Mechanism of Chloride-Dependent Release of Ca^{2+} in the Sarcoplasmic Reticulum of Rabbit Skeletal Muscle

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ABSTRACT We investigated the effect of Cl^- on the Ca^{2+} permeability of rabbit skeletal muscle junctional sarcoplasmic reticulum (SR) using $^{45}Ca^{2+}$ fluxes and single channel recordings. In $^{45}Ca^{2+}$ efflux experiments, the lumen of the SR was passively loaded with solutions of 150 mM univalent salt containing 5 mM $^{45}Ca^{2+}$. Release of $^{45}Ca^{2+}$ was measured by rapid filtration in the presence of extravesicular 0.4–0.8 μM free Ca^{2+} and 150 mM of the same univalent salt loaded into the SR lumen. The rate of release was 5–10 times higher when the univalent salt equilibrated across the SR-contained Cl^- (Tris-Cl, choline-Cl, KCl) instead of an organic anion or other halides (gluconate$^-$, methanesulfonate$^-$, acetate$^-$, HEPES$^-$, Br^-, I^-). Cations (K^+, $Tris^+$) could be interchanged without a significant effect on the release rate. To determine whether Cl^- stimulated ryanodine receptors, we measured the stimulation of release by ATP (5 mM total) and caffeine (20 mM total) and the inhibition by Mg^{2+} (0.8 mM estimated free) in Cl^--free and Cl^--containing solutions. The effects of ATP, caffeine, and Mg^{2+} were the largest in K-gluconate and Tris-gluconate, intermediate in KCl, and notably poor or absent in choline-Cl and Tris-Cl. Procaine (10 mM) inhibited the caffeine-stimulated release measured in K-gluconate, whereas the Cl^- channel blocker clofibric acid (10 mM) but not procaine inhibited the caffeine-insensitive release measured in choline-Cl. Ruthenium red (20 μM) inhibited release in all solutions. In SR fused to planar bilayers we identified a nonselective Cl^- channel ($P_{Cl}:P_{Tris}:P_{Ca} = 1:0.5:0.3$) blocked by ruthenium red and clofibric acid but not by procaine. These conductive and pharmacological properties suggested the channel was likely to mediate Cl^--dependent SR Ca^{2+} release. The absence of a contribution of ryanodine receptors to the Cl^--dependent release were indicated by the lack of an effect of Cl^- on the open probability of this channel, a complete block by procaine, and a stimulation rather than inhibition by clofibric acid. A plug model of Cl^--dependent release, whereby Cl^- removed the inhibition of the nonselective channel by large anions, was formulated under the assumption that nonselective channels and ryanodine receptor channels operated separately from each other in the terminal cisternae. The remarkably large contribution of Cl^- to the SR Ca^{2+} permeability suggested that nonselective Cl^- channels may control the Ca^{2+} permeability of the SR in the resting muscle cell.

INTRODUCTION

The anion Cl^- has been shown to have a profound effect on excitation-contraction coupling of skeletal muscle. Exposure of a skinned skeletal muscle fiber bathed in a Cl^--free solution to Cl^- results in a release of stored Ca^{2+} as inferred by a sudden contracture (Constantin and Podolsky, 1967; Endo and Nakajima, 1973; Stephenson, 1985; Donaldson, 1985; Lamb et al., 1993). This effect is caused by a Cl^--induced depolarization of the transverse tubular membrane followed by transmission to the sarcoplasmic reticulum (SR) membrane of the physiological Ca^{2+} release signal. Analogous experiments have been described in isolated triads consisting of a microsomal preparation enriched in SR terminal cisternae with attached transverse tubules. In the most refined protocol described by Ikemoto et al., (1984), the transverse tubular membrane is polarized by recovery of the Na^+ and K^+ gradients in a Cl^--free solution, and a depolarization is mimicked by replacement of the impermeant anion with Cl^-. This manipulation causes a release of Ca^{2+} from the attached SR terminal cisternae, which has been suggested to occur, as in the skinned muscle protocols, by depolarization of prepolarized transverse tubules (Ikemoto et al., 1984; 1992).

Biochemical studies have shown that Cl^- is highly permeable across the SR membrane (Kometani and Kasai, 1978) and that the anion increases the SR Ca^{2+} permeability in preparations that have a low density of attached transverse tubules such as "light" and "heavy" SR (Kasai and Miyamoto, 1973, 1976; Meissner and McKinley, 1976; Beeler et al., 1979; Ohnishi, 1979; Campbell and Shamoo, 1980; Caswell and Brandt, 1981; Miyamoto and Racker, 1982; Hasselbach and Migala, 1992). A mechanism by which Cl^- may increase the Ca^{2+} permeability of the SR, and which does not involve signal transmission across the triadic junction, was suggested to be a direct SR depolarization (reviewed by Martonosi, 1984). This hypothesis was prompted by observations made in SR loaded with Ca^{2+} in the absence of ionic pump activity that would polarize the transverse tubular membrane. Thus replacement of the extravesicular solution of a passively loaded SR suspension equilibrated in a Cl^--free solution (typically K-gluconate or K-methanesulfonate, or MSF), by a new solution containing Cl^- (typically KCl), resulted in a release of the SR Ca^{2+} content (Kasai and Miyamoto, 1976). The relevance of this observation was later dismissed, as it may have originated from osmotically driven influx of water causing a rupture of SR vesicles (Meissner and McKinley, 1976). Less attention was given to the fact, however, that Ca^{2+} efflux was augmented by Cl^-, albeit less than in the case of the K-gluconate (or K-MSF) to KCl exchange protocol, when Cl^- was present on both sides of the SR membrane all the time (Fig. 1 of Kasai and Miyamoto, 1976; Table 2 of Meissner and McKinley,

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1976). The ionic basis of the Cl^--dependent increase of the SR Ca^{2+} permeability observed in symmetrical solutions of Cl^- is the focus of the present work.

We characterized the influence of Cl^- on Ca^{2+} release from rabbit junctional SR with an interest in establishing whether ryanodine receptors can be modulated by Cl^-. If this is the case, the Cl^--dependent release observed when the SR membrane is exposed to the anion might be explained as a direct effect of Cl^- on the activity of ryanodine receptor channels. On the contrary, we show that the Cl^--dependent SR Ca^{2+} release has a ligand dependence markedly different from that of the ryanodine receptor. Planar bilayer recordings further suggested that a nonselective Cl^- channel, but not the ryanodine receptor channel, had a pharmacological profile appropriate to mediated Cl^--dependent Ca^{2+} release. Part of these results have been published in abstract form (Sukhareva and Coronado, 1993, 1994; Coronado et al., 1994).

MATERIALS AND METHODS

Isolation of rabbit skeletal muscle junctional SR

SR sedimenting in 36% sucrose (w/v) was prepared from fresh rabbit leg and back muscle by discontinuous sucrose density gradient centrifugation (Valdivia et al., 1992b). A total of 15 preparations, each from a separate animal, were used in this study. SR was stored frozen at −80°C in 10% (w/v) sucrose, 0.1 M KCl, 5 mM Na-PIPES, pH 6.8.

[^{3}H]ryanodine binding assay

Duplicate samples (50 μg protein each) were incubated for 120 min at 36°C in 0.1 ml of 7 nM [^{3}H]ryanodine, 0.15 M choline-Cl, 1.25 mM EGTA plus 1 mM Ca(acetate)$_2$, 20 mM Tris-HEPES pH 7.2. The estimated free Ca^{2+} of this solution was ≈1 μM. Other details of the assay were described elsewhere (El-Hayek et al., 1993). Specific [^{3}H]ryanodine binding to the junctional SR fraction under optimal conditions (1 M KCl, 100 μM Ca^{2+}, 5 mM ATP, 100 nM [^{3}H]ryanodine) was previously shown to be 15.5 ± 2.3 pmol/mg (El-Hayek et al., 1993).

$^{45}Ca^{2+}$ content of junctional SR equilibrated in Cl^--free and Cl^--containing solutions

SR samples were equilibrated passively with $^{45}Ca^{2+}$ prepared in each of the following univalent salts (C^+A^-): K-gluconate, Tris-gluconate, Tris-Cl, choline-Cl, or KCl. To remove the sucrose and salt used for SR storage, a thawed aliquot of SR was diluted in 10 volumes of 150 mM C^+A^- and 20 mM Tris-HEPES pH 7.2 and was kept on ice for 1 h. SR was pelleted by centrifugation for 10 min in a benchtop centrifuge (Eppendorf-Brinkmann Instruments, Westbury, NY) at 12,000 rpm and resuspended at a protein concentration of ≈1.5 mg/ml in a $^{45}Ca^{2+}$ loading solution composed of 150 mM C^+A^-, 5 mM ^{45}Ca(acetate)$_2$ (2,000 cpm/nmol), 20 mM Tris-HEPES pH 7.2. Passive loading of $^{45}Ca^{2+}$ was achieved by incubation of the SR sample at room temperature for 2 h. SR samples were washed and loaded with $^{45}Ca^{2+}$ in each of the five univalent salts C^+A^- indicated above. The total $^{45}Ca^{2+}$ content at the end of the 2-h loading period was determined by rapid filtration of aliquots in duplicate for a nominal time of 2 ms at 4 ml/s filtration rate. The filtration solution was composed of 150 mM C^+A^-, 6 mM Mg(acetate)$_2$, 20 μM ruthenium red, 20 mM Tris-HEPES pH 7.2. Filters were rinsed as described below. In experiments described in Figs. 1 and 2 and Table 1, the total $^{45}Ca^{2+}$ content in SR equilibrated in K-gluconate was 59.1 ± 15.5 nmol/mg (n = 30; N = 10) (N is the number of SR preparations; n is the number of $^{45}Ca^{2+}$ loadings); in Tris-gluconate, 49.8 ± 9.0 nmol/mg (n = 17; N = 3); in Tris-Cl, 49.1 ± 22 nmol/mg (n = 18; N = 3); in choline-Cl, 71.7 ± 16.6 nmol/mg (n = 24; N = 12); and in KCl, 69.2 ± 14.2 nmol/mg protein (n = 26; N = 6). The average SR internal volume was 16 μl/mg estimated with [^{3}H]sucrose and was approximately the same in SR equilibrated in each salt. The total $^{45}Ca^{2+}$ content of SR in other experiments is described in the corresponding figure legend.

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

Ca^{2+} release from SR was measured by rapid filtration, a technique in which $^{45}Ca^{2+}$-loaded SR is bound to a nitrocellulose filter and the extravesicular solution bathing the SR is changed by forcing a new solution through the filter (Dupont, 1984; Valdivia et al., 1992b; Hayek et al., 1993). Experiments were done in a Biologic Rapid Filtration Apparatus (Biologic Instruments, Echirolles, France) at a constant filtration rate of 4 ml/sec using 0.8 μm Millipore type AA filters (Bedford, MA). Before each filtration, the extravesicular Ca^{2+} was lowered to an estimated 7 nM free Ca^{2+} by dilution of 40 μl of $^{45}Ca^{2+}$-loaded sample (≈6 μg protein) into 1 ml of 5 mM Mg(acetate)$_2$, 1 mM Na$_2$EGTA, 150 mM C^+A^-, 20 mM Tris-HEPES pH 7.2. The rapid filtration solution contained 150 mM C^+A^- of the same composition used for $^{45}Ca^{2+}$ loading, 1.25 mM Na$_2$EGTA, 1 mM Ca(acetate)$_2$ (estimated 0.4–0.8 μM free Ca^{2+} depending on salt composition), pH 7.2. Ligands to stimulate or inhibit $^{45}Ca^{2+}$ release were added to the rapid filtration solution as indicated in the text. After rapid filtration, all filters were rinsed under mild vacuum with 2 ml of 150 mM K-gluconate, 6 mM Mg(acetate)$_2$, 20 μM ruthenium red, 20 mM Tris-HEPES pH 7.2. Filters were counted for $^{45}Ca^{2+}$ content in 4 ml of scintillation fluid. All filtrations were made in duplicate and averaged. Background $^{45}Ca^{2+}$ was evaluated using ionophore A23187 as described (Valdivia et al., 1992b) and was subtracted from each filter.

