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Regulation of Skeletal Muscle Ca^{2+} Release Channel (Ryanodine Receptor) by Ca^{2+} and Monovalent Cations and Anions*

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The effects of ionic composition and strength on rabbit skeletal muscle Ca^{2+} release channel (ryanodine receptor) activity were investigated in vesicle-$^{45}Ca^{2+}$ flux, single channel and [^{3}H]ryanodine binding measurements. In <0.01 μM Ca^{2+} media, the highest $^{45}Ca^{2+}$ efflux rate was measured in 0.25 M choline-Cl medium followed by 0.25 M KCl, choline 4-morpholineethanesulfonic acid (Mes), potassium 1,4-piperazinediethanesulfonic acid (Pipes), and K-Mes medium. In all five media, the $^{45}Ca^{2+}$ efflux rates were increased when the free [Ca^{2+}] was raised from <0.01 μM to 20 μM and decreased as the free [Ca^{2+}] was further increased to 1 mM. An increase in [KCl] augmented Ca^{2+}-gated single channel activity and [^{3}H]ryanodine binding. In [^{3}H]ryanodine binding measurements, bell-shaped Ca^{2+} activation/inactivation curves were obtained in media containing different monovalent cations (Li^+, Na^+, K^+, Cs^+, and choline$^+$) and anions (Cl^-, Mes^-, and $Pipes^-$). In choline-Cl medium, substantial levels of [^{3}H]ryanodine binding were observed at [Ca^{2+}] <0.01 μM. Replacement of Cl^- by Mes^- or $Pipes^-$ reduced [^{3}H]ryanodine binding levels at all [Ca^{2+}]. In all media, the Ca^{2+}-dependence of [^{3}H]ryanodine binding could be well described assuming that the skeletal muscle ryanodine receptor possesses cooperatively interacting high-affinity Ca^{2+} activation and low-affinity Ca^{2+} inactivation sites. AMP primarily affected [^{3}H]ryanodine binding by decreasing the apparent affinity of the Ca^{2+} inactivation site(s) for Ca^{2+}, while caffeine increased the apparent affinity of the Ca^{2+} activation site for Ca^{2+}. Competition studies indicated that ionic composition affected Ca^{2+}-dependent receptor activity by at least three different mechanisms: (i) competitive binding of Mg^{2+} and monovalent cations to the Ca^{2+} activation sites, (ii) binding of divalent cations to the Ca^{2+} inactivation sites, and (iii) binding of anions to specific anion regulatory sites.

In skeletal muscle, an intracellular Ca^{2+} conducting channel releases Ca^{2+} from the sarcoplasmic reticulum (SR)[1] in response to an action potential, to bring about muscle contraction (1–3). The Ca^{2+} release channels are also known as ryanodine receptors (RyR) because they can bind the plant alkaloid ryanodine with high affinity and specificity. The skeletal muscle RyR has been purified as a 30 S protein complex comprising four large (ryanodine receptor, M_r 565,000) and four small (FK506-binding protein, M_r 12,000) subunits, and shown to be regulated by various endogenous and exogenous effector molecules including Ca^{2+}, Mg^{2+}, ATP, calmodulin, caffeine, and ryanodine (4–6).

Skeletal muscle RyR activity is affected by the ionic strength and composition of the assay media. An increase in KCl or NaCl concentration stimulates Ca^{2+} release from SR vesicles and increases [^{3}H]ryanodine binding (7–11). A stimulation of [^{3}H]ryanodine binding (9) and slowing of single channel gating (12) by sucrose in the presence of salt suggests that the osmolarity and viscosity of the assay media may play a role in determining channel activity. Anions often classified as chaotropic ions (ClO_4^-, SCN^-, I^-, NO_3^-) (13, 14) and inorganic phosphate anions (15) stimulate Ca^{2+} release channel activity and [^{3}H]ryanodine binding, whereas replacement of Cl^- by gluconate$^-$ decreases SR Ca^{2+} release and [^{3}H]ryanodine binding (13). These results suggest that monovalent cations and anions as well as osmolarity or viscosity may modulate skeletal muscle RyR activity. However, the mechanism(s) by which these ions affect the SR Ca^{2+} release channel have remained unclear.

Here, we describe the effects of monovalent cations and anions on $^{45}Ca^{2+}$ efflux from and [^{3}H]ryanodine binding to rabbit skeletal muscle SR vesicles. The effects of ionic strength were also determined in single channel measurements. Our results indicate that RyR activity may be affected by the binding of cations to Ca^{2+} regulatory sites and anions to anion regulatory sites, and that there is a strong functional interaction between the two classes of regulatory sites.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]Ryanodine was purchased from DuPont NEN and $^{45}Ca^{2+}$ from ICN Biomedicals. Unlabeled ryanodine was obtained from Calbiochem, and leupeptin and Pefabloc (a protease inhibitor) from Boehringer Mannheim. All other chemicals were of analytical grade.

Preparation of SR Vesicles—"Heavy" SR vesicle fractions enriched in [^{3}H]ryanodine binding and Ca^{2+} release channel activities were prepared in the presence of protease inhibitors (100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride) as described (16). The maximum number of high-affinity [^{3}H]ryanodine-binding sites determined under optimal binding conditions (17) ranged from 11 to 23 pmol/mg protein, depending on the preparation.

$^{45}Ca^{2+}$ Efflux Measurements—SR vesicles (5–10 mg of protein/ml) were passively loaded for 60 min at 23 °C with 2 mM $^{45}Ca^{2+}$ in media containing 20 mM imidazole, pH 6.8, protease inhibitors (0.2 mM Pefabloc, 20 μM leupeptin), and different salts as described (18). $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 1:300 into efflux media that contained the salt used in the incubation step, and stopped by placing 0.4-ml aliquots at various times on a 0.45-μm filter (type HA, Millipore). Filters were washed with a quench solution containing 20 mM imidazole, pH 6.8, the salt used in the incubation step, 10 mM Mg^{2+}, 20 μM ruthenium red, and 0.2 mM EGTA. Rapid $^{45}Ca^{2+}$ efflux was determined

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[1] The abbreviations used are: SR, sarcoplasmic reticulum; RyR, ryanodine receptor; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; AMP-PCP,adenosine 5′-(β,γ-methyleneltriphosphate; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate.

with a Biologic Rapid Filtration system (Meylan, France). Aliquots of the passively loaded vesicles (about 10 μg of protein) were placed on 0.65 μm (type DA) Millipore filters. The filters were prewashed for 30 s with 3 × 1 ml of a medium containing 20 mM imidazole, pH 6.8, 1 mM choline EGTA, 1 mM $MgCl_2$, and the salt used in the incubation step. Vesicles on the filters were then washed for 0.05–3 s with release media containing 20 mM imidazole, pH 6.8, the salt used in the incubation step, and different concentrations of free Ca^{2+}. Radioactivity remaining with the vesicles on the filters was determined by liquid scintillation counting. The time course of $^{45}Ca^{2+}$ efflux from the Ca^{2+}-permeable vesicle population was obtained by subtracting the amount not readily released (18).

