has been shown to potentiate rather than to inhibit the action of IP_3 (Finch et al., 1991). Thus, the most direct interpretation of this observation is that both pathways are activated but the IP_3-sensitive channels are present in junctional SR vesicles that in addition contain Ca^{2+}-sensitive channels. This conclusion is also supported by the kinetic parameters of the release of $^{45}Ca^{2+}$ summarized in Table 1. Although the presence of IPS_3 in the release medium did not increase the total Ca^{2+} mobilized, the activation of the IP_3-sensitive pathway in the presence of CICR resulted in faster kinetics of release. Thus, when the two triggering agents were combined, the initial release rate increased almost two-fold. This would be expected if the two release channels are present in the same SR membrane, because activation of two pathways in parallel would increase the rate but not the extent of release.

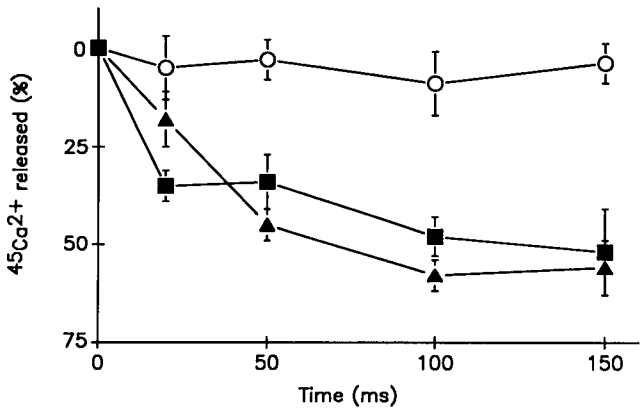

FIGURE 7 Combined release induced by Ca^{2+} and IPS_3. Filtrations were carried out in passively loaded SR in release medium (*triangles*), release medium plus 25 μM IPS_3 (*squares*), and nonrelease medium plus 2.5 mg/ml heparin (*circles*).

TABLE 1 **Kinetic parameters of $^{45}C^{2+}$ release induced by IP_3, IPS_3, and Ca^{2+} in rabbit skeletal muscle heavy SR**

Filtration solution	Release rate	$t_{1/2}$, at t = 150 ms	%Release	n
	nmol mg^{-1} s^{-1}			
NRM	—	—	9 ± 3	5
NRM + 25 μM IP_3	118 ± 36	18 ± 8	30 ± 8	3
NRM + 25 μM IPS_3	117 ± 11	20 ± 2	33 ± 7	3
NRM + 25 μM IPS_3 + 10 μM RR	129 ± 29	17 ± 5	31 ± 6	3
NRM + 25 μM IPS_3 + 2.5 mg/ml heparin	—	—	5 ± 4	4
RM	126 ± 28	31 ± 9	55 ± 7	4
RM + 25 μM IPS_3	205 ± 44	18 ± 5	52 ± 11	3
RM + 10 μM RR	—	—	14 ± 4	3
RM + 2.5 mg/ml heparin	124 ± 14	39 ± 5	68 ± 8	3

Initial release rate (±SD) was calculated as [(nmol $^{45}Ca^{2+}$ mg^{-1} loaded at t = 0) − (nmol $^{45}Ca^{2+}$ mg^{-1} at $t = t_{1/2}$)] × $[t_{1/2}]^{-1}$, which assumed a constant efflux rate between t = 0 and $t = t_{1/2}$. The $t_{1/2}$ (±SD) corresponds to the 50% efflux time between t = 0 and t = 150 ms. The %release corresponds to (filter cpm at time t)/(filter cpm at time t = 0) × 100. The average $^{45}Ca^{2+}$ loaded at t = 0 was 14.2 ± 3 nmol mg^{-1} SR protein (n = 22). n = 1 corresponds to a data set consisting of a single filtration with a duplicate at each of the following times: t = 0, 20, 50, 100, and 150 ms. NRM, nonrelease medium; RM, release medium; RR, ruthenium red.

