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Chloride-Dependent Sarcoplasmic Reticulum Ca^{2+} Release Correlates with Increased Ca^{2+} Activation of Ryanodine Receptors

Bradley R. Fruen,* Patricia K. Kane,* James R. Mickelson,* and Charles F. Louis*§
Departments of *Veterinary PathoBiology and §Biochemistry, University of Minnesota, St. Paul, Minnesota 55108 USA

ABSTRACT The mechanism by which chloride increases sarcoplasmic reticulum (SR) Ca^{2+} permeability was investigated. In the presence of 3 μM Ca^{2+}, Ca^{2+} release from $^{45}Ca^{2+}$-loaded SR vesicles prepared from porcine skeletal muscle was increased approximately 4-fold when the media contained 150 mM chloride versus 150 mM propionate, whereas in the presence of 30 nM Ca^{2+}, Ca^{2+} release was similar in the chloride- and the propionate-containing media. Ca^{2+}-activated [^{3}H]ryanodine binding to skeletal muscle SR was also increased (2- to 10-fold) in media in which propionate or other organic anions were replaced with chloride; however, chloride had little or no effect on cardiac muscle SR $^{45}Ca^{2+}$ release or [^{3}H]ryanodine binding. Ca^{2+}-activated [^{3}H]ryanodine binding was increased ~4.5-fold after reconstitution of skeletal muscle RYR protein into liposomes, and [^{3}H]ryanodine binding to reconstituted RYR protein was similar in chloride- and propionate-containing media, suggesting that the sensitivity of the RYR protein to changes in the anionic composition of the media may be diminished upon reconstitution. Together, our results demonstrate a close correlation between chloride-dependent increases in SR Ca^{2+} permeability and increased Ca^{2+} activation of skeletal muscle RYR channels. We postulate that media containing supraphysiological concentrations of chloride or other inorganic anions may enhance skeletal muscle RYR activity by favoring a conformational state of the channel that exhibits increased activation by Ca^{2+} in comparison to the Ca^{2+} activation exhibited by this channel in native membranes in the presence of physiological chloride ($\leq$10 mM). Transitions to this putative Ca^{2+}-activatable state may thus provide a mechanism for controlling the activation of RYR channels in skeletal muscle.

INTRODUCTION

In both skeletal muscle and cardiac muscle, ryanodine receptor (RYR) Ca^{2+} channels in the sarcoplasmic reticulum (SR) membrane constitute the pathway for the rapid release of Ca^{2+} that triggers muscle contraction (Coronado et al., 1994); however, the primary in vivo mechanisms thought to control the activation of mammalian skeletal (RYR1) and cardiac (RYR2) Ca^{2+} channel isoforms are fundamentally different. The activation of skeletal muscle SR Ca^{2+} release is proposed to be controlled through a mechanism, termed mechanical coupling, in which depolarization-induced conformational changes in transverse tubule voltage sensor molecules are transmitted via protein-protein interactions to RYR1 channels. In contrast, the activation of cardiac SR Ca^{2+} release is proposed to be controlled by local increases in intracellular Ca^{2+} through a mechanism intrinsic to the RYR2 channel, termed Ca^{2+}-induced Ca^{2+} release (Rios and Pizarro, 1991; Schneider, 1994).

Insights into the different mechanisms controlling the activation of RYR channels in skeletal and cardiac muscle may come from understanding the mechanisms by which certain inorganic anions affect SR Ca^{2+} release, as these anions' effects appear to be selective for skeletal muscle RYR1 channels (Louis, 1994). In this regard, anions of the chaotropic series, such as nitrate, perchlorate, and thiocyanate, enhance the activity of RYR1 but not RYR2 channels at concentrations equal to or greater than 5 mM (Ma et al., 1993; Fruen et al., 1994a). The stimulation of RYR1 activity by these anions correlates with their chaotropic properties. Accordingly, chaotropic anions were postulated to act by destabilizing protein-protein interactions that are critical in controlling RYR1 channel activation (Gonzalez and Rios, 1993; Ma et al., 1993).

In addition to the chaotropic anions, inorganic phosphate (P_i), the predominant anion in working skeletal muscle, also selectively increases RYR1 but not RYR2 channel activity (Fruen et al., 1994b). At 5–20 mM, P_i produces an effect on RYR1 channel function similar to that of the chaotropic anions (~2-fold stimulation at 10 mM P_i). P_i, however, is not a chaotrope (Gonzalez and Rios, 1993; Parsegian, 1995), suggesting that this endogenous inorganic anion may enhance RYR1 channel activity through a different mechanism. Indeed, the stimulatory effects of P_i on RYR1 channel activity were postulated to result from the interaction of this anion with a discrete, saturable site on the RYR1 channel protein (Fruen et al., 1994b).

A third mechanism by which inorganic anions may increase skeletal muscle SR Ca^{2+} permeability was recently proposed by Sukhareva and co-workers (1994) based on their studies of the effects of chloride. Consistent with earlier reports (e.g., Campbell and Shamoo, 1980; Hasselbach and Migala, 1992), SR vesicles equilibrated in univalent salts containing chloride in place of an organic anion such as gluconate exhibited an approximately fivefold increased rate of Ca^{2+} release. Chloride, however, had no

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Address reprint requests to Dr. Bradley R. Fruen, Department of Veterinary PathoBiology, University of Minnesota, 1988 Fitch Avenue, Room 295, St. Paul, MN 55108. Tel.: 612-625-3193; Fax: 612-625-0204; E-mail: fruen001@maroon.tc.umn.edu.