Rapid filtration solutions

Solutions for SR wash, loading, dilution, and release were made separately for each of the five univalent salts C^+A^- of Table 1. SR sucrose wash

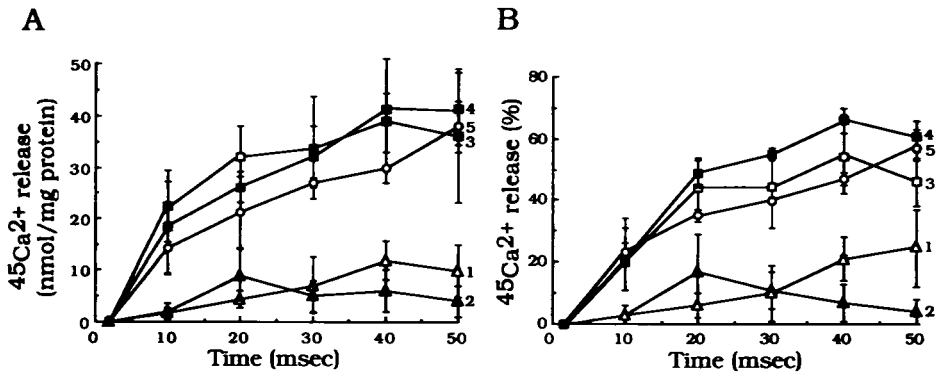

FIGURE 1 Time-resolved efflux of $^{45}Ca^{2+}$ in Cl^--free and Cl^--containing solutions. SR samples were separately equilibrated in $^{45}Ca^{2+}$ loading solutions containing 150 mM C^+A^-. Release was initiated by rapid filtration with reference solution consisting of the same 150 mM C^+A^-. Curve 1 (△): K-gluconate. Curve 2 (▲): Tris-gluconate. Curve 3 (□): Tris-Cl. Curve 4 (■): choline-Cl. Curve 5 (○): KCl. The right and left panels are representations of the same data in nmol/mg $^{45}Ca^{2+}$ release or percent release after exposure to reference solution at t = 0.

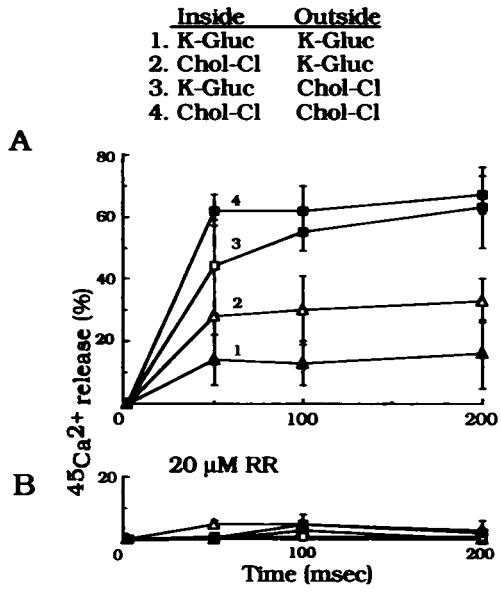

FIGURE 2 Stimulation of Cl^--dependent SR Ca^{2+} release by external Cl^-. SR samples were separately equilibrated in $^{45}Ca^{2+}$-loading solutions containing 150 mM K-gluconate (curves 1 and 3) or 150 mM choline-Cl (curves 2 and 4). (*A*) Release was initiated by rapid filtration with reference solution containing 150 mM K-gluconate (curves 1 and 2) or 150 mM choline-Cl (curves 3 and 4). (*B*) Release was initiated by the same reference solutions containing 20 μM ruthenium red (RR).

solution was composed of 150 mM C^+A^-, 20 mM Tris-HEPES pH 7.2. SR $^{45}Ca^{2+}$ loading solution was composed of 150 mM C^+A^-, 5 mM $Ca(acetate)_2$ plus ≈2,000 cpm/nmol $^{45}CaCl_2$, 20 mM Tris-HEPES pH 7.2. Dilution solution used before rapid filtration was composed of 150 mM C^+A^-, 5 mM $Mg(acetate)_2$, 1 mM Na_2EGTA, 20 mM Tris-HEPES pH 7.2. Rapid filtration solution without ligands other than micromolar free Ca^{2+} was designated reference solution and was composed of 150 mM C^+A^-, 1.25 mM Na_2EGTA, 1 mM $Ca(acetate)_2$, 20 mM Tris-HEPES pH 7.2. Filter rinse solution was composed in all cases of 150 mM K-gluconate, 6 mM $Mg(acetate)_2$, 20 μM ruthenium red, 20 mM Tris-HEPES pH 7.2. In some experiments, the reference solution was supplemented with the stimulatory or inhibitory ligands as described in the text and in Table 1. Solutions containing 150 mM Tris-Cl and 150 mM Tris-gluconate were prepared by mixing 150 mM HCl or 150 mM gluconic acid and solid Tris base to reach pH 7.2. Solutions containing 150 mM K-gluconate, choline-Cl, or KCl were prepared from solid salts.

Calibration of free Ca^{2+}

A Ca^{2+} electrode (Orion Research Co., Boston, MA) was used to estimate the free Ca^{2+} of solutions. To calibrate the Ca^{2+} electrode we used Ca-EGTA buffers calculated by a computer program (Fabiato, 1988) prepared in 150 mM KCl, 20 mM Tris-HEPES pH 7.2. The electrode response was slightly nonlinear in the range of 0.1–10 μM free Ca^{2+} with a slope of 25 mV/pCa unit. Ca^{2+} release reference solutions of nominal 1 μM free Ca^{2+} were prepared for each of the univalent salts of interest by mixing 1.25 mM Na_2EGTA and 1 mM $Ca(acetate)_2$ in 150 mM C^+A^-, 20 mM Tris-HEPES pH 7.2. Voltage output from the Ca^{2+} electrode immersed in each reference solution was: KCl (−120 ± 4 mV), choline-Cl (−131 ± 2 mV), Tris-Cl (−127 ± 1 mV), Tris-gluconate (−117 ± 1 mV), and K-gluconate (−117 ± 2 mV). Using the Ca^{2+} electrode voltage versus pCa curve calibrated in 150 mM KCl, the estimated free Ca^{2+} of the Cl^--free and Cl^--containing solutions were as follows: KCl (1 μM), choline-Cl (0.4 μM), Tris-Cl (0.5 μM), Tris-gluconate (0.8 μM), and K-gluconate (0.8 μM). The free Ca^{2+} concentrations of these solutions were considered closely matched and were not corrected further. Reference solution prepared in 150 mM choline-Cl with free Ca^{2+} adjusted to give a Ca^{2+} electrode reading of −120 mV released the same percentage of SR $^{45}Ca^{2+}$ as a noncorrected choline-Cl reference solution with a Ca^{2+} electrode reading of −131 mV, respectively 56 ± 2% and 60 ± 2% $^{45}Ca^{2+}$ release.

Planar bilayer recordings

Planar bilayer formation and recording was described previously (Coronado et al., 1992; Valdivia et al., 1992a). Bilayers were composed of equimolar concentrations of phosphatidyl ethanolamine and serine dissolved in decane (Aldrich Chemical Co., Milwaukee, WI). SR was added to the *cis* solution at a final concentration of ≈100 μg/ml. Recordings of ryanodine receptors were made in *cis* (cytosolic) solution composed of 250 mM CsCl (or 240 mM $CsCH_3SO_3$, 10 mM CsCl) and 10 mM HEPES titrated with Tris to pH 7.2. The *trans* (lumenal) solution was 50 mM CsCl (or 40 mM $CsCH_3SO_3$, 10 mM CsCl) and 10 mM HEPES titrated with Tris to pH 7.2. The contaminant-free Ca^{2+} of the *cis* chamber was in the range of 1–3.6 μM and was measured by Ca^{2+} electrode. Records were low-pass filtered at 1 KHz and digitized at 3 KHz. Recordings of Cl^- channels were made in *cis* solution composed of 450 mM or 150 mM HCl titrated with Tris base to pH

TABLE 1 Ca^{2+} Release from junctional sarcoplasmic reticulum induced by Cl^--containing and Cl^--free solutions

Salt	Reference solution plus ligand	Release at 10 ms (nmol Ca^{2+}/mg)	Release at 50 ms (nmol Ca^{2+}/mg)
K-gluc (n = 7)	RF	1.7 ± 1	9.8 ± 5
	+ ATP	5.5 ± 3	55.8 ± 10
	+ caffeine	10.8 ± 3	32.1 ± 5
	+ ATP + Mg^{2+}	4.0 ± 3	12.6 ± 1
	+ ATP + RR	2.8 ± 3	7.3 ± 1
	+ RR	N.D.	0.15 ± 0.1
Tris-gluc (n = 3)	RF	1.9 ± 2	4.9 ± 4
	+ ATP	6.9 ± 4	32.0 ± 2
	+ caffeine	14.9 ± 9	30.8 ± 12
	+ ATP + Mg^{2+}	5.6 ± 3	9.1 ± 3
	+ ATP + RR	3.2 ± 2	6.1 ± 4
Tris-Cl (n = 3)	RF	24.4 ± 12	41.3 ± 10
	+ ATP	40.4 ± 4	58.1 ± 2
	+ caffeine	19.9 ± 3	48.0 ± 11
	+ ATP + Mg^{2+}	26.6 ± 5	63.7 ± 3
	+ ATP + RR	4.2 ± 3	9.2 ± 5
Chol-Cl (n = 8)	RF	18.3 ± 9	41.4 ± 7
	+ ATP	29.8 ± 3	55.8 ± 6
	+ caffeine	21.1 ± 5	47.0 ± 7
	+ ATP + Mg^{2+}	14.8 ± 5	45.9 ± 10
	+ ATP + RR	7.2 ± 2	12.2 ± 2
	+ RR	1.5 ± 1	1.0 ± 0.4
KCl (n = 4)	RF	13.7 ± 5	34.4 ± 1
	+ ATP	30.4 ± 13	46.2 ± 19
	+ caffeine	13.7 ± 6	45.1 ± 4
	+ ATP + Mg^{2+}	29.8 ± 6	55 ± 14
	+ ATP + Mg^{2+} (15 mM)	2.5 ± 2	6.5 ± 2
	+ ATP + RR	3.0 ± 2	7.5 ± 3

$^{45}Ca^{2+}$ release at 10 ms and 50 ms was measured in reference solution (RF) consisting of 150 mM C^+A^-, 1 mM $Ca(acetate)_2$, 1.25 mM Na_2EGTA (1 μM free Ca^{2+}), 20 mM Tris-HEPES pH 7.2 plus the following ligands: 5 mM total Na_2ATP (ATP), 20 mM total caffeine (caffeine), 5.1 mM total Mg $(acetate)_2$ plus 5 mM total Na_2ATP to yield 0.8 mM free Mg^{2+} (ATP + Mg^{2+}), and 5 mM Na_2ATP plus 20 μM total ruthenium red (ATP + RR). In K-gluc and chol-Cl solutions ruthenium red was also tested in reference solution without ATP (RR). In KCl solutions, Mg^{2+} was also tested at a total concentration of 20.3 mM $Mg(acetate)_2$ plus 5 mM total Na_2ATP to yield 15 mM free Mg^{2+}. n is number of determinations in duplicate at each time. N.D., not detectable.