DISCUSSION

Comparison of the cellular rate of Ca^{2+} release and the SR efflux rate induced by IP_3

Because the kinetics of release produced by IP_3 were fast and obviously within the time frame of Ca^{2+} release observed in situ (Vergara and Delay, 1986), we were interested in estimating whether the initial Ca^{2+} release rate induced by IP_3 were comparable to rates measured in skeletal muscle fibers by Ca^{2+} indicators during excitation–contraction coupling. For large depolarizations, the release rate in frog fibers measured by the Ca^{2+} indicator antipyrylazo III corresponds to a change of free Ca^{2+} concentration of ~1 to 10 μM/ms (Rios and Brum, 1987; Simon et al., 1989). A typical volume of fiber monitored optically in these experiments is a section of 250-μm length and two thirds of fiber width, which for an average fiber with a diameter of 100 μm translates into ~1.7 nl. Thus, the total Ca^{2+} change over the monitored area is ~1.7 to 17 pmol s^{-1}. Assuming a muscle tissue density of 1.06 g/ml (Mendez and Keys, 1960), an SR content of 5 mg/g muscle (Meissner et al., 1973), and a terminal cisternae or junctional SR content of one third of the total SR (Mobley and Eisenberg, 1975), we estimated the rate of release at ~550 to 5,500

nmol Ca^{2+} mg^{-1} s^{-1}. However, this rate may be underestimated by a factor of 10 due to uncertainties in whether the dye concentration used to report cytosolic Ca^{2+} is exclusively the free dye or a mixture of free and cell-bound dye (Maylie et al., 1987). Thus, the rate of release measured in situ, with the assumptions of the calculation and the uncertainty in the estimation of the concentration of dye, lies within the range of 550–5,500 nmol Ca^{2+} mg^{-1} s^{-1}. Initial release rates calculated in the various solutions employed in this study are shown in Table 1. In particular, the release rates produced by 25 μM IP_3 or IPS_3 (~ 120 nmol Ca^{2+} mg^{-1} s^{-1}) or by IP_3 and Ca^{2+} combined (~ 200 nmol Ca^{2+} mg^{-1} s^{-1}) are close to the lower limits of the cellular measurement. The contribution of CICR to the release rate within cells will be limited by the local concentration of free Mg^{2+}, which is known to block this release mechanism (Endo, 1985), and in the present experiments was kept purposely low. However, because the IP_3-sensitive release was measured in the 5 mM $MgCl_2$-containing nonrelease medium, the IP_3-induced release within cells is expected to be largely insensitive to Mg^{2+}. The calculation thus shows that the opening of IP_3-sensitive channels, even at physiological levels of Mg^{2+}, (Maughan and Godt, 1989) could account for, or contribute to, the massive movement of Ca^{2+} required to initiate muscle contraction. It should be noticed, however, that the experimental milieu used here is simpler than that of muscle cells (Maughan and Godt, 1989); therefore, cellular ions not yet tested in our system could influence the rate of release in a positive or negative manner.

By rapidly quenched flow in actively loaded SR, Ikemoto et al. (1985) were able to induce a release of $^{45}Ca^{2+}$ with a $t_{1/2}$ in the range of 50–70 ms in a medium containing 4 μM free Ca^{2+} plus caffeine. Meissner et al. (1986) used the same technique in passively loaded SR to show that a medium containing adenine nucleotides produced a massive release of stored Ca^{2+} that was essentially "instantaneous" with a $t_{1/2}$ of ~ 10 ms. In previous studies of rapid filtration (Sumbilla and Inesi, 1987; Calviello and Chiesi, 1989), release of $^{45}Ca^{2+}$ in < 100 ms could be induced by 5 μM free Ca^{2+} in the absence of other agents or in solutions that combined micromolar Ca^{2+} and millimolar levels of total Mg^{2+} and ATP. The total released $^{45}Ca^{2+}$ and the $t_{1/2}$ of the efflux measured by Sumbilla and Inesi (1987) in micromolar free Ca^{2+} without other ligands was similar to that reported here. In contrast, the release rates induced by IP_3 reported previously in non-muscle cells were remarkably slow, on the time scale of several seconds. In permeabilized rat basophilic leukemia cells (Meyer et al., 1988), the efflux rate saturated with IP_3 concentrations > 50 nM at a value of ~ 0.3 s^{-1}, which would correspond to a $t_{1/2}$ > 2 s. Thus, even though the response was highly sensitive to the second-messenger concentration, the release rate was 100-fold slower than that found here. A fast activation of IP_3-sensitive Ca^{2+} release in the SR of rabbit skeletal muscle was, therefore, largely unexpected. In support of our finding, however, a recent study in brain synaptosomes (Finch et al., 1991) showed that the rate of release produced by 1 μM IP_3 peaked at ~ 140 ms, consistent with the delay in the activation of release that we observed at low IP_3 concentrations (Fig. 2). Taken together, these results would seem to suggest that the kinetics of IP_3 receptor activation may vary from cell to cell, depending on the need for slow or fast Ca^{2+} signaling.