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significant effect on the open probability of RYR1 channels reconstituted into planar lipid bilayers, and Ca^{2+} release from SR vesicles equilibrated in chloride instead of organic anions showed significantly decreased sensitivity to pharmacological ligands of RYR channels (Sukhareva et al., 1994). Based on these findings, a model was proposed whereby chloride-dependent Ca^{2+} release operated via non-RYR channels. In this model, the equilibration of SR vesicles in salts containing chloride was postulated to increase SR Ca^{2+} permeability by allowing Ca^{2+} release through nonselective ion channels that were blocked when SR vesicles were equilibrated in salts containing larger organic anions (Sukhareva et al., 1994; Patel et al., 1995). The authors concluded that this chloride-dependent Ca^{2+} release pathway may constitute a major undescribed pathway for Ca^{2+} release from the SR.

The surprising magnitude of chloride's effects on SR Ca^{2+} permeability suggest that a more complete description of the underlying mechanism and physiological significance of this anion's effects may have important implications for investigations of the mechanisms controlling SR Ca^{2+} release in muscle. Indeed, although myoplasmic chloride concentrations are estimated to be less than 10 mM (Godt and Maughan, 1988), studies of Ca^{2+} release from isolated SR vesicles have typically been performed in media containing 100–300 mM chloride. Furthermore, supraphysiological chloride concentrations have also been employed in many ion substitution protocols designed to trigger SR Ca^{2+} release via transverse tubule depolarization (e.g., Endo and Nakajima, 1973; Stephenson, 1985; Donaldson, 1985; Lamb et al., 1993). Thus it is possible that chloride may artifactually increase SR Ca^{2+} release in a number of common experimental protocols via mechanism(s) that remain largely undefined.

To further investigate the mechanism of chloride-dependent SR Ca^{2+} release we have now examined $^{45}Ca^{2+}$ efflux and [^{3}H]ryanodine binding to compare chloride's effects on SR Ca^{2+} release channel function in skeletal muscle SR vesicles, cardiac muscle SR vesicles, and after reconstitution into artificial liposomes. The possible relationship of chloride's effects to the effects of chaotropic anions and of P_i have also been considered.

MATERIALS AND METHODS

Materials

Pigs were obtained from the swine genetics herds maintained by the Department of Animal Science at the University of Minnesota Experimental Farm. $^{45}Ca^{2+}$ and [^{3}H]ryanodine were purchased from Dupont NEN (Boston, MA). Unlabeled ryanodine was purchased from Calbiochem (La Jolla, CA). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation of SR vesicles

Skeletal muscle SR vesicles were prepared from porcine longissimus dorsi. Muscle was homogenized in 0.1 M NaCl, 5 mM Tris maleate buffer (pH 6.8), and the membranes that pelleted between 2,600 and 10,000 × *g* were extracted in 0.6 M KCl, 20 mM Tris (pH 6.8), centrifuged at 100,000 × *g* and then resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Tris (pH 6.8). Cardiac muscle SR vesicles were prepared from porcine ventricular tissue. Muscle was homogenized in 10 mM $NaHCO_3$, and membranes that pelleted between 4,000 and 80,000 × *g* were extracted in 0.6 M KCl, 20 mM Tris (pH 6.8), centrifuged at 100,000 × *g*, and then resuspended in 0.3 M sucrose. All isolation buffers contained a mixture of protease inhibitors (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride). SR vesicles were flash-frozen in liquid nitrogen and stored at −70°C.

$^{45}Ca^{2+}$ efflux

Ca^{2+} efflux from SR vesicles was determined in media containing 15 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 7.0) and either 150 mM KCl or 150 mM Kpropionate as the major univalent salt. SR vesicles were pre-equilibrated separately in KCl or Kpropionate media for 30 min on ice, then pelleted at 100,000 × *g* and resuspended (~15 mg protein/ml) in the same univalent salt media supplemented with 5 mM ^{45}Ca(acetate)$_2$ (~20,000 cpm/nmol). Passive loading of SR vesicles with $^{45}Ca^{2+}$ was achieved by incubation for 2.5 h at 22°C. The total amount of $^{45}Ca^{2+}$ loaded was not significantly different in the different univalent salt media for either skeletal muscle SR vesicles (50 ± 4.9 nmol/mg in KCl versus 42 ± 2.6 nmol/mg in Kpropionate; $n = 6$, $p = 0.14$, independent Student's *t*-test) or cardiac SR vesicles (11 ± 2.4 nmol/mg in KCl versus 11 ± 1.3 nmol/mg in Kpropionate; $n = 4$). $^{45}Ca^{2+}$-loaded vesicles (2 μl) were placed on the side of a polystyrene tube containing 200 μl of a Ca^{2+} release medium (150 mM KCl or Kpropionate, 15 mM PIPES, pH 7.0, and a Ca(acetate)$_2$-EGTA buffer set to give the desired Ca^{2+} concentration; Brooks and Storey, 1992). Ca^{2+} release was initiated with rapid mixing and stopped at various times (1–5 s using a metronome) by rapid dilution into 12 ml of a release-inhibiting medium (150 mM Kpropionate, 15 mM PIPES, pH 7.0, 10 mM EGTA, 5 mM $MgCl_2$, 20 μM ruthenium red) followed immediately by filtration onto 0.45 μM Millipore HA membranes. The fraction of total loaded $^{45}Ca^{2+}$ that was not released after 15-s incubations in release media containing 150 KCl, 10 mM ATP, and 10 μM Ca^{2+} (i.e., maximum RYR activation) was considered background and was subtracted from all determinations.

[^{3}H]Ryanodine binding

SR vesicles were pre-equilibrated separately in 150 mM KCl or 150 mM Kpropionate for 60 min on ice, then diluted (0.2 mg protein/ml) into media containing 150 mM of the same univalent salt, 15 mM PIPES, pH 7.0, 100 nM [^{3}H]ryanodine, and a Ca(acetate)$_2$-EGTA buffer set to give the desired Ca^{2+} concentration. After a 90-min incubation at 36°C, SR vesicles were collected on Whatman GF/B filters and washed with 8 ml ice-cold 150 mM Kgluconate. Estimates of the maximum [^{3}H]ryanodine binding capacity (B_{max}) of each SR vesicle preparation were determined in media containing 500 mM KCl, 10 mM ATP, and 10 μM Ca^{2+}. Nonspecific binding was measured in the presence of 20 μM nonradioactive ryanodine.