7.2. The *trans* solution was composed of 50 mM HCl titrated with Tris base to pH 7.2 in all cases. In some experiments, the *cis* solution was composed of 220 mM $CaCl_2$ and 10 mM HEPES-Tris pH 7.2, whereas the *trans* solution was 20 mM $CaCl_2$ and 10 mM HEPES-Tris pH 7.2. Records were low-pass filtered at 0.1 KHz and digitized at 0.5 kHz.

Clofibric acid and procaine solutions

Stock solutions of clofibric acid and procaine were prepared in 95% methanol at concentrations of 300 mM and 1 M, respectively. In planar bilayer recordings, the final concentration of methanol in the *cis* planar bilayer solution at 10 mM drug concentration was ≈1–3% (v/v). In control recordings, methanol had no effect on single channel activity up to a final *cis* solution concentration of ≈5% (v/v). The stock clofibric acid solution was neutralized with solid Tris base, and the stock solution of procaine was neutralized with HCl. Appropriate controls were performed with a pH electrode. In rapid filtrations, the pH of reference solutions containing clofibric acid or procaine were adjusted at the moment of use.

Chemicals and abbreviations

Na_2ATP, procaine, and clofibric acid were from Sigma Chemical Co. (St. Louis, MO). Ruthenium red (ruthenium III chloride oxide) was from Alpha Products (Andover, MA). $^{45}Ca^{2+}$ (1 Ci/mmol) was from Du Pont-New England Nuclear (Wilmington, DE). Potassium chloride, choline chloride, potassium gluconate, cesium chloride, cesium MSF, calcium acetate, magnesium acetate, Tris base and gluconic acid were from Sigma Chemical Co. Choline chloride was purchased monthly, and older batches were discarded. EGTA (ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N',N'*-tetra acetic acid); HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid); MES (2-[*N*-morpholino] ethane-sulfonic acid); PIPES (piperazine-*N,N'*-bis-2-ethanesulfonic acid); Tris (Tris[hydroxymethyl] aminomethane).

Protein assay

Protein concentration was determined using a Bio-Rad Kit (Richmond, CA) with bovine serum albumin as standard.

RESULTS

Ca^{2+} release rate in the presence and absence of Cl^-

As shown in Fig. 1 we investigated the effect of Cl^- on the SR Ca^{2+} permeability by comparing the amount of $^{45}Ca^{2+}$ released as a function of time in five samples of SR, each separately equilibrated in a Cl^--free (curves 1, 2) or a Cl^--containing (curves 3, 4, 5) univalent salt solution. The total $^{45}Ca^{2+}$ content was approximately the same in all cases, and the values were indicated in Materials and Methods. Release was stimulated by a rapid change in extravesicular 7 nM free Ca^{2+} and 5 mM total Mg^{2+} to extravesicular 0.4–1 μM free Ca^{2+} and nominally 0 Mg^{2+}, as delivered by the reference solution. The composition and concentration of the univalent salt inside the SR and in the reference solution was the same in each case. The $^{45}Ca^{2+}$ content at $t = 0$ ms was established by delivering the reference solution to the SR sample for a nominal time of 2 ms. The $^{45}Ca^{2+}$ content at $t = 0$ did not change when 20 μM ruthenium red was included in the reference solution during the filtration. Comparison of the five curves indicated that Ca^{2+} release proceeded much faster, and reached a greater percentage of the total $^{45}Ca^{2+}$ initially loaded into the lumen, when the SR and the reference solution contained 150 mM Cl^-. In SR equilibrated in KCl (Fig. 1, curve 5), release reached ≈0.012 μmol $^{45}Ca^{2+}$/mg in 10 ms (*A*) and was equivalent to ≈20% of total $^{45}Ca^{2+}$ content (*B*). Release in the same solution was ≈0.035 μmol $^{45}Ca^{2+}$/mg at 50 ms and was equivalent to ≈50% of the total $^{45}Ca^{2+}$ content. The total $^{45}Ca^{2+}$ released from KCl-equilibrated SR during a filtration time of 9 s, the longest time attainable by the instrument, was typically 65–70% of the $^{45}Ca^{2+}$ content at $t = 2$ ms (not shown). The data shown in Fig. 1 indicated that $^{45}Ca^{2+}$ release from this pool in KCl-equilibrated SR was extremely fast and was almost complete in 50 ms, the longest filtration time employed here. The release rate in KCl, after subtraction of a background release measured in the presence of ruthenium red (Table 1), varied between 1.1 μmol Ca^{2+} mg^{-1} s^{-1} in the interval between 2 and 10 ms and 0.5 μmol Ca^{2+} mg^{-1} s^1 in the interval between 20 and 50 ms. The release rates in KCl were in agreement with reports of others using the same technique (Sumbilla and Inesi, 1987; Calviello and Chiesi, 1989). A high rate of release, like that in KCl, was also observed when SR was equilibrated in choline-Cl (curve 4) or Tris-Cl (curve 3). This result demonstrated that the chemical nature (organic versus inorganic) of the monovalent cation bathing the SR did not greatly affect the Ca^{2+} release rate. However, a significantly lower rate of release was observed when Cl^- was replaced by gluconate$^-$ with either $Tris^+$ (curve 2) or K^+ (curve 1) as cations. In both instances, the release was no more than 20% of the $^{45}Ca^{2+}$ content at 50 ms. The release rate in K-gluconate was approximately linear in the interval between 2 and 50 ms and was ≈0.15 μmol mg^{-1} s^{-1}, a value 3–7 times lower than those measured in KCl.

The difference in release rates between the Cl^--free and the Cl^--containing solutions could not be explained by differences in the free Ca^{2+} of the reference solution nor by differences in the $^{45}Ca^{2+}$ content of the different preparations. For example, the free Ca^{2+} of the reference solution in K-gluconate and KCl were closely matched (0.8 μM and 1.0 μM, respectively); however, the releases at 10 ms differed ≈8-fold. In another case, the $^{45}Ca^{2+}$ content of SR preparations equilibrated in Tris-gluconate and Tris-Cl were identical (49.8 ± 9 nmol/mg and 49.1 ± 22 nmol/mg, respectively), and the free Ca^{2+} of the respective reference solutions were nearly identical (0.8 vs. 0.5 μM; see Materials and Methods), yet releases at 10 ms differed ≈13-fold. Furthermore, when the free Ca^{2+} of the reference solution in 150 mM choline-Cl was matched by Ca^{2+} electrode to that of the reference solution in 150 mM K-gluconate, the release in choline-Cl was unchanged (see Materials and Methods).

To better define the mechanism of Ca^{2+} release stimulated by Cl^-, we investigated whether Cl^- enhanced the release rate by interacting with a preferential face of the SR membrane. This was done, as shown in Fig. 2, by exposing SR equilibrated in K-gluconate or choline-Cl^- to reference solutions of either kind. In these experiments, we extended the time course of release from 50 to 200 ms to ensure that a component of release was not inadvertently missed by the

shorter time scale chosen for the previous figure. Curves 1 and 4 of Fig. 2 *A* represent releases from SR containing K-gluconate or choline-Cl on both sides of the membrane. The small release in the Cl^--free salt and the much larger release in the Cl^--containing salt were essentially completed in 50 ms, and the SR $^{45}Ca^{2+}$ content was invariant with time during the next 150 ms. Exposure of SR without Cl^- in the lumen to external Cl^- (curve 3) resulted in an increase in the release rate. The extent of release at 200 ms was similar to that observed in symmetrical Cl^- solutions. On the other hand, exposure of SR containing Cl^- in the lumen to external Cl^--free conditions (curve 2) resulted in a much smaller release than that induced by external Cl^-. Controls shown in Fig. 2 *B* indicated that ruthenium red blocked release in all cases. From these results we concluded that external Cl^- was much more effective than internal Cl^- in increasing the SR Ca^{2+} permeability. Because Cl^- was more effective in releasing Ca^{2+} when present on the membrane side opposite to that where Ca^{2+} is stored, a mechanism of stimulation of release in which Cl^- movement compensated for the charge movement of Ca^{2+} was considered unlikely.

As shown in Fig. 3, we further investigated the contribution of charge compensation and two other possible mechanisms, Ca^{2+}-Ca^{2+} change and a nonspecific SR leakage, to Ca^{2+} release stimulated by Cl^-. As shown in Fig. 3 *A*, the relatively slower and smaller release of $^{45}Ca^{2+}$ observed in SR equilibrated in K-gluconate could not be increased by 1 μg/ml valinomycin (curves 1 and 3 of Fig. 3 *A*). As this is a large concentration of K^+ carrier, which increased the K^+ conductance of a planar bilayer at least five orders of magnitude (not shown), this result suggested that Ca^{2+} fluxes in the Cl^--free solution were not kinetically limited by the movement of charge across the SR membrane that is necessary to compensate the Ca^{2+} flux. Similarly, no effect of valinomycin was observed in symmetrical solutions of KCl (curves 2 and 4 of Fig. 3 *A*) or when K-gluconate was present in the SR lumen and choline-Cl was present in the myoplasmic face (curves 5 and 6 of Fig. 3 *B*). The latter result is also significant because it argues against the development of a significant membrane diffusion potential during the stimulation of release by Cl^-. We also considered the possibility that the Ca^{2+} flux stimulated by Cl^- did not represent a net flux but corresponded to a Ca^{2+}-Ca^{2+} exchange reaction of the type catalyzed by the SR Ca^{2+} pump. If this were the case, K^+-valinomycin would not have stimulated release because there was no net movement of charge. To determine the contribution of Ca^{2+}-Ca^{2+} exchange to the total flux, isotopic $^{45}Ca^{2+}$ was added to the reference solution at the same specific activity present in the $^{45}Ca^{2+}$ loading solution. If Ca^{2+}-Ca^{2+} exchange were stimulated by Cl^-, the total Ca^{2+}