Single Channel Measurements—Single channel measurements were performed by fusing proteoliposomes containing the purified skeletal muscle Ca^{2+} release channel with Mueller-Rudin-type bilayers as described (19). Single channels were recorded in symmetric KCl buffers containing the additions indicated in the text. Electrical signals were filtered at 4 kHz, digitized at 20 kHz, and analyzed as described (19).

[^{3}H]Ryanodine Binding—Unless otherwise indicated, samples were incubated at 12 °C with 1 nM [^{3}H]ryanodine in media containing 20 mM imidazole, pH 7.2, 0.2 mM Pefabloc, 20 μM leupeptin, and the indicated salt, 0.45 mM 1,2-bis(2-aminophenoxy)ethanetetraacetic acid, 0.9 mM nitrilotriacetic acid, and Ca^{2+} concentrations to yield the indicated free Ca^{2+} concentrations. Nonspecific binding was determined using a 1000-fold excess of unlabeled ryanodine. A relatively low incubation temperature of 12 °C was used to minimize receptor inactivation during the binding reaction. At 12 °C, an incubation time of 90–120 h was generally sufficient to obtain close to maximum [^{3}H]ryanodine binding (see "Results"). After 90–120 h, aliquots of the samples were diluted with 20 volumes of ice-cold water and placed on Whatman GF/B filters soaked with 2% polyethyleneimine. Filters were washed with three 5-ml volumes of ice-cold 0.1 M KCl, 1 mM K-Pipes, pH 7.0, medium, and the radioactivity remaining on the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

Other Biochemical Assays—Protein concentrations were determined by the Lowry method using bovine serum albumin as the protein standard. Free Ca^{2+} concentrations were obtained by including in the solutions the appropriate amounts of Ca^{2+} and Ca^{2+} chelators as determined using the stability constants and computer program published by Shoenmakers *et al.* (20). Free Ca^{2+} concentrations of >1 μM were verified with the use of a Ca^{2+} selective electrode (World Precision Instruments, Inc., Sarasota, FL), except in the 1 M solutions in which the Ca^{2+} electrode measurements were not possible at <10 μM free Ca^{2+} because of limited electrode selectivity.

Data Analysis—The Ca^{2+} dependence of [^{3}H]ryanodine binding was analyzed assuming that the skeletal muscle Ca^{2+} release channel possesses cooperatively interacting high-affinity Ca^{2+} activation and low-affinity Ca^{2+} inactivation binding sites. A simple scheme used to describe the Ca^{2+} dependence of channel activity in media containing inorganic monovalent cations was,

$$R \underset{}{\overset{\mu M\ Ca^{2+}}{\rightleftharpoons}} A_{Ca} \overset{mM\ Ca^{2+}}{\rightleftharpoons} {}_{Ca}I_{Ca}$$

SCHEME 1

In the above scheme (Scheme 1), the Ca^{2+} release channel is assumed to have high-affinity Ca^{2+} activation and low-affinity Ca^{2+} inactivation sites. The channel is present in its closed Ca^{2+}-free form, designated R at $[Ca^{2+}] < 0.1$ μM, and its Ca^{2+}-activated (A_{Ca}) and Ca^{2+}-inactivated ($_{Ca}I_{Ca}$) forms at μM and mM Ca^{2+} concentrations, respectively. The tetrameric Ca^{2+} release channel contains cooperatively interacting Ca^{2+} activation sites and Ca^{2+} inactivation sites (see "Results"), however, only one Ca^{2+} activation and one Ca^{2+} inactivation site are shown.

Ryanodine binding (and, by extension, channel activity) was fitted by the product of an activation and an inactivation variable, each related to $[Ca^{2+}]$ by the Hill formalism,

$$B = B_o([Ca^{2+}]^{n_a}/([Ca^{2+}]^{n_a} + K_a^{n_a}))(1 - [Ca^{2+}]^{n_i}/([Ca^{2+}]^{n_i} + K_i^{n_i})) \quad \text{(Eq. 1)}$$

where B is the [^{3}H]ryanodine binding value at a given $[Ca^{2+}]$, B_o is the binding maximum, K_a and K_i are Hill activation and inactivation constants, and n_a and n_i are the respective Hill coefficients. In the calculations, B_o was included as one of the variables.

In choline$^+$ media, [^{3}H]ryanodine binding was fitted according to the equation,

$$B = B_o([Ca^{2+}]^{n_a} + [choline^+])/([Ca^{2+}]^{n_a} + [choline^+] + K_a^{n_a})$$
$$(1 - [Ca^{2+}]^{n_i}/([Ca^{2+}]^{n_i} + K_i^{n_i})) \quad \text{(Eq. 2)}$$

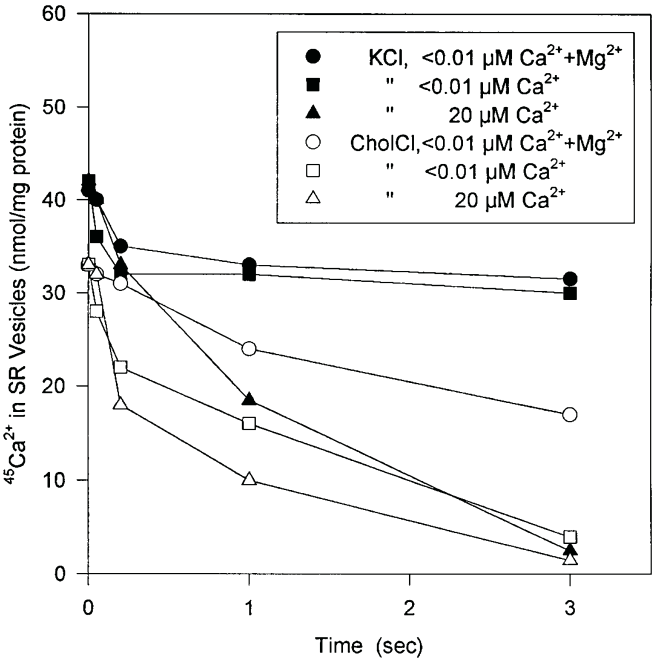

FIG. 1. **Time course of $^{45}Ca^{2+}$ efflux from SR vesicles in KCl and choline-Cl media.** SR vesicles (5 mg of protein/ml) were incubated for 60 min at 24 °C with 2 mM $^{45}Ca^{2+}$ in media containing 20 mM imidazole, pH 6.8, protease inhibitors (0.2 mM Pefabloc, 20 μM leupeptin), and 0.25 M of either KCl (*solid symbols*) or choline-Cl (*open symbols*). $^{45}Ca^{2+}$ efflux was determined in efflux media containing 20 mM imidazole, pH 6.8, 0.25 M of either KCl (*solid symbols*) or choline-Cl (*open symbols*), 0 or 5 mM Mg^{2+}, and the indicated concentrations of free Ca^{2+}. Amounts of $^{45}Ca^{2+}$ remaining with Ca^{2+}-permeable vesicles were determined with the use of a rapid filtration apparatus as described under "Experimental Procedures."

which formalizes the assumption that choline$^+$ is a weak, noncooperative Ca^{2+} agonist of the Ca^{2+} release channel.