Isolation and reconstitution of RYR from skeletal muscle SR

RYR was isolated on sucrose gradients from 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS)-solubilized skeletal muscle SR essentially as described (Shomer et al., 1992). For reconstitution of the isolated RYR channel complex into liposomes, gradient fractions containing RYR protein were pooled, concentrated, and diluted (0.1 mg SR protein/ml) into media containing 50 mM NaCl, 50 mM KCl, 20 mM Tris (pH 7.4), 8 mg/ml phosphatidylcholine, 1% CHAPS and dialyzed for 48 h at 4°C against media containing 50 mM NaCl, 50 mM KCl, 20 mM Tris (pH 7.4), 2 mM β-mercaptoethanol, 1 mM EGTA, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. Reconstituted RYR-containing liposomes were collected by centrifugation at 150,000 × *g* for 30 min and stored frozen at

−70°C. Before use, vesicles were slowly thawed, then resuspended into either 150 mM KCl or 150 mM Kpropionate, and sonicated for 2 min in a bath sonicator.

RESULTS

Ca^{2+}-induced Ca^{2+} release from skeletal and cardiac muscle SR vesicles

To characterize the effect of chloride on SR Ca^{2+} permeability, we initially compared the amount of Ca^{2+} released as a function of time from SR vesicles equilibrated in either 150 mM KCl or 150 mM Kpropionate (Fig. 1). $^{45}Ca^{2+}$-loaded SR vesicles were transferred to release media containing either low Ca^{2+} (~30 nM) or 3 μM Ca^{2+}, so that effects of chloride on SR vesicle Ca^{2+} permeability could be ascribed to either Ca^{2+}-independent or Ca^{2+}-dependent pathways. When $^{45}Ca^{2+}$-loaded skeletal muscle SR vesicles were transferred to release media containing ~30 nM Ca^{2+}, Ca^{2+} release was slow (<15% of initially loaded $^{45}Ca^{2+}$ released in 5 s) and was similar in chloride- and propionate-containing media (Fig. 1 *A*; open symbols). $^{45}Ca^{2+}$ release was activated when the vesicles were transferred to release media containing 3 μM Ca^{2+} (Fig. 1 *A*; closed symbols); however, the extent of this activation was dependent on the anionic composition of the media. Thus, whereas 3 μM Ca^{2+} activated relatively little additional $^{45}Ca^{2+}$ release from skeletal muscle SR vesicles equilibrated in propionate-containing media, in chloride-containing media the extent of Ca^{2+}-activated $^{45}Ca^{2+}$ release was increased approximately fourfold (58 ± 5.1% versus 14 ± 2.7% released in 2 s, $p < 0.01$).

We also examined the effect of chloride on cardiac muscle SR Ca^{2+} permeability. When $^{45}Ca^{2+}$-loaded cardiac SR vesicles were transferred to a release medium containing ~30 nM Ca^{2+}, less than 30% of the initially loaded $^{45}Ca^{2+}$ was released in 5 s (Fig. 1 *B*; open symbols), whereas when the release medium contained 3 μM Ca^{2+}, approximately 60% of the initially loaded $^{45}Ca^{2+}$ was released within 1 s (Fig. 1 *B*; closed symbols). In contrast to skeletal muscle SR vesicles, substituting chloride with propionate did not affect the amount of $^{45}Ca^{2+}$ released from cardiac SR at either of the two Ca^{2+} concentrations examined.

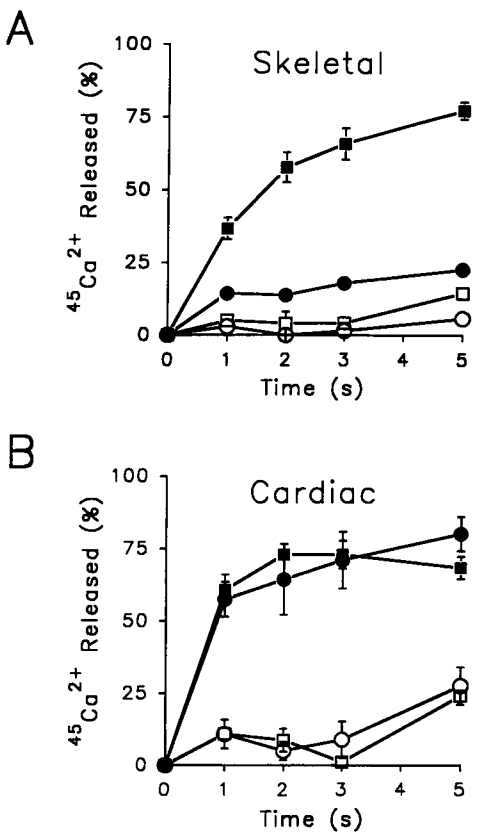

FIGURE 1 Ca^{2+}-activated Ca^{2+} release from SR vesicles equilibrated in either 150 mM KCl (■,□) or 150 mM Kpropionate (●,○). $^{45}Ca^{2+}$-loaded SR vesicles prepared from skeletal muscle (*A*) and cardiac muscle (*B*) were transferred to release media containing either ~30 nM Ca^{2+} (○,□) or 3 μM Ca^{2+} (●,■), as described in Materials and Methods. Data are means ± SE from four experiments (four skeletal and three cardiac muscle SR vesicle preparations).