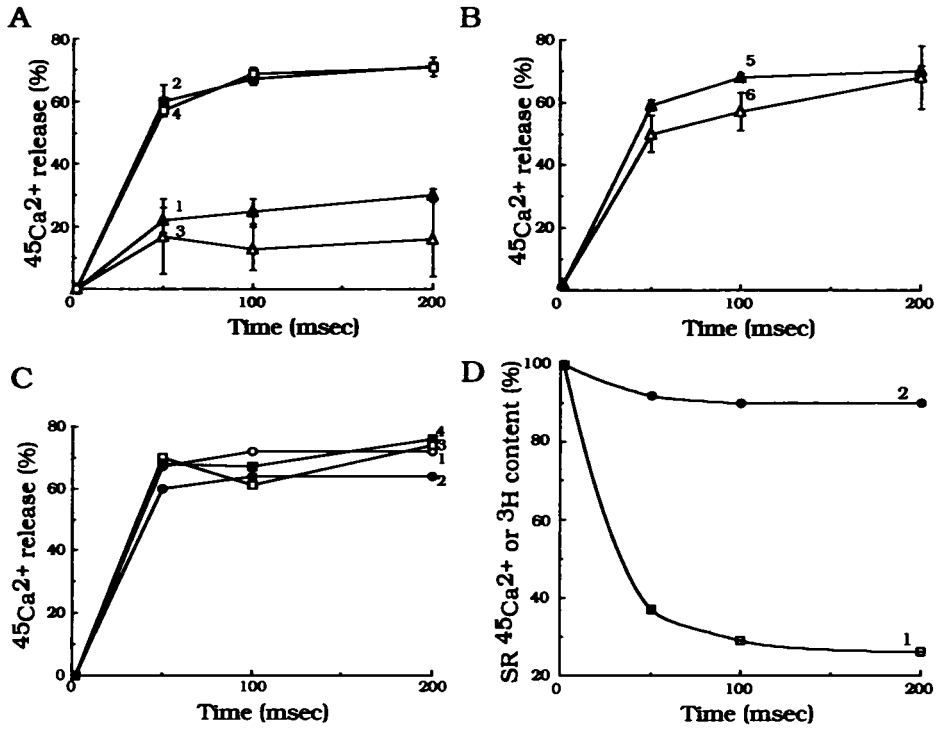

FIGURE 3 Controls of Cl^--dependent Ca^{2+} release in symmetrical and asymmetrical monovalent solutions. (*A*,*B*) SR samples were separately equilibrated in $^{45}Ca^{2+}$ loading solutions containing 150 mM K-gluconate (curves 1, 3, 5, and 6) or 150 mM KCl (curves 2 and 4). Release was initiated by rapid filtration with reference solution containing 150 mM K-gluconate (curves 1 and 3), 150 mM KCl (curves 2 and 4) or 150 mM choline-Cl (curves 5 and 6). Reference solutions contained 1 μg/ml valinomycin in curves 1, 2, and 5. (*C*) SR was equilibrated in $^{45}Ca^{2+}$ loading solution containing 150 mM choline-Cl (curves 1 through 4). Release was initiated by rapid filtration with reference solutions containing 150 mM choline-Cl, 1.25 mM Na_2EGTA, 1 mM $Ca(acetate)_2$ (curves 1 and 3) or 150 mM choline-Cl, 1.25 mM Na_2EGTA, 1 mM $Ca(acetate)_2$ plus 2000 cpm/nmol $^{45}Ca^{2+}$ (curves 2 and 4). Reference solutions contained 10 μM thapsigargin in curves 3 and 4. (*D*) SR samples were separately equilibrated in $^{45}Ca^{2+}$ loading solution containing 150 mM K-gluconate (curve 1) or 150 mM K-gluconate with nonisotopic 5 mM $Ca^{2+}(acetate)_2$ plus 10 mM[^{3}H]sucrose (curve 2). Release was initiated by rapid filtration with reference solution containing 150 mM choline-Cl (curves 1 and 2).

flux would be reduced by external isotopic $^{45}Ca^{2+}$, because the probability of exchange of a hot or cold Ca^{2+} from the lumen with a hot or cold Ca^{2+} from the external solution would be the same (Sumbilla and Inesi, 1987). On the contrary, releases in SR equilibrated in choline-Cl were the same in the absence or in the presence of external isotope (curves 1 and 2 of Fig. 3 *C*) and in the absence or presence of the Ca^{2+} pump inhibitor thapsigargin (curves 3 and 4 of Fig. 3 *C*). These results indicated that the release stimulated by Cl^- represented a net Ca^{2+} flux and was not caused by a Ca^{2+}-Ca^{2+} exchange reaction. Other Ca^{2+} pump inhibitors that had no effect on the Cl^--dependent release were cyclopiazonic acid (100 μM) and quercetin (20 μM), indicating that the Ca^{2+} pump was unlikely to mediate the observed changes in Ca^{2+} permeability. Finally, the Cl^--induced release did not represent a nonspecific leakage, given that other compounds trapped in the SR lumen such as [^{3}H]sucrose (Fig. 3 *D*, curve 2) could not be released after exposure of SR to Cl^-.

Anion selectivity of Cl^--dependent Ca^{2+} release

Inasmuch as gluconate$^-$ was used to replace Cl^- in most cases, it became important to determine whether the low Ca^{2+} permeability of the SR seen in reference solution containing gluconate$^-$ was caused by a deleterious property of the organic anion rather than by the specific absence of Cl^-. We therefore explored whether anions other than gluconate$^-$ could also reduce the SR Ca^{2+} permeability. Fig. 4 shows rapid filtrations carried out for 100 ms in SR equilibrated in $^{45}Ca^{2+}$-loading solution containing 150 mM univalent anion with $Tris^+$ as cation (*top*) or K^+ as cation (*bottom* and *inset*). Release was initiated by reference solution prepared in the same salt (*hatched bars*) or in reference solution plus 20 μM ruthenium red (*filled bars*). Ca^{2+} release was two to four times higher in the presence of Cl^- than in the presence of any other organic anion used to replace Cl^-, regardless of whether K^+ or $Tris^+$ was present in the salt. In particular, the release in the presence of MSF^- or $HEPES^-$ was essentially the same as that described above in the presence of gluconate$^-$. Furthermore, the inset indicates that Cl^- could not be replaced by other halides in the series. Thus Br^- and I^-, like the rest of the organic anions, were not able to support Ca^{2+} release to the same extent as Cl^-. The anion potency series that emerged from these studies was $Cl^- \gg HEPES^- \approx MSF^- \approx$ gluconate$^-$ > acetate$^-$ > Br^- > I^- and was essentially the same in the top ($Tris^+$) and bottom (K^+) panels of Fig. 4. From these results we concluded that Cl^- facilitated SR Ca^{2+} release and that the specific absence of Cl^- from the reference solution resulted in inhibition of a component of Ca^{2+} release.

Effect of ryanodine receptor ligands on Cl^--dependent Ca^{2+} release

To determine whether Ca^{2+} release in Cl^--free and Cl^--containing solutions occurred by activation of ryanodine receptors, in Fig 5 we compared releases in reference solution in the absence (curve 1) and presence (curves 2–5) of ryanodine receptor ligands. The ligand dependence of Ca^{2+} release measured in SR equilibrated in 150 mM K-gluconate was typical of ryanodine receptor mediated fluxes. The agonists ATP, at a concentration of 5 mM (Fig. 5 *A*, curve 2), and caffeine, at a concentration of 20 mM (curve 3), increased the release rate measured between 2 ms and 20 ms from the value measured in reference solution (0.15 μmol mg^{-1} s^{-1}) to 0.5 μmol mg^{-1} s^{-1} (3.3-fold) and 1.5 μmol mg^{-1} s^{-1} (10-fold), respectively. The inhibitor Mg^{2+} at a free concentration of 0.8 mM (curve 4) reduced the release rate stimulated by ATP in the same time range to 0.38 μmol mg^{-1} s^{-1} (4-fold). The blocker ruthenium red at a concentration of 20 μM (curve 5) reduced the ATP-stimulated release to a rate slightly lower, on the average, than that of the reference solution. Ruthenium red in the absence of ATP reduced the release rate to no more than 0.04 μmol mg^{-1} s^{-1} (Table 1). Except for the free Mg^{2+} in curve 4, all ligands were present at concentrations that produced the highest stimulation or inhibition. The stimulation by ATP was in quantitative agreement with that reported by Calviello and Chiesi (1989) using the same flux technique. Moreover, the pattern of release

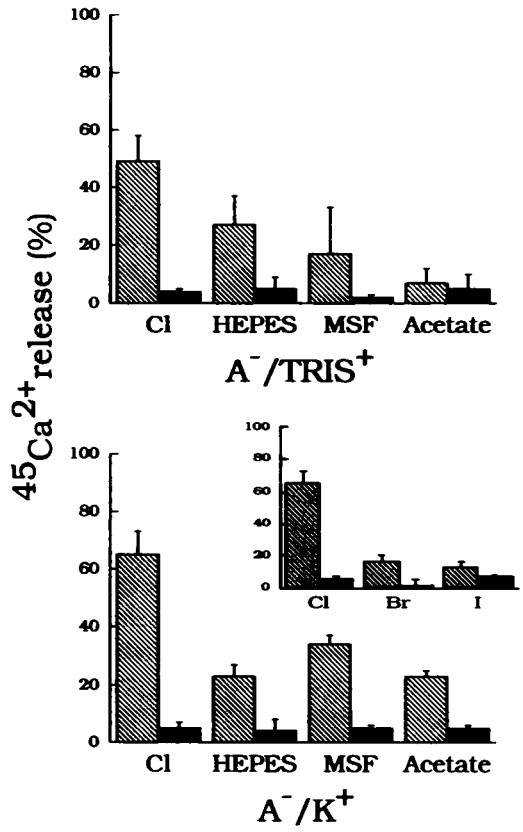

FIGURE 4 Anion selectivity of Cl^--induced SR Ca^{2+} release. SR samples were separately equilibrated in $^{45}Ca^{2+}$ loading solution containing 150 C^+A^- of the indicated composition. Rapid filtrations were carried out for 100 ms in reference solutions containing 150 mM $Tris^+A^-$ (*top*) or 150 mM K^+A^- (*inset* and *bottom*) of the same composition loaded into the SR lumen. Filled bars represent release in reference solutions containing 20 μM ruthenium red.

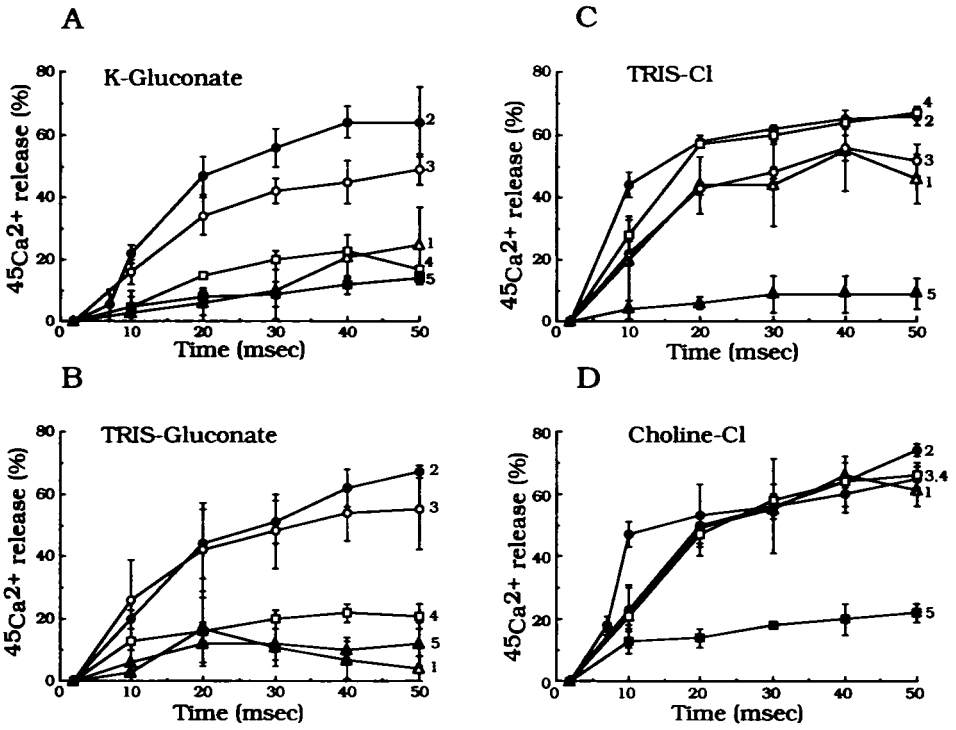

FIGURE 5 Ligand dependence of $^{45}Ca^{2+}$ release in Cl^--free and Cl^--containing reference solutions. SR samples were separately equilibrated in $^{45}Ca^{2+}$ loading solutions containing 150 mM C^+A^- indicated in each panel (*A-D*). Release was initiated by rapid filtration with reference solution containing the same 150 mM C^+A^- loaded into the SR lumen. The following ligands were added to each reference solution. Curve 1 (△): no additions. Curve 2 (●): 5 mM Na_2ATP. Curve 3 (○): 20 mM caffeine. Curve 4 (□): 5 mM Na_2ATP, 5.1 mM $Mg(acetate)_2$. Curve 5 (▲): 5 mM Na_2ATP, 20 μM ruthenium red. The $^{45}Ca^{2+}$ content at t = 2 ms of curves 1–5 of each panel was not significantly different. These data were pooled and labeled 0% release. $^{45}Ca^{2+}$ content at t = 2 ms was 59.1 ± 15.5 nmol/mg (*A*); 49.8 ± 9.0 nmol/mg (*B*); 49.1 ± 22 nmol/mg (*C*); 71.7 ± 16.6 nmol/mg (*D*).

produced by the five solutions, including the quenching of the ATP-stimulated release by ruthenium red (curve 5), agreed with the results of Meissner et al. (1986) and were consistent with a Ca^{2+} flux mediated entirely by ryanodine receptors.