In the competition studies, [^{3}H]ryanodine binding was fitted with the equations,

$$B = B_o([Ca^{2+}]^{n_a} + [choline^+])/([Ca^{2+}]^{n_a} + [choline^+] + K_{Ca,eff}{}^{n_a}) \quad \text{(Eq. 3)}$$

$$K_{Ca,eff}{}^{n_a} = K_{Ca}{}^{n_a}((K_i^{n_i} + [I]^{n_i})/K_i^{n_i}) \quad \text{(Eq. 4)}$$

where B is the [^{3}H]ryanodine binding value at a given $[Ca^{2+}]$, B_o the binding maximum in the absence of the inhibitor (I), K_{Ca} the Ca^{2+} activation constant, and K_i the inhibition constant of the inhibitor.

Results are given as means ± S.D. with the number of experiments in parentheses. Unless otherwise indicated, significance of differences of data was analyzed with Student's unpaired t test. Differences were regarded to be statistically significant at $p < 0.05$.

RESULTS

SR Vesicle-$^{45}Ca^{2+}$ Efflux Measurements—In preliminary experiments, the effects of ionic composition on Ca^{2+} release channel activity were assessed in SR vesicle-$^{45}Ca^{2+}$ efflux measurements. Fig. 1 illustrates the $^{45}Ca^{2+}$ efflux behavior of vesicles diluted into KCl or choline-Cl media. Vesicles were passively loaded with 2 mM $^{45}Ca^{2+}$ in 0.25 M KCl (*closed symbols*) or 0.25 M choline-Cl (*open symbols*) medium and then diluted into the same medium containing 5 mM Mg^{2+} (an inhibitor of the Ca^{2+} release channel) and <0.01 μM free Ca^{2+}, <0.01 μM free Ca^{2+}, or 20 μM free Ca^{2+}. $^{45}Ca^{2+}$ efflux was slow when vesicles were diluted into media containing <0.01 μM Ca^{2+} and 5 mM Mg^{2+}. Omission of Mg^{2+} from <0.01 μM Ca^{2+} media increased the $^{45}Ca^{2+}$ efflux rate to a greater extent in choline-Cl than KCl medium. In the presence of 20 μM Ca^{2+}, similar $^{45}Ca^{2+}$ efflux rates were observed. In both media, the vesicles released half their $^{45}Ca^{2+}$ contents in less than 1 s.

Table I summarizes $^{45}Ca^{2+}$ efflux data obtained in media containing either K^+ or choline$^+$ as a cation and Cl^-, Mes^-, or

TABLE I
Effect of ionic composition on SR $^{45}Ca^{2+}$ release

$^{45}Ca^{2+}$ efflux rates were determined as described in the legend to Fig. 1 in either 0.25 M choline$^+$ or 0.25 M K^+ media. Ca^{2+}-permeable vesicles released half their $^{45}Ca^{2+}$ stores at the indicated times. Data are the averages of two experiments done in triplicate.

Composition of $^{45}Ca^{2+}$ efflux media	Choline-Cl	Choline-Mes	KCl	K-Mes	K-Pipes
			$^{45}Ca^{2+}$ efflux, $t_{1/2}$ (s)		
<0.01 μM Ca^{2+} + 5 mM Mg^{2+}	100	130	170	230	120
<0.01 μM Ca^{2+}	0.7	17	14	150	80
20 μM Ca^{2+}	0.4	3	0.8	8	5
1 mM Ca^{2+}	1.6	40	1.5	70	16

Pipes$^-$ as an anion. In the presence of 5 mM Mg^{2+} at <0.01 μM Ca^{2+}, a time of 100 s or more was required for the vesicles to release half their $^{45}Ca^{2+}$ stores. Omission of Mg^{2+} from the low Ca^{2+} media resulted in a significant increase in the $^{45}Ca^{2+}$ efflux rates. The highest rate was measured in choline-Cl medium followed by KCl, choline-Mes, K-Pipes, and K-Mes medium. In all five media, the $^{45}Ca^{2+}$ efflux rates were increased when the free [Ca^{2+}] was raised from <0.01 to 20 μM, and decreased as the free [Ca^{2+}] was further raised to 1 mM. In agreement with previous vesicle ion flux measurements (10, 11, 13, 15, 18, 21, 22), these results suggest that the Ca^{2+} release channel is activated by micromolar concentrations of Ca^{2+}, inhibited by millimolar concentrations of Ca^{2+}, and furthermore, that the channel's activity is profoundly affected by the ionic composition of the Ca^{2+} efflux media.

Ca^{2+}-dependence of [^{3}H]Ryanodine Binding in Media of Different Ionic Composition—The dependence of Ca^{2+} release channel activity on [Ca^{2+}] and ionic composition was studied in greater detail by measuring high-affinity [^{3}H]ryanodine binding to SR vesicles. Ryanodine is a neutral plant alkaloid that is widely used to monitor the activity of the Ca^{2+} release channel (4–6). Measurements of $^{45}Ca^{2+}$ flux in SR vesicles and of single channels in planar lipid bilayers have shown that ryanodine activates the SR Ca^{2+} release channel at low (submicromolar) concentrations by causing the formation of an open subconductance state, and fully closes the channel at high (micromolar) concentrations. [^{3}H]Ryanodine binding studies have confirmed the presence of high- and low-affinity binding sites, and furthermore, have shown that [^{3}H]ryanodine binds with high specificity to the Ca^{2+} release channel. As a general rule, conditions that open the channel, such as the presence of micromolar Ca^{2+}, millimolar adenine nucleotide, or high ionic strength, were found to increase the affinity of [^{3}H]ryanodine binding to the high-affinity site.