Chloride effects on [^{3}H]ryanodine binding

The effects of chloride on SR vesicle Ca^{2+} permeability suggested that chloride may selectively enhance a Ca^{2+}-dependent Ca^{2+} release pathway in skeletal muscle SR. We therefore examined the possibility that chloride's effects on SR Ca^{2+} permeability were correlated with effects on Ca^{2+}-dependent RYR channel activity by using [^{3}H]ryanodine as a specific ligand for the open state of RYR channels (Chu et al., 1990; Meissner and El-Hashem, 1992). The Ca^{2+} dependence of [^{3}H]ryanodine binding to skeletal muscle SR vesicles followed similar bell-shaped curves in both chloride- and propionate-containing media, with optimal Ca^{2+}-dependent stimulation occurring at micromolar Ca^{2+} concentrations (Fig. 2 *A*). However, the extent of stimulation by Ca^{2+} was increased approximately fourfold in chloride- versus propionate-containing media (36 ± 3.4% B_{max} in chloride versus 9.3 ± 1.3% B_{max} in propionate, at 6 μM Ca^{2+}, $p < 0.01$).

The Ca^{2+} dependence of [^{3}H]ryanodine binding to cardiac muscle SR vesicles was also determined (Fig. 2 *B*). In comparison to skeletal muscle SR, [^{3}H]ryanodine binding to cardiac SR vesicles was stimulated to a greater extent by micromolar Ca^{2+}. Moreover, the maximum Ca^{2+}-dependent stimulation of [^{3}H]ryanodine binding to cardiac muscle SR was similar in chloride- and propionate-containing media (87 ± 2.4% B_{max} in chloride versus 81 ± 5.4% B_{max} in propionate, at 20 μM Ca^{2+}, $p = 0.4$). Thus, consistent with the results of our $^{45}Ca^{2+}$ release experiments, only skeletal muscle SR showed pronounced differences in [^{3}H]ryanodine binding in chloride- versus propionate-containing media.

To further verify that differences in skeletal muscle SR [^{3}H]ryanodine binding in chloride- versus propionate-containing media reflected effects on SR Ca^{2+} permeability, we

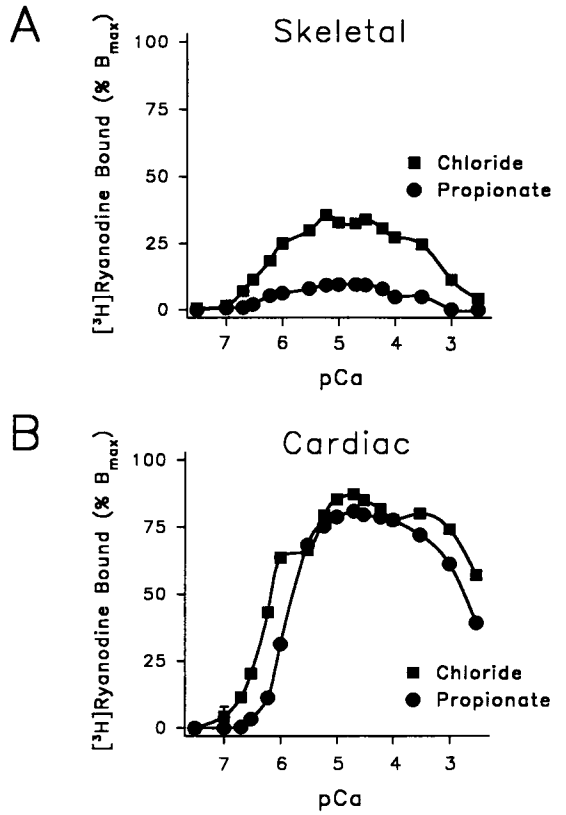

FIGURE 2 Ca^{2+} dependence of [^{3}H]ryanodine binding to SR vesicles equilibrated in KCl (■) or Kpropionate (●). [^{3}H]Ryanodine binding to skeletal muscle SR vesicles (*A*) and cardiac muscle SR vesicles (*B*) was determined as described in Materials and Methods. Data are presented as percentages of B_{max} as determined for each SR preparation within the same experiment (9.9 ± 0.5 pmol/mg and 3.9 ± 0.5 pmol/mg, for three skeletal and three cardiac muscle SR preparations, respectively).

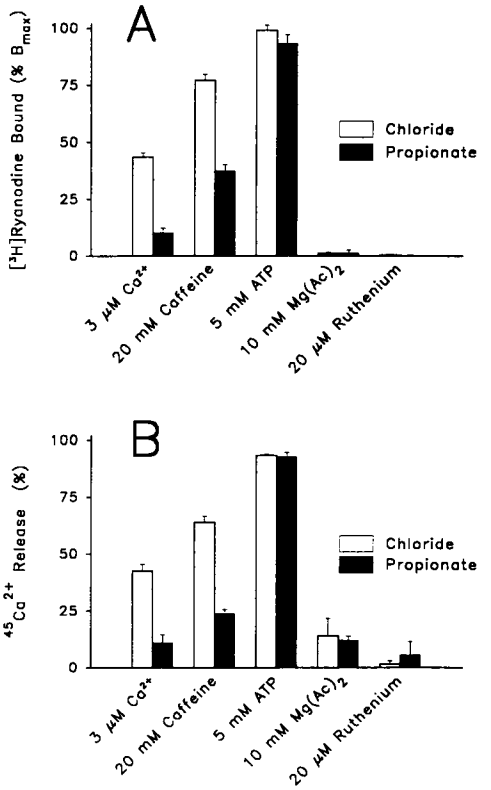

FIGURE 3 Comparison of the effects of RYR channel activators and inhibitors on skeletal muscle SR [^{3}H]ryanodine binding (*A*) and $^{45}Ca^{2+}$ release (*B*) in chloride- and propionate-containing media. Experiments were as described in Materials and Methods in the presence of 3 μM Ca^{2+} and additional activators or inhibitors, as indicated. $^{45}Ca^{2+}$ release (*B*) was determined 1 s after dilution of SR vesicles into release media. All data are means ± SE from three skeletal muscle SR vesicle preparations.