Fig. 5 *B* shows that the same pattern of curves 1–5 was obtained in SR equilibrated with $Tris^+$ as the main monovalent cation, meanwhile keeping $gluconate^-$ as the main anion. This suggested that the nature of the monovalent cation was of little consequence for a ryanodine receptor-mediated SR Ca^{2+} release. On the other hand, replacement of $gluconate^-$ by Cl^- while maintaining $Tris^+$ as cation (Fig. 5 *C*) resulted in a pattern of Ca^{2+} release that was significantly different from that of ryanodine receptor-mediated release. The release rate measured in reference solution between 2 and 20 ms in SR equilibrated in Tris-Cl was ≈2 μmol mg^{-1} s^{-1}, a value 13 times higher than that measured in K-gluconate. Furthermore, ATP could only stimulate this high basal rate to ≈3.6 μmol mg^{-1} s^{-1}. In addition, the chosen free Mg^{2+} of 0.8 mM did not inhibit the ATP-stimulated release. Thus, the Mg^{2+} curve in Fig. 5 *C* (curve 4) was essentially identical to the nonstimulated release (curve 1) and to the nucleotide- and caffeine-stimulated releases (curves 2 and 3). The same change in ligand dependence was observed when $choline^+$ replaced $Tris^+$ (Fig. 5 *D*), demonstrating that these were modifications introduced by Cl^- and not by a specific Cl^-/cation pair. However, an important point of similarity with the Ca^{2+} releases from SR equilibrated in the Cl^--free solutions was the fact that releases in Tris-Cl and choline-Cl, like those in $gluconate^-$ salts, were blocked by ruthenium red (curve 5).

Average Ca^{2+} release stimulated or inhibited by ryanodine receptor ligands at the shortest (10 ms) and longest (50 ms) experimental time for SR equilibrated in each of the salts described above, and in SR equilibrated in KCl, are shown in Table 1. Releases in different salts can be compared directly without normalization because the $^{45}Ca^{2+}$ content after passive loading were approximately similar (see Materials and Methods). It is important to emphasize that ruthenium red blocked release in the presence of ATP to approximately the same extent in all salts. Ruthenium red also blocked Ca^{2+} release in Cl^--free (K-gluconate) and Cl^--containing (choline-Cl) reference solutions in the absence of ATP. This observation validated the use of ruthenium red as an effective Ca^{2+} release-quenching agent to determine the total $^{45}Ca^{2+}$ content of the SR in all cases. As the data in Table 1 indicate, the pattern of changes in the release rate expected of a ryanodine receptor-mediated release, such as the stimulation of release by ATP or caffeine and subsequent inhibition by Mg^{2+}, was much more pronounced in SR equilibrated in K-gluconate or Tris-gluconate than in SR equilibrated in KCl, which is the standard choice of univalent salt in SR $^{45}Ca^{2+}$ release experiments (Meissner et al., 1986; Palade, 1987; Calviello and Chiesi, 1989). At 50 ms, the stimulation of release by caffeine relative to the release seen in reference solution was 5.7-fold and 6.5-fold, respectively, in K-gluconate and Tris-gluconate but no more than 1.4-fold in KCl. Based on these observations we formulated the hypothesis that the total SR Ca^{2+} release was comprised of two components, a Ca^{2+} release component mediated by ryanodine receptors and a component activated when the reference solution contained Cl^-. The presence of a Cl^--dependent pathway in parallel with ryanodine receptors would explain the comparatively lower stimulation of release by ATP and caffeine observed in the presence of Cl^-.

Inhibition of Cl^--dependent release by Mg^{2+} and ruthenium red

Since Mg^{2+} and ruthenium red blocked the ryanodine receptor mediated release as well as the Cl^--mediated release, it became important to determine the apparent affinity of inhibition of each component of release by these ligands. A lower affinity of inhibition by Mg^{2+} in the presence of Cl^- was suggested by the fact that 0.8 mM free Mg^2 was sufficient to block ATP-stimulated release in Cl^--free salts, but a higher concentration was necessary to cause the same inhibition in KCl (Table 1). Fig. 6 shows dose-dependent inhibition of release in SR equilibrated in K-gluconate (curve 1) or two Cl^--containing salts, KCl (curve 2) and choline-Cl (curve 3). To achieve approximately the same release with each salt in the absence of Mg^{2+} or ruthenium red, we supplemented the reference solutions with 5 mM total Na_2ATP and increased the free Ca^{2+} to 10 μM. In these conditions, ~80% of the loaded $^{45}Ca^{2+}$ was released by each reference solution within 100 ms, which was the filtration time chosen for this study. Fig. 6 *A* shows that the half-inhibitory concentration of free Mg^{2+} in SR equilibrated in K-gluconate was ~1 mM, whereas it increased to 5–10 mM in the Cl^--containing SR. On the other hand, the half-inhibitory concentration of ruthenium red (Fig. 6 *B*) in SR equilibrated in K-gluconate was ~0.8–1 μM and increased to 3–5 μM in the Cl^--containing SR. Thus, releases in the presence of Cl^- were significantly less sensitive to inhibition by free Mg^{2+} and ruthenium red than those in Cl^--free SR.

Lack of an effect of Cl^- on the open probability ryanodine receptor channel

A stimulation of the ryanodine receptor channel by Cl^- could account for the higher concentration of Mg^{2+} required to inhibit release in the presence of Cl^-. This stems from the fact that if a given reference solution stimulated the Ca^{2+} release channel to a much higher open probability, a higher concentration of Mg^{2+} would be needed to reduce the open probability to a background level, and thus reduce the release rate. We therefore investigated whether Cl^- increased the activity of ryanodine receptor channels by comparing single channel recordings in planar bilayers in high- and low-Cl^- solutions. Fig. 7 shows two separate recordings performed in salt gradients consisting of 250 mM CsCl or 240 mM CsMSF plus 10 mM CsCl in the myoplasmic-equivalent chamber and 50 mM CsCl or 40 mM CsMSF plus 10 mM CsCl in the lumen-equivalent chamber at 0 mV holding potential. A low concentration of CsCl was deemed necessary in the case of recordings in CsMSF to stabilize the Ag/AgCl electrodes. Channel activity in the high or low Cl^- solutions was sparse and typical of skeletal-type channels (see Coronado et al., 1992). Open events were usually brief, with a population mean of 0.5–1 ms in duration. The free Ca^{2+} of the myoplasmic-equivalent solution measured by Ca^{2+} electrode was in the range of 1–4 μM and was the same in both cases. Occasional long openings are shown at the bottom of each panel to indicate that the mean open-channel current was the same in both solutions. This was expected, as the Cs^+ gradient was the same in both cases and anions do not permeate through the channel (Smith et al., 1988). In Table 2 we show the average open probability of 12 separate recordings made in each of the two solutions. Each of the 24 recordings consisted of no less than 60 s with no less than 700 open events collected from five different SR preparations. Differences between the sample means were not statistically significant and indicated that Cl^- in the concentration range of 10–240 mM had no effect on ryanodine receptor open probability. To verify that the high Cl^- solution had in fact stimulated Ca^{2+} release, we performed rapid filtrations in SR equilibrated in

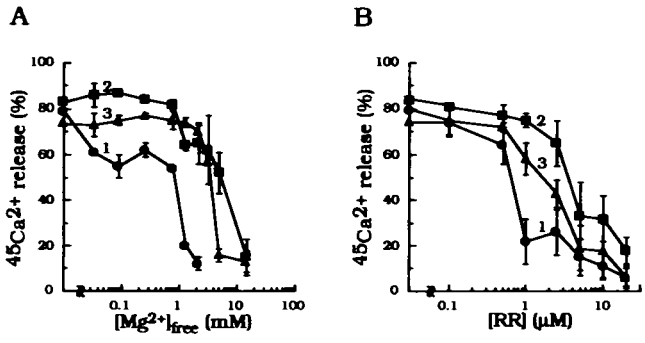

FIGURE 6 Inhibition of Cl^--dependent Ca^{2+} release by Mg^{2+} and ruthenium red. SR samples were separately equilibrated in $^{45}Ca^{2+}$ loading solution containing 150 mM K-gluconate (curve 1), 150 mM KCl (curve 2) or 150 mM choline-Cl (curve 3). Release was initiated by rapid filtration for 100 ms with reference solution containing 150 mM K-gluconate (curve 1), 150 mM KCl (curve 2), or 150 mM choline-Cl (curve 3). (*A*) Reference solution of pCa 5, 5 mM total Na_2ATP, and $Mg(acetate)_2$ to yield the specified free Mg^{2+} concentration. (*B*) Reference solution of pCa 5, 5 mM total Na_2ATP, and specified concentration of ruthenium red.

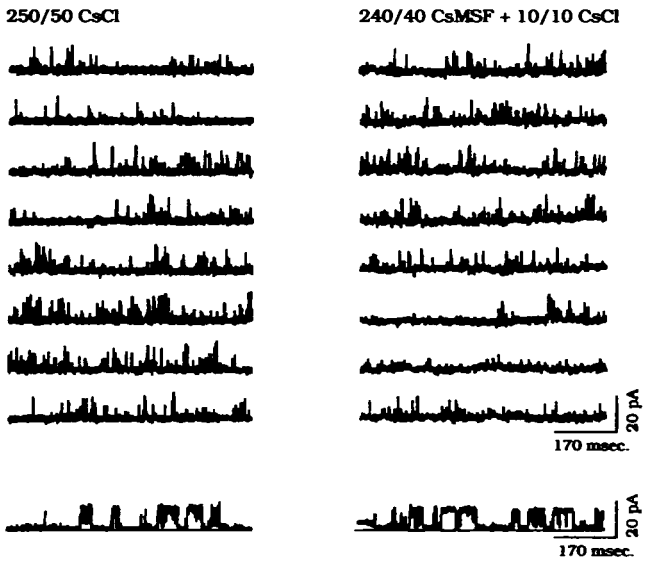

FIGURE 7 Ryanodine receptor channel recordings in low and high Cl^-. Two separate recordings are shown in *cis* 250 mM CsCl and *trans* 50 mM CsCl (*left*); and in *cis* 240 mM CsMSF, 10 mM CsCl and *trans* 40 mM CsMSF, 10 mM CsCl (*right*). Holding potential was 0 mV. Thin line under each trace indicates average baseline.