In preliminary experiments, the time course of specific [^{3}H]ryanodine binding to SR vesicles was determined as described under "Experimental Procedures" at 12 °C in a 0.25 M KCl medium containing 20 μM free Ca^{2+}. [^{3}H]Ryanodine binding was slow occurring with a time constant of 42 ± 4 h (n = 3) (not shown). In the [^{3}H]ryanodine binding experiments described below an incubation time of 90–120 h was used to obtain close to equilibrium binding levels.

Fig. 2*A* compares the Ca^{2+} dependence of [^{3}H]ryanodine binding to SR vesicles incubated in media containing four of the five ion combinations tested in $^{45}Ca^{2+}$ efflux experiments (Table I). In control experiments, the buffer (−salt) and a nonionic solute (0.5 M sucrose) with an osmolarity comparable to that of the salts were used. The highest level of binding was measured in 0.25 M choline-Cl medium followed by KCl and choline-Mes medium. In the three media, bell shaped Ca^{2+} activation/inactivation curves were obtained, with the maximally activating Ca^{2+} concentrations ranging from about 1 to 100 μM. In choline-Cl medium, but not in the other media, substantial levels of binding were observed at [Ca^{2+}] <10^{-8} M. This result agrees with the $^{45}Ca^{2+}$ flux measurements which also suggest that the Ca^{2+} release channel is partially activated in choline-Cl media containing a low [Ca^{2+}]. Replacement of Cl^- by Mes$^-$ in choline$^+$ and K^+ media resulted in reduced levels of [^{3}H]ryanodine binding. Reduced levels of binding were also observed when Cl^- was replaced by Pipes$^-$ in choline$^+$ and K^+ media (not shown). Low levels of [^{3}H]ryanodine binding (<0.05 pmol/mg protein) were measured in 0.5 M sucrose medium, whereas in the buffer solution (−salt) only close to background levels of binding could be detected. These results suggest that sucrose can activate the RyR to a limited extent. Comparison of [^{3}H]ryanodine binding data of Fig. 2*A* with $^{45}Ca^{2+}$ efflux measurements (Table I) shows a qualitatively similar dependence on [Ca^{2+}], thus supporting the idea that under the above ionic conditions [^{3}H]ryanodine binding correlated well with channel activity.

Fig. 2*B* illustrates the Ca^{2+} activation/inactivation profiles of [^{3}H]ryanodine binding in 0.25 M Cl^- media with Li^+, Na^+, K^+, and Cs^+ as the cation. The binding levels were lowest in LiCl medium, intermediate in the KCl and NaCl media, and highest in CsCl medium.

Data of Fig. 2, *A* and *B*, suggest that Ca^{2+} activates and inhibits [^{3}H]ryanodine binding by binding to high-affinity Ca^{2+} activation and low-affinity Ca^{2+} inactivation sites. Furthermore, the data suggest that the Ca^{2+} binding affinities are dependent on the ionic composition of the binding media. We were able to describe the Ca^{2+} dependence of [^{3}H]ryanodine binding by the scheme and Equations 1 and 2 given under "Experimental Procedures." Equation 1 provided a good fit (lines) to [^{3}H]ryanodine binding data determined in the presence of an inorganic monovalent cation (Na^+, K^+, Cs^+, Fig. 2, *A* and *B*). Binding levels in K-Mes (Fig. 2*A*), LiCl (Fig. 2*B*), and KPipes (not shown) media were too low to yield meaningful fits. Data in choline$^+$ media could be best fitted assuming that choline$^+$ was a weak, noncooperative Ca^{2+} agonist of the Ca^{2+} release channel (Equation 2). A [choline$^+$] of 0.25 M was equivalent to a [Ca^{2+}] of 0.014 ± 0.007 μM (n = 11) in stimulating [^{3}H]ryanodine binding. Table II shows the averaged Hill constants and coefficients of several experiments. The data suggest that changes in the apparent affinity as well as cooperativity of the Ca^{2+}-activating and Ca^{2+}-inactivating sites contribute to the different levels of [^{3}H]ryanodine binding observed in Fig. 2, *A* and *B*. The significance of the changes evidenced in Table II will be discussed specifically for each intervention.

Scatchard analysis indicated the presence of a single high-affinity [^{3}H]ryanodine-binding site (not shown). Changes in binding affinity (K_D) without major changes in B_{max} value were observed in KCl, K-Mes, and choline-Mes media (all at 20 μM Ca^{2+}) and choline-Cl medium (at <0.01 and 20 μM Ca^{2+}) (Table III). These results suggest that the different binding values of Fig. 2, *A* and *B*, reflect changes in binding affinity rather than the number of binding sites.

Effects of Increasing Concentrations of KCl and Choline-Cl—The effects of ionic strength on Ca^{2+} release channel activity were assessed in single channel (Fig. 3) and [^{3}H]ryanodine binding measurements (Fig. 4). In Fig. 3, purified skeletal muscle Ca^{2+} release channels were incorporated into planar lipid bilayers and recorded at [KCl] ranging from 0.15 to 1.0 M. The free Ca^{2+} in the cis bilayer chamber (SR cytoplasmic side)