compared the actions of various RYR channel activators and inhibitors on chloride-stimulated [^{3}H]ryanodine binding and $^{45}Ca^{2+}$ release. In the presence of 3 μM Ca^{2+}, [^{3}H]ryanodine binding to skeletal muscle SR was increased approximately fourfold in chloride- as compared to propionate-containing media, and the addition of caffeine (20 mM) further increased Ca^{2+}-stimulated [^{3}H]ryanodine binding to a similar extent in the two different media (Fig. 3 *A*). With the addition of ATP (5 mM), [^{3}H]ryanodine binding was near-maximally activated in both chloride- and propionate-containing media (99 ± 2.2% B_{max} in chloride versus 92 ± 3.8% B_{max} in propionate, $p = 0.2$), whereas the addition of Mg^{2+} (10 mM) or ruthenium red (20 μM) fully blocked [^{3}H]ryanodine binding in both chloride- and propionate-containing media. The effects of these various RYR channel modulators on skeletal muscle SR [^{3}H]ryanodine binding in chloride- versus propionate-containing media (Fig. 3 *A*) were well correlated with their effects on skeletal muscle SR $^{45}Ca^{2+}$ release (Fig. 3 *B*). Thus, differences in skeletal muscle SR [^{3}H]ryanodine binding in chloride- versus propionate-containing media appeared to accurately reflect differences in SR Ca^{2+} permeability in media containing these two different anions.

Effects of other organic and inorganic anions on [^{3}H]ryanodine binding

The effects of substituting chloride with organic anions other than propionate were also examined to rule out the possibility that propionate had a specific inhibitory effect on skeletal muscle RYR activity (Fig. 4). [^{3}H]Ryanodine binding to skeletal muscle SR vesicles in chloride-containing media was therefore compared to that in media containing each of four different univalent organic anions (all at 150 mM). In the presence of 30 nM Ca^{2+}, [^{3}H]ryanodine binding did not exceed nonspecific binding in any of these different media (data not shown). In the presence of 3 μM Ca^{2+}, [^{3}H]ryanodine binding in chloride-containing media was 2- to 10-fold greater than in media containing any of the organic anions used to substitute for chloride (Fig. 4). The relative effectiveness of these different univalent anions in increasing Ca^{2+}-stimulated [^{3}H]ryanodine binding was chloride > methanesulfonate > gluconate > propionate >

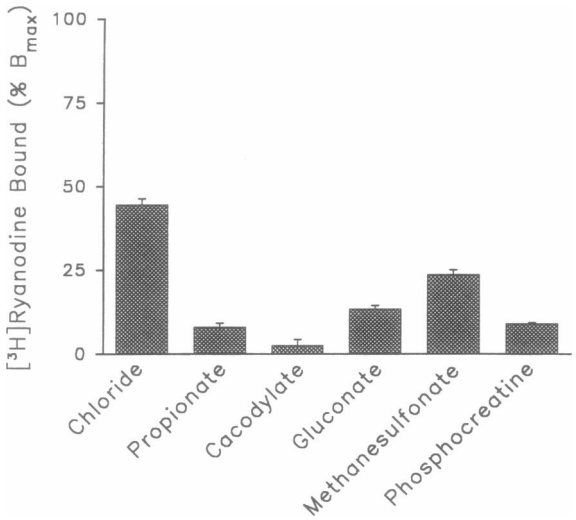

FIGURE 4 Decreased Ca^{2+}-stimulated [^{3}H]ryanodine binding in media containing various organic anions. Skeletal muscle SR [^{3}H]ryanodine binding was determined in media containing 3 μM Ca^{2+}, and either chloride or one of four different univalent organic anions, all at 150 mM. The divalent phosphocreatine was included at a concentration of 57 mM, to produce an ionic strength equivalent to 150 mM univalent salt. Data are means ± SE from three SR vesicle preparations.

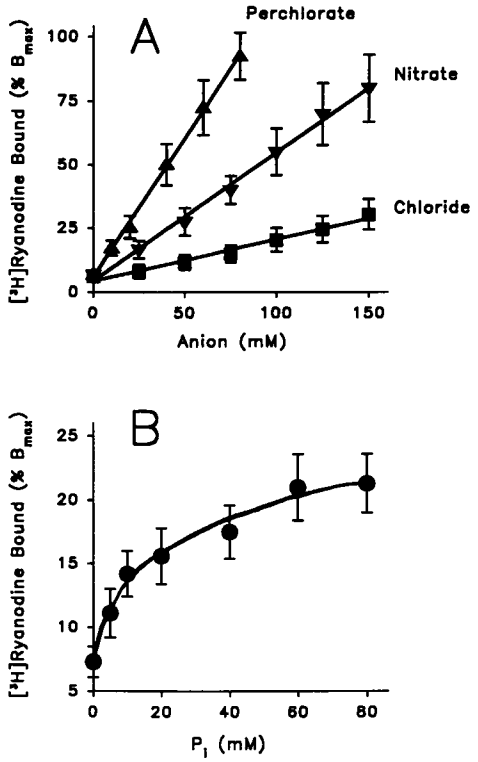

FIGURE 5 Increased Ca^{2+}-stimulated [^{3}H]ryanodine binding in the presence of various inorganic anions. Skeletal muscle SR [^{3}H]ryanodine binding was determined in the presence of 150 mM propionate, 3 μM Ca^{2+}, and the indicated concentrations of perchlorate (▲), nitrate (▼), and chloride (■; *A*); or P_i (*B*). Experiments were performed as described in Materials and Methods, except that all anions were added as sodium salts, thereby avoiding the formation of $KClO_4$ precipitate. Data are means ± SE from three SR vesicle preparations.

cacodylate. The effect of phosphocreatine was also examined, as phosphocreatine constitutes the predominant anion in resting skeletal muscle (Godt and Maughan, 1988). Ca^{2+}-stimulated [^{3}H]ryanodine binding in phosphocreatine-containing media was approximately one-fifth of that in chloride-containing media (9.0 ± 0.3% B_{max} in phosphocreatine versus 44 ± 1.9% B_{max} in chloride, $p < 0.01$). These results thus indicate that in media containing a variety of organic anions, Ca^{2+}-stimulated [^{3}H]ryanodine binding to skeletal muscle SR is markedly decreased relative to that in media containing chloride as the major anion.