TABLE 2 Cl^--dependent Ca^{2+} release and ryanodine receptor open probability in low and high Cl^-

Reference Solution	$^{45}Ca^{2+}$ Release Flux, nmol/mg (n = 4)		
	250 mM CsMSF	240 mM CsMSF +10 mM CsCl	250 mM CsCl
pCa 6	24.5 ± 3	29.5 ± 3	57.9 ± 0.3
pCa ≈6	34.3 ± 0.4	30.6 ± 2	57.4 ± 0.2
pCa 6 + RR	3.2 ± 2	8.6 ± 2	21.3 ± 7
pCa ≈6 + RR	0.7 ± 1	16.4 ± 2	14.7 ± 5

Cis solution pCa ≈6	Ryanodine Receptor Open Probability, p_o (n = 12)	
	240 mM CsMSF +10 mM CsCl	250 mM CsCl
Po	0.10 ± 0.08	0.106 ± 0.05
Total recording time (s)	1240	1061

$^{45}Ca^{2+}$ loading solution was 5 mM $^{45}Ca(acetate)_2$, 10 mM HEPES-TRIS pH 7.2 plus the indicated CsMSF and CsCl concentration. Reference solution (pCa 6) was 1.25 mM Na_2EGTA, 1 mM $Ca(acetate)_2$, 10 mM HEPES-TRIS pH 7.2 plus the indicated CsMSF and CsCl concentration. Release solution with contaminant-free Ca^{2+} (pCa ≈6) was 10 mM HEPES-TRIS pH 7.2 plus the indicated CsMSF and CsCl concentration without EGTA or added Ca $(acetate)_2$. The total $^{45}Ca^{2+}$ content of SR equilibrated in loading solution containing 250 mM CsMSF was 87 ± 2 nmol/mg. In SR equilibrated in 240 CsMSF plus 10 mM CsCl was 75 ± 16 nmol/mg. In SR equilibrated in 250 mM CsCl was 89 ± 11 nmol/mg. Ruthenium red (RR) concentration was 20 μM and was added to the reference solution. n, number of rapid filtrations (t = 100 ms) each made in duplicate, or number of planar bilayer recordings each containing a single ryanodine receptor channel. *Cis* and *trans* planar bilayer recording solutions were described in Fig. 4.

the same high- and low-Cl^- solutions used for single channel recordings. Table 2 showed that $^{45}Ca^{2+}$ release in SR equilibrated in 250 mM $CsCl^-$ was significantly higher than that of SR equilibrated in 240 mM CsMSF plus 10 mM $CsCl^-$. In addition, $^{45}Ca^{2+}$ release in the low Cl^- solution was similar to that observed in 250 mM CsMSF in the absence of added Cl^-. These results indicated that Cl^--dependent Ca^{2+} release was indeed stimulated in the high-Cl^- solution but was not present in the low-Cl^- solution. If the ryanodine receptor had mediated the Cl^--dependent release detected by rapid filtration in the high-Cl^- solution, the open probability of channels recorded in this solution should have been higher than that of channels recorded in the low-Cl^- solution. This was not the case; therefore, the Cl^--dependent release was unlikely to have originated from Cl^--mediated changes in ryanodine receptor open probability.

Inhibition of Cl^--dependent Ca^{2+} release by clofibric acid and not by procaine

A pharmacological separation of the Cl^--dependent Ca^{2+} release and the ryanodine receptor-mediated Ca^{2+} release was possible using the local anesthetic procaine, which is known to block ryanodine receptors (Xu et al., 1993), and clofibric acid, which is known to block muscle Cl^- channels (De Luca et al., 1992). To separate the relative contribution of the ryanodine receptor and non-ryanodine receptor pathways, ex-

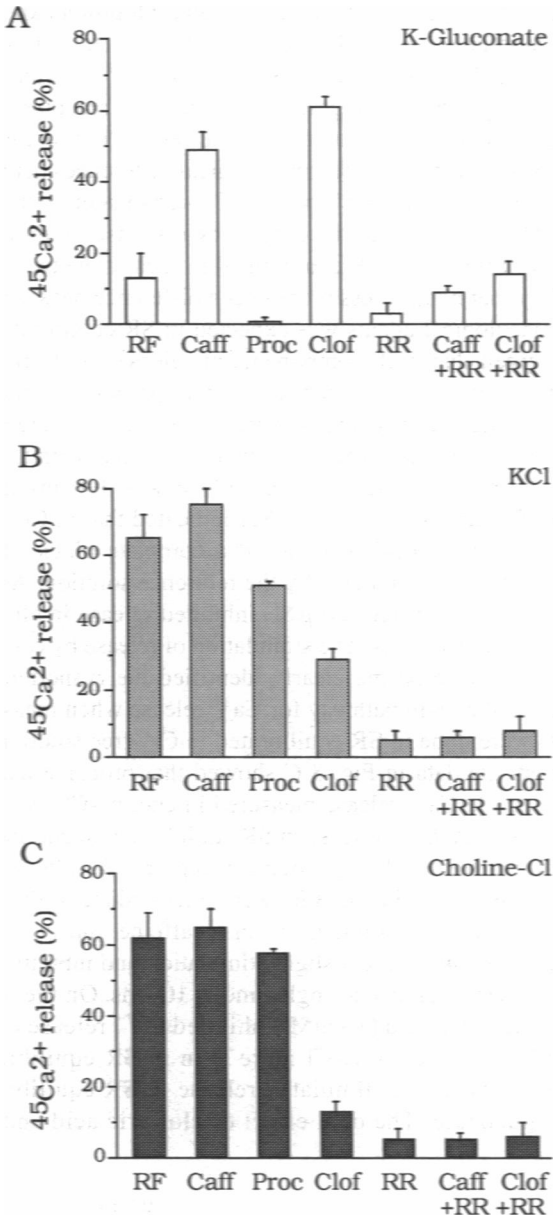

FIGURE 8 Pharmacological profile of Cl^--dependent Ca^{2+} release. SR samples were separately equilibrated in $^{45}Ca^{2+}$-loading solution containing 150 mM K-gluconate (*A*), 150 mM KCl (*B*), or 150 mM choline-Cl (*C*). Release was initiated by rapid filtration for 100 ms with reference solution containing 150 mM K-gluconate (*A*), 150 mM KCl (*B*) or 150 mM choline-Cl (*C*). Additions to the reference solutions were: none (RF); 20 mM caffeine (Caff); 10 mM procaine (Proc); 10 mM clofibric acid (Clof); 20 μM ruthenium red (RR); 20 mM caffeine plus 20 μM ruthenium red (Caff+RR); or 10 mM clofibric acid plus 20 μM ruthenium red (Clof+RR). Total $^{45}Ca^{2+}$ content labeled 100% corresponds to 70 ± 5 nmol/mg (*A*), 77 ± 13 nmol/mg (*B*) and 80 ± 6 nmol/mg (*C*). Error bars represent S.D. of three determinations each in duplicate.

periments of Fig. 8 were performed in SR equilibrated in K-gluconate (*A*) and separately in SR equilibrated in KCl (*B*) or choline-Cl (*C*). The rationale for selecting these salts is found in the ligand-dependence of Ca^{2+} release when the SR is equilibrated in K-gluconate, choline-Cl, or KCl (Table 1). Ca^{2+} release from SR equilibrated in K-gluconate adhered

closely to the pattern of stimulation and inhibition expected of ryanodine receptors. However, the Ca^{2+} release from SR equilibrated in choline-Cl lacked modulation by ryanodine receptor ligands and suggested that ryanodine receptors only made a small contribution to the total release in this case. It was thus expected that pharmacological differences between the ryanodine receptor and the Cl^--dependent pathways would become apparent by comparing responses to procaine and clofibric acid in SR equilibrated in each of the two salts. On the other hand, a less pronounced difference between the pharmacological agents was expected in SR equilibrated in KCl, given that both components of release would be operative in this salt. To simplify the interpretation of the results, all ligands and drugs were present in the reference solution exclusively. Thus, they were in contact with the SR only during the rapid filtration, which was 100 ms in all cases. The data shown in Fig. 8 *A* indicated that in Cl^--free SR, procaine (10 mM) produced a complete block of the release initially stimulated by the reference solution. As expected, ruthenium red (20 μM) inhibited release in all conditions and in all salts. The stimulation of release by caffeine and block by procaine clearly identified the ryanodine receptor as the main pathway for Ca^{2+} release when measurements were done in SR equilibrated in Cl^--free solution. In contrast, the data in Fig. 8 *C* showed that procaine had no effect on the Ca^{2+} release measured in choline-Cl. We thus concluded that Ca^{2+} release in SR equilibrated in choline-Cl was not mediated by ryanodine receptors. Results in SR equilibrated in KCl (Fig. 8 *B*) were intermediate to those in K-gluconate and choline-Cl in that caffeine and procaine, respectively, produced a slight stimulation and inhibition of release as measured at a single time of 100 ms. On the other hand, clofibric acid (10 mM) inhibited Ca^{2+} release in SR equilibrated in choline-Cl more than in SR equilibrated in KCl, whereas it stimulated release in SR equilibrated in K-gluconate. The dual effect of clofibric acid and the absence of inhibition of release by procaine in SR equilibrated in choline-Cl clearly demonstrated that the Cl^--dependent release was pharmacologically distinct from the ryanodine receptor-mediated Ca^{2+} release. Other Cl^- channel blockers that were tested but had no effect on Ca^{2+} release at concentrations of 1.5–10 mM were anthracene-9-carboxylic acid, diphenylaminecarboxylate; 4,4′-diisothiocyanostilbene-2,2-disulfonic acid), 5-nitro-2-(3-phenylpropylaminobenzoic) acid, niflumic acid, and gallic acid.

A nonselective SR Cl^- channel blocked by ruthenium red and clofibric acid

To identify a channel that could potentially mediate the Cl^--dependent release, we searched for SR Cl^- channels that were sensitive to ruthenium red. At least two types of Cl^- channels have been observed in SR fused to planar bilayers. Low conductance types have been reported to have unit conductances in the range of 55–116 pS in recording solutions containing 100–500 mM Cl^- (Rousseau et al., 1988; Rousseau, 1989; Kawano et al., 1992). Larger conductance types display multiple states in the range of 200–400 pS in 100 mM Cl^- (Tanifuji et al., 1987). A preliminary screening revealed that the large conductance channels were insensitive to ruthenium red up to a concentration of 60 μM. Thus, we focus on the low-conductance anionic channels of SR. Fig. 9 shows an anion selective channel that was frequently recorded in solutions consisting of *cis* 450 mM Cl^- and *trans* 50 mM Cl^- with $Tris^+$ as the cation. The unit current was ~1 pA at 0 mV, and the reversal potential was +10 mV. The kinetics of opening and closing consisted of a rapid flickering interspersed with long closed events lasting several seconds. Substate conductances were also present, but these were difficult to resolve. As shown by the bottom records, addition of 20 μM ruthenium red to the *cis* solution resulted in a reduction of

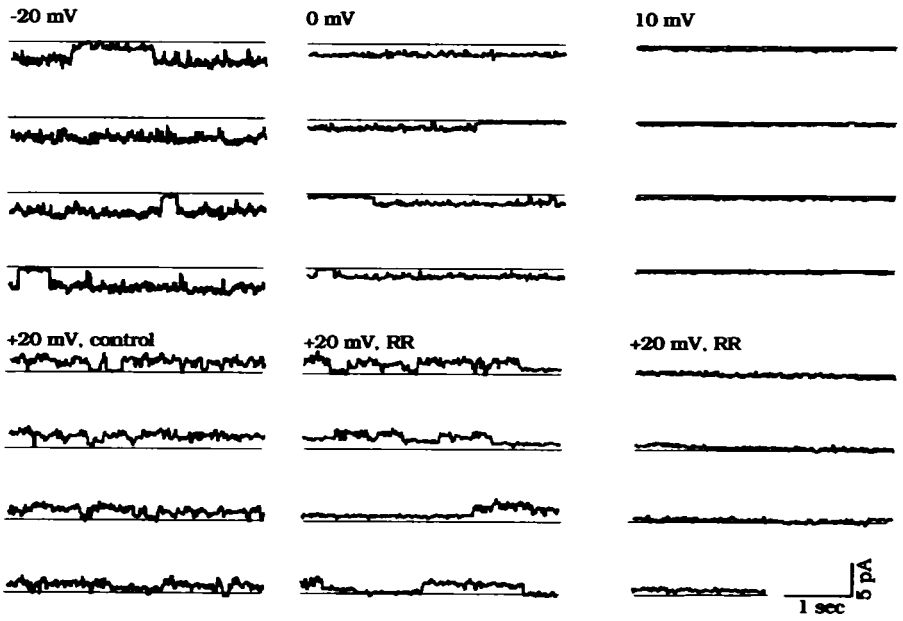

FIGURE 9 Single channel recordings of nonselective Cl^- channels. *Cis* solution was composed of 450 mM Tris-Cl. The *trans* solution was composed of 50 mM Tris-Cl. Ruthenium red (20 μM) was added to the *cis* solution. Bottom center and right panels are consecutive recordings following addition of ruthenium red.

the open probability immediately after addition and stirring of the *cis* solution (bottom center) and a complete block at longer times of exposure (bottom right).