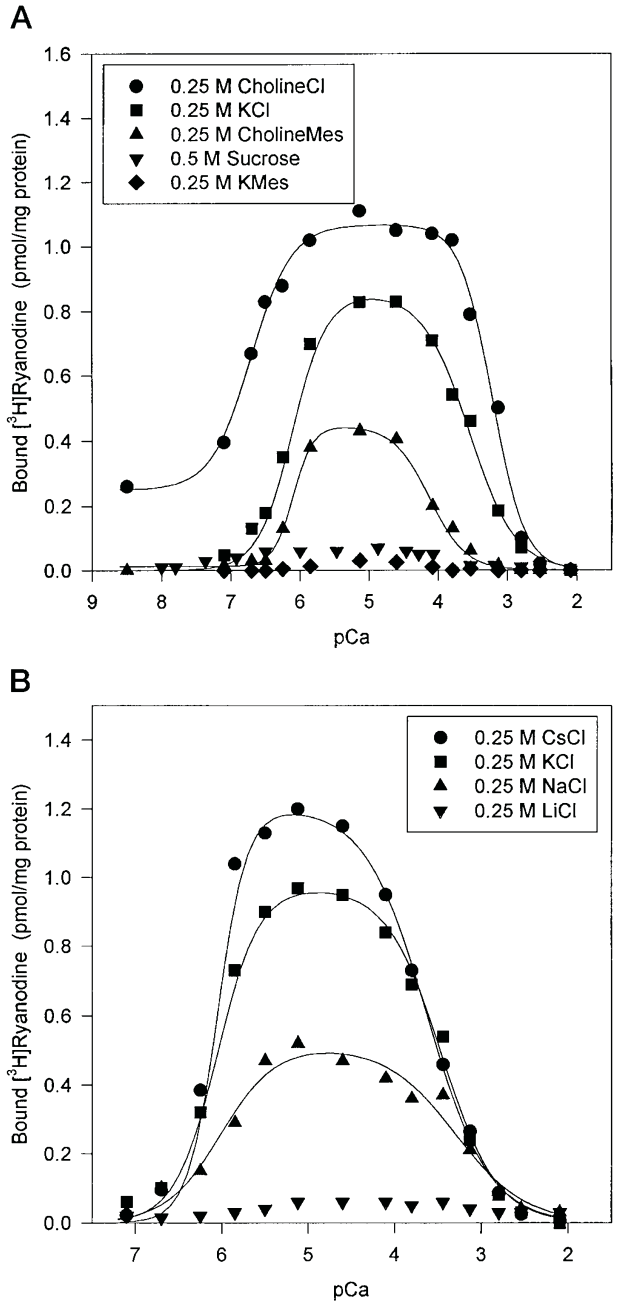

FIG. 2. **Ca^{2+} dependence of [^{3}H]ryanodine binding in media of different ionic composition.** Specific [^{3}H]ryanodine binding was determined as described under "Experimental Procedures" in media containing the indicated salts and concentrations of free Ca^{2+}. Continuous lines for data in Na^+, K^+, and Cs^+ media (*A* and *B*) were obtained by fitting data with Equation 1 under "Experimental Procedures." Data in K-Mes and sucrose (*A*) and LiCl (*B*) medium could not be well fitted by the above equation. Continuous lines for data in choline$^+$ media (*A*) were obtained assuming that choline$^+$ is a weak Ca^{2+} agonist of the Ca^{2+} release channel and fitting data with Equation 2 under "Experimental Procedures." Derived Hill constants and coefficients are summarized in Table II.

was maintained at 20 μM, as at this level of free Ca^{2+} close to maximum ryanodine binding was observed (Fig. 2). Inspection of the four current traces of Fig. 3*A* and the plot of mean P_o as a function of [KCl] (Fig. 3*B*) shows that an increase in [KCl] from 0.15 to 0.5 M resulted in a major increase in channel activity. No further significant increase in channel open probability (P_o) was seen when [KCl] was raised from 0.5 to 1.0 M. Similar increases in channel activity with regard to [KCl] were obtained when channels were recorded at +40 or −40 mV holding potential (Fig. 3*B*). These results suggest that skeletal muscle Ca^{2+} release channel activity is highly sensitive to the ionic strength of the recording solutions.

Fig. 4*A* shows that comparable increases in the [^{3}H]ryanodine binding levels were obtained when the [KCl] in the binding media was raised from 0.1 to 0.25 M, 0.5 M, and 1.0 M. At all four [KCl], bimodal Ca^{2+} activation/inactivation curves were obtained, with [^{3}H]ryanodine binding being maximal in the micromolar Ca^{2+} concentration range. Equation 1 under "Experimental Procedures" provided a good fit to the observed concentration dependence. Inspection of the derived Hill constants and coefficients (Table II) shows that an increase in [KCl] significantly decreased the apparent affinity and increased the cooperativity of Ca^{2+} binding to the inactivation site(s). The Hill constant (K_i^{Ca}) for Ca^{2+} increased from 50 to 5600 μM as the [KCl] was increased from 0.1 to 1.0 M. A linear correlation coefficient of 0.95 (n = 21) also indicates that an increase in [KCl] from 0.1 to 1.0 M resulted in a highly significant increase of K_i^{ca}. The effect of [KCl] on the Ca^{2+} activation site(s) was more complex. [^{3}H]Ryanodine binding was activated by Ca^{2+} with Hill constants (K_a^{Ca}) increasing from 0.43 μM at 0.1 M KCl to 0.92 μM at 0.25 M KCl, and decreasing then to 0.81 μM at 0.5 M KCl, and 0.42 μM at 1 M KCl. Hill activation coefficients (n_a) of 1.4–1.7 at elevated [KCl] suggest that Ca^{2+} activated [^{3}H]ryanodine binding by cooperative interactions involving at least two Ca^{2+}-binding sites.

An increase in [KCl] raises the concentration of an ion pair where the anions appear to increase the affinity of the Ca^{2+} activation site for Ca^{2+}, and the cations reduce [^{3}H]ryanodine binding by competing with Ca^{2+} for the Ca^{2+} activation sites (see below). The effects of ionic strength on [^{3}H]ryanodine binding were therefore also assessed in choline-Cl media to avoid the presence of an inhibitory cation. As observed for the KCl media, the binding levels at the different [Ca^{2+}] increased as the [choline-Cl] was raised (Fig. 4*B*). At micromolar [Ca^{2+}], 0.5 and 1.0 M choline-Cl were similarly effective in causing [^{3}H]ryanodine binding. As observed for the KCl media, an increase in [choline-Cl] from 0.1 to 1.0 M caused a large decrease in the apparent Ca^{2+} affinity of the Ca^{2+} inactivation sites (Table II). A linear correlation coefficient of 0.91 (n = 24) indicates that the increase in K_i was highly significant. Two important differences were, however, that substantial levels of [^{3}H]ryanodine binding were measured at [Ca^{2+}] $<10^{-8}$ M in the choline-Cl but not KCl media, and second that the apparent Ca^{2+} affinity of the Ca^{2+} activation sites monotonously increased as the [choline-Cl] was raised from 0.1 to 1.0 M. A linear correlation coefficient of 0.78 (n = 24) indicates that the increase in affinity was highly significant.

Effects of AMP and Caffeine on Ca^{2+}-dependence of [^{3}H]Ryanodine Binding—Ca^{2+}-gated Ca^{2+} release channel activity is affected by various endogenous and exogenous effectors such as adenine nucleotides and caffeine (4–6). In this study, we used AMP rather than ATP or a nonhydrolyzable ATP analog because AMP, in contrast to adenine triphosphates, binds Ca^{2+} with a negligible affinity. Fig. 5 shows that the addition of AMP to 0.25 M KCl medium resulted in an increase in [^{3}H]ryanodine binding. This increase could be accounted for by a small (not significant) increase in the apparent affinity of the receptor activation sites and 3–4-fold (significant) decrease in the apparent affinity of the inactivation sites for Ca^{2+} (Fig. 5, Table II). Caffeine (20 mM) shifted the Ca^{2+} activation curve to the left, by increasing the apparent affinity of the Ca^{2+} activation and Ca^{2+}-inactivation sites by a factor of 15 and 1.7, respectively (Fig. 5, Table II). An additional effect of caffeine was to decrease the cooperativity of Ca^{2+} activation and inactivation.