To examine the relationship of chloride-dependent stimulation of skeletal muscle RYR activity to stimulation by other inorganic anions, we compared the concentration dependence of the stimulation of SR vesicle [^{3}H]ryanodine binding by chloride to the stimulation by the chaotropic anions perchlorate and nitrate (Fig. 5 *A*) and to that by P_i (Fig. 5 *B*). Like perchlorate and nitrate, chloride-dependent stimulation of [^{3}H]ryanodine binding to skeletal muscle SR vesicles was linear and did not saturate over the entire concentration range examined (up to 150 mM). In addition, the relative effectiveness of each of these three inorganic anions in stimulating [^{3}H]ryanodine binding was in keeping with their relative chaotropic strength (perchlorate > nitrate > chloride). In contrast, P_i-dependent stimulation of skeletal muscle SR vesicle [^{3}H]ryanodine binding appeared saturable, suggesting an interaction of P_i with specific activation sites in the SR vesicles (Fig. 5 *B*). Thus, although stimulation by P_i exceeded that by chloride at all concentrations less than or equal to 80 mM, this difference was most pronounced at lower P_i concentrations (e.g., 16 ± 2.2% B_{max} at 20 mM P_i versus 8.1 ± 2.7% B_{max} at 25 mM chloride, $p = 0.05$).

[^{3}H]Ryanodine binding to skeletal muscle RYR reconstituted into liposomes

To determine whether the effects of chloride may be altered after reconstitution of the RYR1 protein, we examined Ca^{2+}-activated [^{3}H]ryanodine binding to RYR purified from skeletal muscle SR and reconstituted into artificial liposomes. Micromolar Ca^{2+} stimulated [^{3}H]ryanodine binding to the reconstituted RYR protein in both chloride- and propionate-containing media (Fig. 6 *A*). In contrast, [^{3}H]ryanodine binding to the native skeletal muscle SR vesicles was stimulated by micromolar Ca^{2+} in chloride-containing media, but was only minimally stimulated by Ca^{2+} in propionate-containing media (Fig. 6 *B*). Thus, in propionate-containing media, Ca^{2+}-dependent [^{3}H]ryanodine binding to reconstituted RYR was increased more than fourfold relative to native SR (53 ± 10% B_{max} versus 12 ± 1.4% B_{max}, at 3 μM Ca^{2+}, $p < 0.01$). Furthermore, the marked difference in Ca^{2+}-dependent [^{3}H]ryanodine binding to native skeletal muscle SR in chloride- versus propi-

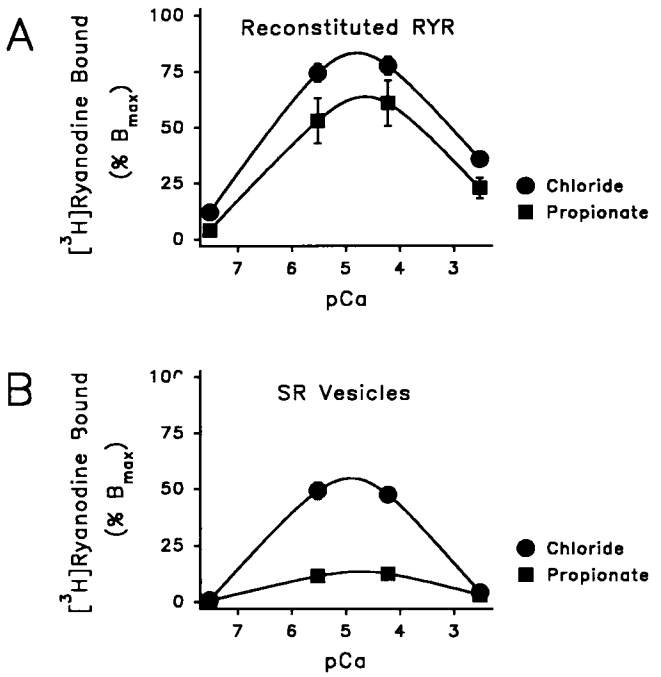

FIGURE 6 Increased Ca^{2+}-stimulated [^{3}H]ryanodine binding to skeletal muscle RYR reconstituted into liposomes. The Ca^{2+} dependence of [^{3}H]ryanodine binding to skeletal muscle RYR reconstituted into artificial liposomes (*A*) or to skeletal muscle SR vesicles prepared from the same animal (*B*) was determined in media containing either 150 mM KCl (■) or 150 mM Kpropionate (●), as described in Materials and Methods. Data are means ± SE from four preparations (B_{max} = 227 ± 38 pmol/mg and 8.4 ± 1.0 pmol/mg, for *A* and *B*, respectively).

onate-containing media was diminished after reconstitution of RYR protein into liposomes.

DISCUSSION

In a number of common experimental protocols measuring SR Ca^{2+} release, the major fraction of release may be dependent on the presence of supraphysiological concentrations of chloride. Nevertheless, the mechanism of this chloride-dependent Ca^{2+} release and its potential relevance to physiological mechanisms controlling SR Ca^{2+} release in vivo have remained in question. For example, the stimulatory effects of chloride on Ca^{2+} release from SR vesicles have been variously ascribed to osmotic lysis of the vesicles (Meissner and McKinley, 1976), to SR membrane depolarization (Campbell and Shamoo, 1980), and to a direct activation of RYR channels (Hasselbach and Migala, 1992). Most recently it has been proposed that the effects of chloride on SR Ca^{2+} release may be evidence for a major, previously unrecognized Ca^{2+} release pathway in skeletal muscle SR that is distinct from the well-characterized RYR channels (Sukhareva et al., 1994; Patel et al., 1995; Sukhareva and Coronado, 1996).