We further characterized the ionic permeability of this channel to determine whether Ca^{2+} could serve as a current carrier. A current-voltage curve of the nonselective channel in the Tris-Cl gradient is shown in Fig. 10 *A*. The slope conductance in the voltage range of the reversal potential was ~200 pS and decreased to 50 pS at negative potentials. The reversal potential, intermediate between the $Tris^+$ and the Cl^- equilibrium potential indicated by the arrows, indicated a permeability ratio $P_{Cl}/P_{Tris} = 1/0.5$. In the same solutions, it was possible to record ryanodine receptor $Tris^+$ current with a slope conductance of ~30 pS. Ryanodine receptor channels were identified in these solutions by the brief open time that was increased by 5 mM Na_2ATP and blocked by 1 μM ruthenium red (not shown). A current-voltage curve of the nonselective channel in recording solutions consisting of *cis* 440 Cl^- and *trans* 40 Cl^- with Ca^{2+} as cation is shown in Fig. 10 *B*. The slope conductance in the voltage range of the reversal potential was ~150 pS and decreased to 50 pS at negative potentials. The reversal potential indicated a permeability ratio $P_{Cl}/P_{Ca} = 1/0.3$. Thus the channel displayed a significant permeability toward divalent cations. Although rarely, it was also possible to record ryanodine receptor Ca^{2+} current in these solutions with a single channel slope conductance of ~90 pS. Recordings of nonselective channels and ryanodine receptor channels in the $CaCl_2$ solutions at +20 mV are shown at the bottom. At this voltage, Ca^{2+} is the main current carrier in both cases. These recordings clearly identified a novel channel sensitive to ruthenium red and permeable to Ca^{2+} that was altogether different from the ryanodine receptor channel.

Given that clofibric acid increased Ca^{2+} release in K-gluconate whereas it decreased release in choline-Cl, we investigated the effect of this drug on the ryanodine receptor and the nonselective Cl^- channel. Ryanodine receptors shown in Fig. 11 were recorded in standard CsMSF solutions at 0 mV. Open probability during the control period averaged 0.11 and increased to 0.28 following addition of pH-neutralized clofibric acid to the *cis* solution. Thus clofibric acid produced a significant stimulation of the ryanodine receptor. The bottom traces show a blockade by procaine of a separate ryanodine receptor that had control activity similar to that described above. Procaine added to the *cis* solution reduced the open probability of this channel approximately 10-fold to less than 0.01. The requirement of millimolar concentrations of procaine for an effective block of the channel was the same as that reported by Xu et al. (1993) using 250 mM KCl as the recording solution. Nonselective Cl^- channels shown in the same figure were recorded in 150 Cl^- with $Tris^+$ as cation at −40 mV. Addition of clofibric acid to the same control channel shown on the top recordings resulted in a dramatic reduction of the single channel current. On the

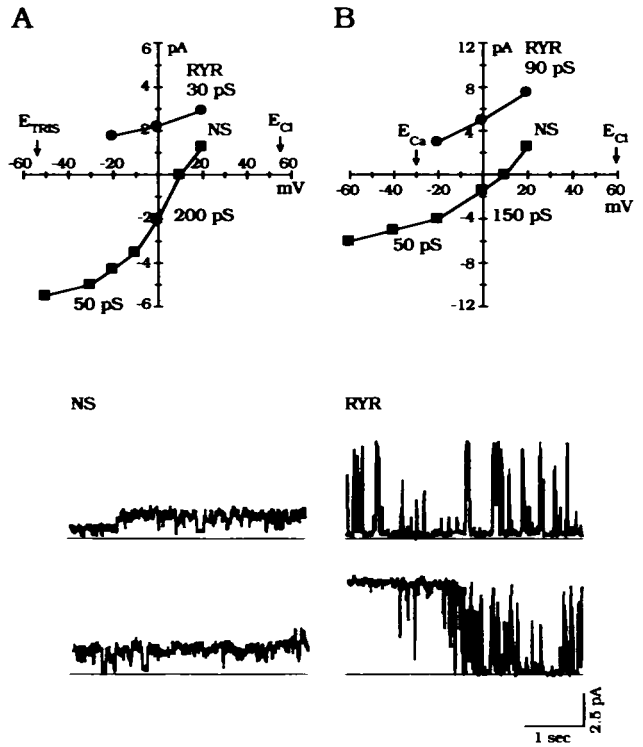

FIGURE 10 Current-voltage curves of nonselective Cl^- channel and ryanodine receptor channel in identical solutions. (*A*) Current-voltage curves of ryanodine receptor channel (RYR) and nonselective Cl^- channel (NS) in *cis* 450 mM Tris-Cl and *trans* 50 mM Tris-Cl. (*B*) Current-voltage curves of ryanodine receptor channel (RYR) and nonselective Cl^- channel (NS) in *cis* 220 mM $CaCl_2$ and *trans* 20 mM $CaCl_2$. Slope conductance of the nonselective channel is specified at positive potentials and potentials more negative than −30 mV. Single channel recordings are at +20 mV in the $CaCl_2$ solutions of (*B*).

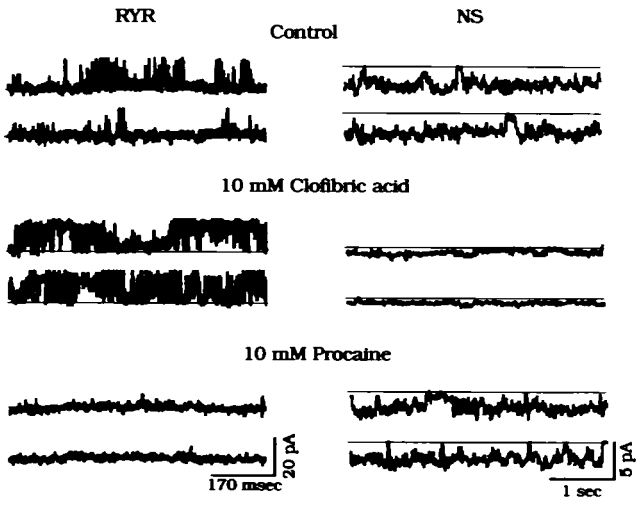

FIGURE 11 Effect of clofibric acid and procaine on ryanodine receptor and nonselective Cl^- channels. Top and middle records show a ryanodine receptor channel at 0 mV in *cis* 240 CsMSF, 10 mM CsCl and *trans* 40 CsMSF, 10 CsCl before (control) and after addition of 10 mM pH-neutralized clofibric acid to the *cis* solution. Bottom recordings with *cis* 10 mM procaine are from a separate channel with a similar control activity. Top and middle records show a nonselective Cl^- channel at −40 mV in *cis* 150 Tris-Cl and *trans* 50 mM Tris-Cl before (control) and after addition of 10 mM pH-neutralized clofibric acid to the *cis* solution. Bottom recordings with *cis* 10 mM procaine are from a separate channel.

other hand procaine had no discernible effect on the gating or conductive properties of this channel. These results demonstrated that the opposite effects of clofibric acid observed on Ca^{2+} release in SR equilibrated in Cl^--free and Cl^--containing solutions were likely to arise from the separate effects of clofibric acid on two different channel populations. In K-gluconate, clofibric acid would stimulate release by increasing the activity of ryanodine receptors, whereas in choline-Cl, clofibric acid would inhibit release by reducing the single channel current of nonselective Cl^- channels. A mechanism by which the stimulation of ryanodine receptor channel in SR equilibrated in choline-Cl by clofibric acid may not contribute to the Ca^{2+} flux, is described further below.

Co-localization of the Cl^--dependent Ca^{2+} release pathway and ryanodine receptors

To determine the location of the Cl^--dependent Ca^{2+} release pathway in the SR, we fractionated total SR by discontinuous sucrose gradient centrifugation into three fractions previously identified as "light," "intermediate," and "heavy" SR (Meissner, 1984). As shown by the filled bars in Fig. 12, [^{3}H]ryanodine binding was the highest in the 36% (w/v) "heavy" fraction with a binding activity of 0.8 ± 0.08 pmol/mg and was the lowest in the 18% (w/v) "light" fraction with a binding activity of 0.22 ± 0.06 pmol/mg. The binding activity was measured in reference solution in choline-Cl and did not reflect the total number of high-affinity [^{3}H]ryanodine binding sites, inasmuch as stimulatory ligands were not present in this solution. Previous studies have shown that the actual binding site density of the "heavy" fraction in our preparation reaches a near maximum of ≈15 pmol/mg under optimal concentrations of stimulatory ligands (El-Hayek et al., 1993). $^{45}Ca^{2+}$ release was measured in the three SR fractions after equilibration in K-gluconate and choline-Cl. To identify ryanodine receptor activity, measurements were also made in the presence and absence of 20 mM caffeine. The data indicated (Fig. 12, *hatched bars*) that Cl^--dependent Ca^{2+} release was absent in "light" SR and was progressively more prominent in "intermediate" and "heavy" SR fractions. Caffeine did not stimulate the Cl^--dependent release but significantly stimulated release in K-gluconate (*empty bars*). Moreover, this effect was progressively more prominent in the heavier SR fractions. From these results it became clear that the Ca^{2+} release stimulated by Cl^- co-purified with the SR terminal cisternae and appeared to be absent in the "light" SR fraction.

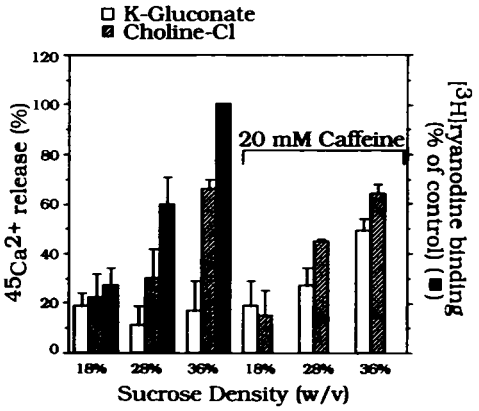

FIGURE 12 Cl^--induced $^{45}Ca^{2+}$ release in light, intermediate, and heavy SR. Total SR sedimenting at 18% ("light SR"), 28% ("intermediate SR"), and 36% ("heavy SR") sucrose was equilibrated in $^{45}Ca^{2+}$-loading solution containing 150 mM choline-Cl (*hatched bars*) or 150 mM K-gluconate (*open bars*). $^{45}Ca^{2+}$ release was induced by rapid filtration during 50 ms with reference solution containing 150 mM choline-Cl (*hatched bars*) or 150 mM K-gluconate (*open bars*). *Filled bars* represent specific [^{3}H]ryanodine binding activity at a concentration of 7 nM [^{3}H]ryanodine in reference solution containing choline-Cl. 100% refers to binding activity in heavy SR. *Error bars* represent S.D. of three determinations each in duplicate.