Interaction of Mg^{2+} and Monovalent Cations with High-af-

TABLE II

Ca^{2+}-dependence of [^{3}H]ryanodine binding in media of different ionic strength and composition

Ca^{2+}-dependence of [^{3}H]ryanodine binding was determined as described in the legends to Figs. 2, 4, and 5. Derived Hill constants and coefficients were obtained as indicated in the legends of the figures. Values are the mean ± S.D. of indicated number of experiments.

Composition of assay media	Derived Hill constants and coefficients				Number of experiments
	K_a^{Ca}	n_a	K_i^{Ca}	n_i	
	μM		μM		
0.1 M KCl	0.43 ± 0.25	1.6 ± 0.3	49 ± 27	1.0 ± 0.1	3
0.25 M KCl	0.92 ± 0.21[a]	1.7 ± 0.3	255 ± 107[a]	1.3 ± 0.2	12
0.5 M KCl	0.81 ± 0.23	1.6 ± 0.2	1226 ± 296[a,b]	1.8 ± 0.3[a,b]	3
1.0 M KCl	0.42 ± 0.03[b]	1.4 ± 0.1	5635 ± 1174[a,b,c]	1.5 ± 0.2[a]	3
0.1 M Choline-Cl	0.22 ± 0.10	1.8 ± 0.2	220 ± 129	1.4 ± 0.2	4
0.25 M Choline-Cl	0.16 ± 0.05	1.5 ± 0.3	695 ± 109[d]	2.1 ± 0.3[d]	11
0.5 M Choline-Cl	0.09 ± 0.07	1.3 ± 0.6	1915 ± 271[d,e]	2.0 ± 0.2[d]	4
1.0 M Choline-Cl	0.02 ± 0.02[d,e]	0.9 ± 0.5[d]	6108 ± 2327[d,e,f]	1.9 ± 0.3[d]	5
0.25 M NaCl	1.30 ± 0.46	1.4 ± 0.1	523 ± 144[b]	1.3 ± 0.2	3
0.25 M KCl	0.92 ± 0.23	1.7 ± 0.2	255 ± 107	1.3 ± 0.2	12
0.25 M CsCl	0.73 ± 0.02	2.2 ± 0.1[b]	290 ± 86	1.4 ± 0.3	3
0.25 M Choline-Cl	0.16 ± 0.05	1.5 ± 0.3	695 ± 109	2.1 ± 0.3	11
0.25 M Choline-Mes	0.94 ± 0.37[e]	2.6 ± 0.4[e]	60 ± 15[e]	1.5 ± 0.2[e]	3
0.25 M Choline-Pipes	0.60 ± 0.20[e]	2.0 ± 0.3	118 ± 41[e]	1.1 ± 0.2[e]	3
0.25 M KCl	0.92 ± 0.23	1.7 ± 0.2	255 ± 107	1.3 ± 0.2	12
0.25 + 5 mM AMP	0.77 ± 0.04	2.0 ± 0.4	854 ± 113[b]	1.4 ± 0.4	4
0.25 + 20 mM Caffeine	0.06 ± 0.05[b]	0.9 ± 0.3[b]	154 ± 33	1.0 ± 0.1	4

[a] Significantly different ($p < 0.05$) from 0.1 M KCl.
[b] Significantly different ($p < 0.05$) from 0.25 M KCl.
[c] Significantly different ($p < 0.05$) from 0.5 M KCl.
[d] Significantly different ($p < 0.05$) from 0.1 M choline-Cl.
[e] Significantly different ($p < 0.05$) from 0.25 M choline-Cl.
[f] Significantly different ($p < 0.05$) from 0.5 M choline-Cl.

TABLE III

Effects of ionic composition on B_{max} and K_D values of [^{3}H]ryanodine binding

Skeletal muscle SR vesicles were incubated with [^{3}H]ryanodine (0.5 to 300 nM) at 12 °C for 96 h in the presence of the indicated salts and free [Ca^{2+}]. B_{max} and K_D values were determined by Scatchard analysis. Data are the averages of two experiments.

Assay medium	[^{3}H]Ryanodine binding	
	B_{max}	K_D
	pmol/mg protein	*nM*
0.25 M KCl, 20 μM Ca^{2+}	12.4	3.4
0.25 M K-Mes, 20 μM Ca^{2+}	10.3	82
0.25 M Choline-Mes, 20 μM Ca^{2+}	12.4	11.6
0.25 M Choline-Cl, 20 μM Ca^{2+}	10.8	1.8
0.25 M Choline-Cl, <0.01 μM Ca^{2+}	10.3	22.0

finity Ca^{2+} Activation Sites—We considered the possibility that monovalent cations inhibit [^{3}H]ryanodine binding by competing with Ca^{2+} for the high-affinity Ca^{2+} activation sites. Initially, we tested the effects of Mg^{2+}, which is known to inhibit Ca^{2+} release channel activity by interacting with the Ca^{2+} activation sites (5). In these studies, we took advantage of the observation that significant levels of [^{3}H]ryanodine binding were observed in choline-Cl media containing submicromolar [Ca^{2+}] (Fig. 4*B*). It could be argued that the effects of the monovalent cations are best studied in the absence of another monovalent cation. However, at submicromolar [Ca^{2+}] the presence of an activating anion (≥0.25 M Cl^-) is required to observe satisfactory levels of [^{3}H]ryanodine binding (Fig. 2).

The inhibitory effects of Mg^{2+} (Fig. 6) and K^+ (Fig. 7) were assessed in 0.5 M choline-Cl media at different free Ca^{2+} concentrations that were expected to partially activate [^{3}H]ryanodine binding but to have only negligible inhibitory effects. To interpret the [^{3}H]ryanodine binding data, three simple alternative types of inhibition were considered, namely that Mg^{2+} and K^+ were competitive, noncompetitive, and uncompetitive inhibitors. The binding data could not be fitted assuming non- or uncompetitive inhibition (not shown) but could be well fitted when it was assumed that Mg^{2+} (Fig. 6) and K^+ (Fig. 7) inhibited [^{3}H]ryanodine binding by a competitive mechanism (formalized by Equations 3 and 4 under "Experimental Procedures"), according to which the two cations bind to the Ca^{2+} activation site, but fail to activate the channel. Table IV summarizes the derived Ca^{2+} activation and inhibition constants and coefficients for Mg^{2+} and four monovalent cations (Li^+, Na^+, K^+, and Cs^+). Similar Ca^{2+} activation constants and Hill coefficients were obtained in all five media. Among the cations tested, Mg^{2+} was most effective in inhibiting [^{3}H]ryanodine binding (K_i = 0.013 mM). For the monovalent cations, the order of effectiveness was $Li^+ > Na^+ > K^+ > Cs^+$. Hill coefficients of ~1.6 suggested that the monovalent cations inhibited the channel by a cooperative interaction involving at least two cations. The higher affinity of Na^+ for the Ca^{2+} activating site(s) provided at least a partial explanation for the observation that higher [Ca^{2+}] were required to half-maximally activate [^{3}H]ryanodine binding in NaCl than in KCl or CsCl media (Table II). The inhibitory effects of Mg^{2+} and the monovalent cations were also tested in 0.5 M choline-Cl media containing 5 mM AMP. Table IV shows that the addition of AMP resulted in a 1.1–2.2-fold increase in the affinity of the Ca^{2+} activation sites for Ca^{2+}. No major changes in the inhibition constants and coefficients were observed.