Chloride-dependent SR Ca^{2+} release is mediated by skeletal muscle RYR1 channels

In the present study we have further examined the possible role of RYR channels in mediating chloride-dependent SR Ca^{2+} release. Our results indicate that chloride-dependent Ca^{2+} release from skeletal muscle SR is Ca^{2+} dependent. Accordingly, $^{45}Ca^{2+}$ release from skeletal muscle SR vesicles in the presence of 30 nM Ca^{2+} was not significantly increased when vesicles were equilibrated in media containing 150 mM chloride versus 150 mM propionate, whereas $^{45}Ca^{2+}$ release in the presence of 3 μM Ca^{2+} was increased approximately fourfold in chloride- versus propionate-containing media (Fig. 1 *A*). $^{45}Ca^{2+}$ release activated by 20 mM caffeine was also increased in chloride-containing media (~2.5-fold; Fig. 3 *B*), so that in contrast to the findings of Sukhareva et al. (1994), release was activated by caffeine both in the presence and in the absence of chloride. Increases in $^{45}Ca^{2+}$ release in chloride-containing media were reflected by similar increases in [^{3}H]ryanodine binding to skeletal muscle SR vesicles (Figs. 2 *A* and 3 *A*). Chloride, however, had little effect on cardiac muscle SR $^{45}Ca^{2+}$ release or [^{3}H]ryanodine binding (Figs. 1 *B* and 2 *B*). Our results thus indicate a close correlation of chloride-dependent increases in SR Ca^{2+} release with increased activation of skeletal muscle RYR1 channels, and thereby support the conclusion of Hasselbach and Migala (1992) that chloride's effects are the result of a direct action of this anion on the functional activity of the RYR channel complex. Although this conclusion would appear to conflict with the reported failure of chloride to significantly affect the single-channel open probability of RYR1 channels in planar lipid bilayers (Sukhareva et al., 1994), we also found that chloride-dependent stimulation of [^{3}H]ryanodine binding was diminished when RYR1 protein was reconstituted into artificial liposomes (Fig. 6). Thus it is possible that chloride's effects are most apparent on RYR1 channels in intact SR membranes and may be similarly diminished when these channels are diluted into planar lipid bilayers or reconstituted into liposomes. Clarification of the factors responsible for the decreased chloride activation of RYRs in bilayers and in proteoliposomes will require further studies, however.

Chloride and chaotropic anions may increase SR Ca^{2+} release through a common mechanism

The effects of chloride on RYR1 channels resemble the effects of nitrate and perchlorate, chaotropic anions that act as potentiators of excitation-contraction coupling in skeletal muscle (reviewed in Melzer et al., 1995). Accordingly, the effect of each of these inorganic anions was to increase the Ca^{2+}-dependent activation of skeletal muscle RYR1 channels, while having little or no effect on cardiac muscle RYR2 channels (Figs. 1 and 2; Fruen et al., 1994a). Furthermore, the concentration dependence of the stimulation of skeletal muscle SR [^{3}H]ryanodine binding exhibited by each of these inorganic anions was linear over a wide range

of concentrations, and the relative effectiveness of these different anions in stimulating binding was correlated with their relative chaotropic strength (perchlorate > nitrate > chloride; Fig. 5 *A*). These results support the view (Gonzalez and Rios, 1993) that these inorganic anions exert their effects not by interacting with a discrete site on the RYR1 channel, but rather through a mechanism that depends on their chaotropic properties. According to this view, a number of inorganic anions of different molecular size and structure may increase RYR1 channel activity through a common mechanism, presumably related to their ability to destabilize protein-protein interactions within the RYR1 channel complex or otherwise affect the conformational stability of the RYR1 channel protein.

We have previously shown that the endogenous inorganic anion P_i also increases RYR1 channel activity (Fruen et al., 1994b). At lower concentrations (1–20 mM) the effects of P_i and chaotropic anions on RYR1 channels were similar in terms of their magnitude, dependence on micromolar Ca^{2+}, and specificity for skeletal as compared to cardiac muscle RYRs (Fruen et al., 1994a). However, the present results indicate a difference in the concentration dependence of stimulation of [^{3}H]ryanodine binding to skeletal muscle SR by P_i and other anions at higher concentrations. Thus, whereas the concentration dependence of stimulation by chloride, nitrate, and perchlorate was linear up to 150 mM, the major stimulatory effect of P_i occurred at concentrations less than or equal to 40 mM and largely plateaued at higher concentrations (Fig. 5). These data suggest that, in contrast to chaotropic anions, P_i may activate RYR1 channels by preferentially interacting with specific sites on skeletal muscle RYR1 channels. In vivo these sites may thus be largely saturatable by P_i concentrations generated during muscle activity ($\geq$30 mM).

The anionic composition of resting skeletal muscle may not support Ca^{2+}-induced Ca^{2+} release

Concentrations of inorganic anions capable of enhancing RYR1 Ca^{2+} activation are not likely to exist in the myoplasm of resting skeletal muscle. In resting frog skeletal muscle, for example, the concentration of chloride has been estimated at only 2.2 mM and that of P_i at 1.4 mM (reviewed by Godt and Maughan, 1988). The anionic composition of the resting myoplasm is instead dominated by organic anions, with phosphocreatine being the major species (estimated concentration of approximately 50 mM in resting frog muscle; Godt and Maughan, 1988). Our results indicate that under anionic conditions resembling those in resting muscle, skeletal muscle SR vesicle RYR1 channels may exhibit only minimal activation by Ca^{2+}. For example, in media containing phosphocreatine as the major anion, Ca^{2+}-activated [^{3}H]ryanodine binding to skeletal muscle SR vesicles was approximately one-fifth of that in chloride-containing media (Fig. 4) and one-tenth of that in nitrate- or perchlorate-containing media (Fig. 5). Comparable changes in the anionic composition of the media had little effect on the extent of Ca^{2+} activation of cardiac RYR2 channels (Figs. 1 and 2; Fruen et al., 1994a). These data demonstrate that differences in the extent of Ca^{2+} activation of skeletal and cardiac muscle RYR isoforms are most pronounced in media composed primarily of more physiological organic anions. By the same token, differences in the Ca^{2+} activation of skeletal and cardiac muscle RYRs are less apparent in media containing more chaotropic inorganic anions, such as chloride, nitrate, or perchlorate.