DISCUSSION

We investigated the mechanism by which Cl^- increased the Ca^{2+} permeability of the SR membrane. Most flux experiments were done maintaining the internal and external concentrations of Cl^- and those of the replacing anion, as well as those of the cation in the salt, constant and equal. The purpose of eliminating monovalent ion gradients was to avoid an osmotic flow of water into the SR lumen, which would have resulted in an artifactual release of lumenal $^{45}Ca^{2+}$ (Meissner and McKinley, 1976). In our hands however, this osmotic phenomenon did not occur within the 2–200-ms time frame of the present experiments. The results indicated that the Ca^{2+} permeability of the junctional SR membrane is significantly and specifically increased by Cl^- as brought about by a pharmacologically distinct Cl^--dependent Ca^{2+} release mechanism. We also considered the alternative hypothesis that Cl^- may have directly stimulated or increased the sensitivity of ryanodine receptors to cytosolic ligands. The contribution of ryanodine receptors to the release induced by Cl^- was ruled out 1) by single channel recordings demonstrating that Cl^- did not increase the open probability of the ryanodine receptor; 2) by the pharmacological profile of Cl^--dependent release, which differed in a significant manner from that of the ryanodine receptor; and 3) by the recording of a novel nonselective Cl^- channel leaky to Ca^{2+} and blocked by clofibric acid but not by procaine and thus pharmacologically competent to underlie Cl^--dependent Ca^{2+} release.

The Cl^--dependent Ca^{2+} release pathway, like the ryanodine receptor, was enriched in the heavy SR membrane fraction. It would thus appear that the two pathways operate side by side and mobilize Ca^{2+} from the same pool. This conclusion is also strengthened by the measurements in Table 1, which show that Ca^{2+} release in KCl does not amount to the sum of those separately measured in K-gluconate and choline-Cl. Earlier reports concerning Cl^--mediated Ca^{2+} release from various SR preparations gave conflicting results with regard to its location. Campbell and Shamoo (1980) suggested two pharmacologically distinct Cl^--induced Ca^{2+} release mechanisms located in the "light" and "heavy" SR,

respectively. On the other hand, Caswell and Brandt (1981) suggested a location on the SR terminal cisternae exclusively, whereas Meissner and McKinley (1976) did not observe marked regional differences in the Cl^--dependent release. In retrospect, however, it would appear that not all Cl^--dependent release mechanisms initially reported had a common molecular origin. Meissner and McKinley (1976) concluded that releases in "light" and "heavy" SR were caused by osmotic swelling of vesicles, which is expected to occur when an impermeant anion is present inside the SR and the SR-permeant KCl salt is suddenly presented to the myoplasmic face of the SR. In contrast, Campbell and Shamoo (1980) identified an osmotic swelling-induced component in the "light" SR but a pharmacologically sensitive component unrelated to swelling in the "heavy" SR. The Cl^--dependent release reported by Campbell and Shamoo (1980) in heavy SR is likely to be related to the phenomenon under study here.

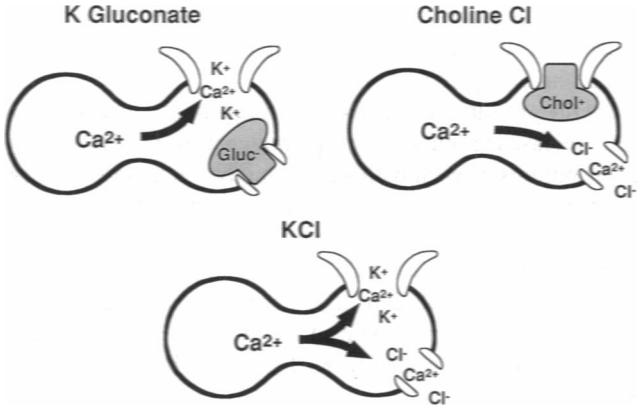

FIGURE 13 Plug model of Cl^--dependent SR Ca^{2+} release. A nonselective Cl^- channel is postulated to mediate Cl^--dependent Ca^{2+} release. Cl^- and Ca^{2+} move through the channel as a salt pair. Cl^--induced SR Ca^{2+} release is explained by the unplugging of the nonselective channel when a relatively impermeant anion (gluconate$^-$) is replaced by Cl^-.

A plug model for Cl^--dependent SR Ca^{2+} release

The block of ryanodine receptor channels by procaine in SR fused to planar bilayers is in agreement with previous data obtained in purified channel preparations (Xu et al., 1993), Ca^{2+} fluxes in SR equilibrated in KCl (Antoniu et al., 1985; Palade, 1987), as well as Ca^{2+}-induced Ca^{2+} release in intact muscle fibers (Klein et al., 1992). On the other hand, clofibric acid (2-[p-clorophenoxy] propionic acid) was previously known only to block the skeletal muscle sarcolemmal Cl^- current (De Luca et al., 1992). Thus the inhibition of the nonselective Cl^- channel by clofibric acid but not procaine strongly suggested the participation of this channel and not the ryanodine receptor in Cl^--dependent release. That a Cl^- channel could have a sizable permeability for Ca^{2+} and thus have a large impact on the Ca^{2+} permeability of the SR is not altogether surprising. Many Cl^- channels found in surface membranes and in skeletal muscle and neurons are not exclusively permeable to Cl^- and reveal a generalized permeability toward inorganic cations (Blatz and Magleby, 1985; Franciolini and Nonner, 1987; Woll et al., 1987). Permeability ratios P_{Cl}/P_{C+} in the range of 3–9 are commonly reported for muscle Cl^- channels (Blatz and Magleby, 1985; Woll et al., 1987). However, the permeability of Cl^- channels toward large organic anions such as gluconate$^-$ and others used in this study is low (see Table III of Franciolini and Nonner, 1987). The vastly different ligand-dependence of Ca^{2+} release seen in SR equilibrated in K-gluconate or choline-Cl (Table 1) could be explained as shown in Fig. 13 by making straightforward assumptions about the conductive properties of ryanodine receptors and nonselective Cl^- channels. We would have to accept the notion that the nonselective channel, even if permeable to divalent cations, could still be physically narrow and subjected to single-file diffusion (Hille, 1984). Following the suggestion of Franciolini and Nonner (1987) made for the movement of monovalent cations in the neuronal Cl^- channels, we suggest that Ca^{2+} may be able to flow through this pore as in a single file along with Cl^- and other small cations. When Cl^- is replaced by gluconate$^-$ or by another anion with a low permeability, the pathway may not be available for Ca^{2+} efflux by virtue of being effectively blocked or plugged most of the time by the slow passage through the pore of the anion used to replace Cl^-. The possibility that Cl^- may also play a structural role in stabilizing the open state cannot be discarded at this point. The latter may be significant when it is considered that Cl^- could not be replaced by other halides (Fig. 4) which, like Cl^-, are also small in size. In the context of this model, Cl^--dependent Ca^{2+} release is brought about by the removal from the SR of a less permeable anion and its replacement by Cl^- allowing the nonselective channel to conduct Ca^{2+} and Cl^- at a high rate.

The same plug model applied to Ca^{2+} flow through the ryanodine receptor can explain the fact that stimulation of Ca^{2+} release by caffeine and ATP was more pronounced in SR equilibrated in K-gluconate than in KCl and was almost absent in SR equilibrated in choline-Cl (Table 1). The ryanodine receptor channel is ideally selective for cations over anions but the pore displays little discrimination between small monovalent cations of the alkali series and group IIA divalent cations (Smith et al., 1988). However, large organic cations of the tetraalkylammonium series display a low permeability and have been shown to reduce K^+ and divalent cation flow (Tinker et al., 1992). Choline$^+$ and Tris$^+$ also belong to this group, inasmuch as the conductance of choline$^+$ and Tris$^+$ through the ryanodine receptor is $\approx$20 times lower than the K^+ conductance and $\approx$7 times lower than the Ca^{2+} conductance (Smith et al., 1988). Thus, when SR is equilibrated in choline-Cl, the ryanodine receptor pathway would operate at a turnover rate slower than that of the Cl^--dependent pathway because the ryanodine receptor would be plugged most of the time by choline$^+$ (Fig. 13). It is important to indicate that even if the ryanodine receptor conducts Ca^{2+} at a reduced rate in the presence of an organic cation, the total flux could still display the ligand dependence attributable to a ryanodine receptor mediated release if the block of the Cl^-

channel was more efficient than the block of the ryanodine receptor. The latter would appear to be the case in SR equilibrated in Tris-gluconate (Fig. 5).

In SR equilibrated in KCl the plug model predicts that Ca^{2+} release is funneled through both pathways. The relative contributions of each pathway to the total release would depend on the choice of reference solution (with or without Cl^-) and the level of stimulation of ryanodine receptors in that solution. Because the Cl^--dependent pathway is open all the time, the stimulation of Ca^{2+} release by caffeine and ATP relative to that produced by the reference solution is lower in SR equilibrated in KCl than in SR equilibrated in K-gluconate.

Implications for excitation-contraction coupling

The stimulation of the $^{45}Ca^{2+}$ efflux rate by Cl^- was surprisingly large and suggested that the Cl^--dependent pathway, in the absence of ryanodine receptor stimulation, dominates the SR Ca^{2+} permeability. According to Table 1, $^{45}Ca^{2+}$ release in reference solution at 50 ms reached between 30 and 40 nmol/mg in the presence of Cl^- but was only 5–10 nmol/mg in the absence of Cl^-. Release in K-gluconate reached 30–50 nmol/mg, and thus became comparable in magnitude to release rates in Cl^- only when ryanodine receptors were fully activated by ATP or caffeine. The relative contribution of the Cl^--dependent pathway can also be determined based on the effect of procaine, a drug that selectively blocks the ryanodine receptor and does not have any effect on the nonselective Cl^- channel. It would appear that the Cl^--dependent Ca^{2+} release is about two times larger than that mediated by ryanodine receptors because, according to Fig. 8, procaine blocks about one-third of the flux measured in SR equilibrated in KCl. Also, it is important to mention that the Cl^--dependent release, unlike the ryanodine receptor-mediated release, was independent of the free Ca^{2+} of the reference solution in the range of pCa 9 to 6 (not shown). The latter observation and the larger contribution of the Cl^--dependent component to the total flux makes it likely that the Cl^--dependent pathway dominates the SR Ca^{2+} permeability in the resting muscle cell.

A possible function of the Cl^--dependent pathway could be to provide a pathway for charge compensation during Ca^{2+} release and therefore serve as a mechanism to enhance the SR Ca^{2+} release rate. However, there are several observations suggesting that the release induced by Cl^- does not serve this purpose. First, Ca^{2+} release in the absence of Cl^- was not stimulated by K^+-valinomycin and therefore it did not appear likely that charge compensation was in fact a rate-limiting step for Ca^{2+} release in Cl^--free SR. Second, external Cl^- stimulated release much more than internal Cl^-. The opposite was expected to be the case if Cl^- stimulated release by a mechanism involving charge compensation. Finally, it has been argued that the Ca^{2+} release channel itself, because it has a large and nonselective cationic conductance and because it has a reversal potential close to 0 mV in physiological solutions, may serve as its own pathway for charge compensation (Smith et al., 1988). Although the role of nonselective Cl^- channel in the Ca^{2+} homeostasis of the muscle cell is unclear at this point, the fact that the channel is always open is significant, as this condition will likely affect the Ca^{2+} loading capacity of the SR and the rate of recycling of Ca^{2+} across the SR membrane.

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