Interaction of Divalent Cations with Low-affinity Inhibitory Sites—The decline of SR Ca^{2+} release activity and [^{3}H]ryanodine binding at elevated [Ca^{2+}] indicates that the Ca^{2+} release channel possesses low affinity inactivation sites (Figs. 1, 2, and 4). The divalent cation specificity of these sites was tested in media that contained 0.1 M KCl, 0.5 M KCl, or 0.5 M choline-Cl, 5 mM AMP, a close to maximally activating [Ca^{2+}] (Fig. 4; 20 μM at 0.1 M and 50 μM at 0.5 M), and different concentrations of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}. Essentially identical inhibition patterns were obtained for the four divalent cations (Fig. 8). At 0.1 M KCl, the divalent cations inhibited [^{3}H]ryanodine binding

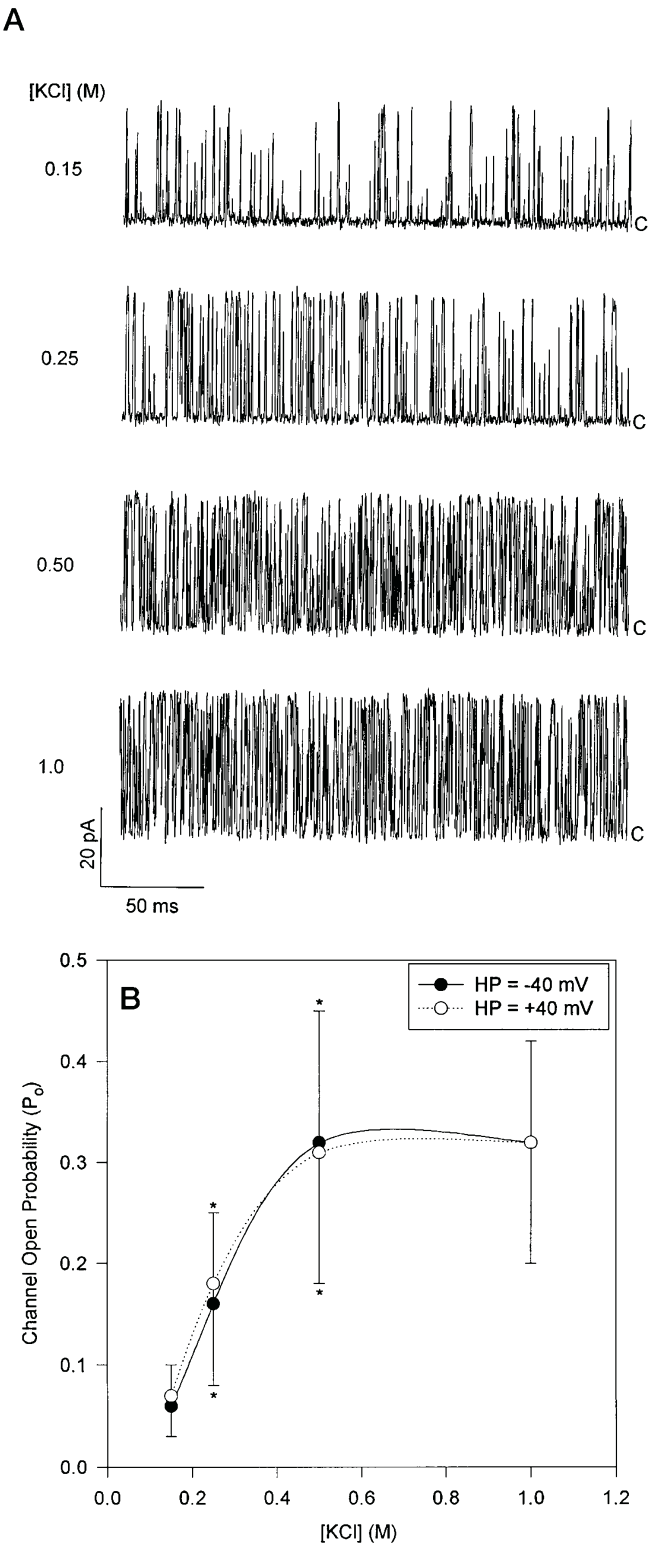

FIG. 3. **Effect of [KCl] on RyR channel activity.** *A*, recordings of a single Ca^{2+} release channel at increasing [KCl] at a holding potential of +40 mV. All recordings are from a single experiment. Single-channel currents, shown as upward deflections from the closed levels (marked *C*), were recorded in symmetrical media containing 10 mM K-Hepes, pH 7.3, 100 μM EGTA, 120 μM $CaCl_2$ (20 μM free Ca^{2+}) and the indicated concentrations of KCl. Top recording: P_o = 0.05. Second recording: P_o = 0.10. Third recording: P_o = 0.30. Bottom recording: P_o = 0.45. *B*, mean P_o from channels recorded as in *A* (at both +40 and −40 mV holding potentials) as a function of [KCl]. Data points are mean ± S.D. of eight experiments. *, values at 0.15 M KCl significantly different from those at 0.25 M KCl, and at 0.25 M KCl from those at 0.5 M KCl, as determined by Student's paired *t* test.