Interestingly, the activation of skeletal muscle RYR channels by Ca^{2+} was also increased after reconstitution of RYR1 channel protein into artificial liposomes (Fig. 6). Similarly, Ma et al. (1993) reported an increase in the affinity of [^{3}H]ryanodine binding upon reconstitution of purified RYR protein into liposomes. These results suggest that the Ca^{2+} activation of skeletal muscle RYR1 channels may be enhanced not only in the presence of chloride and other inorganic anions, but also when RYR1 protein is diluted into an artificial membrane lipid environment. This effect may explain why RYR1 channels in planar lipid bilayers exhibit significant Ca^{2+} activation, even in the absence of chloride or other inorganic anions (e.g., Rousseau et al., 1987; Gyorke et al., 1994). We suggest that an enhanced Ca^{2+} activation of RYR1 channels may therefore be an important consideration not only when one is interpreting SR vesicle experiments performed in nonphysiological chloride-containing media, but also when RYR1 protein reconstituted either in liposomes or in planar lipid bilayers is being used.

Anion effects may reflect alternative RYR1 conformations with distinct Ca^{2+} activation properties

Among the molecular components controlling SR Ca^{2+} release in muscle, the skeletal muscle RYR1 protein exhibits a particular sensitivity to changes in the anionic composition of the medium. This is most clearly demonstrated in comparisons of anion effects on the functional activity of the skeletal muscle RYR1 and cardiac muscle RYR2 isoforms (Figs. 1 and 2; Fruen et al., 1994a). That transverse tubule voltage sensors may also be less sensitive than RYR1 channels to the direct effects of anions is indicated by the absence of significant effects of millimolar perchlorate on dihydropyridine binding to isolated skeletal muscle membrane vesicles (Gallant et al., 1993; Ma et al., 1993) and on L-type Ca^{2+} currents in ventricular myocytes (Ma et al., 1993). Although the precise molecular mechanism by which anions selectively effect RYR1 channel activity remains to be defined, it is thought that an important mechanism of regulating ion channels may involve the stabilization of the channel protein in conformations with different activation properties (e.g., Delcour and Tsien, 1993). Thus the sensitivity of RYR1 channel activation to anions may be a

reflection of this channel's ability to easily assume alternative conformational states depending on the anionic composition of the media.

Evidence that conformational changes in the RYR1 protein are important in controlling RYR1 channel activation has been provided by the fluorescent conformational probe studies of Ikemoto and co-workers (Kang et al., 1992; Yano et al., 1995a). These studies indicate that the RYR1 protein undergoes a rapid conformational change in response to transverse tubule depolarization; this conformational change precedes and is distinct from channel opening but may be a prerequisite for channel activation (Yano et al., 1995a). Importantly, perchlorate (15–30 mM) produced a conformational change that appeared equivalent to that produced by depolarization (Yano et al., 1995b). Thus a tenable hypothesis is that inorganic anions enhance RYR1 channel activity by facilitating a conformational transition in the RYR1 protein that is an integral step in controlling RYR1 activation in situ.

The present results indicate that any effects that anions may have on the conformational state of the skeletal muscle RYR1 protein are likely linked to major changes in the Ca^{2+} activation properties of the RYR1 channel. Thus media containing primarily organic anions appear to favor a conformational state of the RYR1 protein that is characterized by being unresponsive to activation by Ca^{2+}, whereas media containing high concentrations of inorganic/chaotropic anions (perchlorate > nitrate > chloride) appear to favor a conformational state that, like RYR2, is more fully activated by micromolar Ca^{2+}. This increased activation by micromolar Ca^{2+} in media containing inorganic anions may also be associated with a decreased sensitivity of RYR1 channels to inactivation by millimolar concentrations of Mg^{2+}, procaine, or Ca^{2+} (Sukhareva et al., 1994; Ogawa, 1994; Fruen et al., 1994b).

The possibility that RYR1 channels may assume alternative Ca^{2+} activation states is also supported by recent studies of Ca^{2+}-dependent RYR1 channel activity in planar lipid bilayers (Percival et al., 1994; Copello et al., 1996). These studies suggest that RYR1 channels may interconvert between discrete high-activity and low-activity states that differ in the extent to which they are activated by Ca^{2+} in the absence of ATP. In contrast to this heterogeneous Ca^{2+}-dependent activation observed for RYR1 channels, cardiac RYR2 channels (Copello et al., 1996) and avian RYRβ channels (Percival et al., 1994) appeared less heterogeneous and displayed a consistent Ca^{2+}-activatable state. These studies thus suggest that the capacity of the RYR1 channel to exhibit different Ca^{2+} activation states may be a defining characteristic of this RYR isoform in lipid bilayers. Furthermore, our results suggest that manipulations of the anionic composition of the media may provide an effective means of stabilizing these different activation states in SR vesicle preparations.

In summary, we have further examined the mechanism by which chloride anions affect SR Ca^{2+} release. Our results indicate that the effects of various anions, including chloride, are primarily a function of their effects on RYR1 channels. We suggest that these effects may be attributable to the influence of the anionic composition of the media on the conformational stability of the RYR1 channel protein, and that media of different anionic composition may thus favor alternative conformational states of the RYR1 protein with different functional properties. In this view, media containing supraphysiological concentrations of chloride or other inorganic/chaotropic anions may favor a conformational state of the channel that is characterized by a marked increase in its activation by Ca^{2+}. In vivo, conformational transitions to this putative Ca^{2+}-activatable state may be an integral mechanism controlling SR Ca^{2+} release in skeletal muscle and may be mediated by protein-protein interactions, by phosphorylation, or by endogenous ligands such as ATP and P_i.

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