Implications for excitation–contraction coupling

The kinetics of activation and inactivation of ryanodine and IP_3 receptors under various ionic conditions prevailing in skeletal muscle (Maughan and Godt, 1989), and the interactions that may occur between the two channels when present in junctional SR, are presently unknown. However, a possible colocalization of Ca^{2+}-sensitive and IP_3-sensitive Ca^{2+} release channels in the same membrane may have several implications. A small component of the total release of Ca^{2+} from the SR of fast twitch muscle is essentially instantaneous and follows the time course of the depolarizing stimulus (Kovacs et al., 1979; Schneider and Simon, 1988; Simon et al., 1989). A much larger release, with kinetics of hundreds of milliseconds, has also been described in continuously depolarized fibers (Brum et al., 1988). This complexity in the time course of release makes it unlikely that a single channel type is entirely responsible for the observed Ca^{2+} release waveform. In chromaffin cells and neurons (Supattapone et al., 1988; Ellisman et al., 1990; Malgaroti et al., 1990; Opsichuck et al., 1990), IP_3 and ryanodine receptors coexist in the same cell. Hence, their coexistence in skeletal muscle would not be entirely surprising and may account for the complexity in the intracellular Ca^{2+} release waveform produced by depolarization. Berridge and Gallione (1988) suggested that an IP_3-mediated release of Ca^{2+} in a localized region of the cytoplasm may rapidly propagate through the entire cell by a self-sustaining wave of CICR. For this model to apply to skeletal muscle, the amount of IP_3 produced should be large enough to activate IP_3-sensitive channels in the vicinity of the terminal cisternae, which is the presumed site of Ca^{2+} release during excitation–contraction coupling (Vergara et al., 1986). The fast kinetics of activation of IP_3 receptors would thus ensure a rapid accumulation of Ca^{2+} that could trigger the activation of ryanodine receptor channels (Fabiato, 1985; Imagawa et al., 1987; Inui et al., 1987;

Beuckelmann and Wier, 1988; Lai et al., 1988; Niggli and Lederer, 1990). The location of ryanodine receptors exclusively in the terminal cisternae SR (Block et al., 1988; Wagenknecht et al., 1989), and their high density (Smith et al., 1986), would contribute to the generation of a wave of CICR that could quickly spread over the entire terminal. Alternatively, IP_3 receptors could contribute to excitation–contraction coupling after the initial release of SR Ca^{2+} had occurred. For example, the morphological connection of ryanodine receptors to the transverse tubular membrane (Block et al., 1988; Wagenknecht et al., 1989), and the activation of phospholipase C by Ca^{2+} (Tiger et al., 1989; Baird and Nahorski, 1990), suggests a cascade in which charge movement would activate ryanodine receptors (Takeshima et al., 1989), and the local increase in cytosolic Ca^{2+} would in turn stimulate production of IP_3 (Tiger et al., 1989; Baird and Nahorski, 1990). The activation of IP_3-sensitive channels after Ca^{2+}-dependent activation of phospholipase C may thus serve to sustain SR Ca^{2+} release beyond the duration the initial depolarizing stimulus. Regardless of the mechanism of activation in situ, we expect IP_3-sensitive channels to have a major impact in the Ca^{2+} homeostasis of skeletal muscle since, as shown in Table 1, the amount of Ca^{2+} mobilized by the IP_3-sensitive pathway is more than half of that mobilized by ryanodine receptors.

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