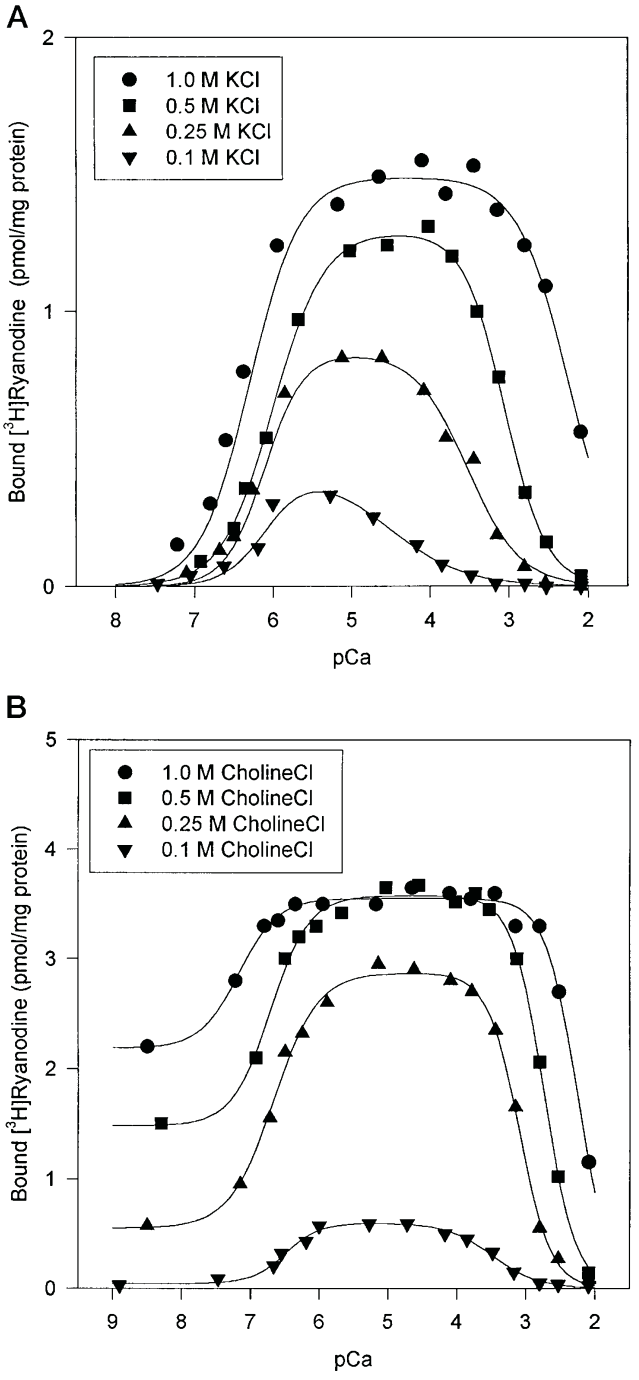

FIG. 4. **Ca^{2+} dependence of [^{3}H]ryanodine binding at different [KCl] and [choline-Cl].** Specific [^{3}H]ryanodine binding was determined as described under "Experimental Procedures" in media containing the indicated concentrations of KCl (*A*) and choline-Cl (*B*), and free Ca^{2+}. Continuous lines in *A* and *B* were obtained by fitting experimental data according to Equations 1 and 2 under "Experimental Procedures," respectively. According to Equation 2, the calculated effective [choline-Cl] were 0.01, 0.02, 0.03, and 0.01 μM in 0.1, 0.25, 0.5, and 1.0 M choline-Cl media, respectively. Derived Hill constants and coefficients are summarized in Table II.

with a $K_i \sim 0.1$ mM and $n_i \sim 1.2$. In 0.5 M KCl media, higher divalent cation concentrations were required to inhibit [^{3}H]ryanodine binding ($K_i \sim 1.35$ mM, $n_i \sim 1.1$). In 0.5 M choline-Cl media, the K_i and n_i values increased to ~3.3 mM and 2.2, respectively. Hill coefficients of greater than 2 indicated that the divalent cations inhibited [^{3}H]ryanodine binding by a cooperative interaction in choline-Cl medium. Taken together, the results of Fig. 8 suggest that the low-affinity inhibitory ryanodine

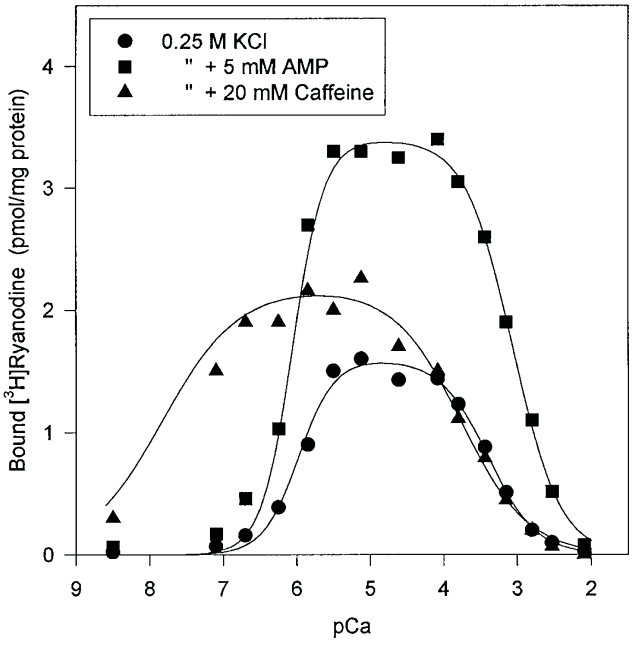

FIG. 5. **Effects of AMP and caffeine on Ca^{2+} dependence of [^{3}H]ryanodine binding.** Specific [^{3}H]ryanodine binding was determined as described under "Experimental Procedures" in 0.25 M KCl media containing the indicated concentrations of free Ca^{2+}, AMP, and caffeine. Continuous lines were obtained by fitting data with Equation 1 under "Experimental Procedures." Derived Hill constants and coefficients are summarized in Table II.

receptor sites have a broad divalent cation specificity. However, in agreement with the data of Figs. 2 and 4, the efficacy of divalent cations in inhibiting [^{3}H]ryanodine binding was dependent on the ionic strength and composition of the assay media.

Evidence for Anion Regulatory Site(s)—Data of Figs. 1 and 2*A* suggest that the activity of the SR Ca^{2+} release channel is not only affected by the cationic but also by the anionic composition of the assay media. In both figures lower levels of channel activity were observed when Cl^- was replaced by a buffer such as Mes^- or $Pipes^-$. The effects of the two buffers on [^{3}H]ryanodine binding were further investigated in media that either contained a different [Cl^-] (0.1–1 M) but a constant [Ca^{2+}] (Fig. 9) or a constant [Cl^-] (0.5 M) but different [Ca^{2+}] (Fig. 10). In media containing 20 μM Ca^{2+} (Fig. 9), we used $Pipes^-$ rather than Mes^- as the buffer anion because [Ca^{2+}] binds Mes^- with $K_D \sim 0.2$ M. In the experiments using low concentrations of Ca^{2+} (Fig. 10), we preferred to use Mes^- because it is more fully present in its anionic form at pH 7.2. In Fig. 9, the effects of $Pipes^-$ on [^{3}H]ryanodine binding were examined in the presence of K^+ because in the presence of K-Pipes but not choline-Pipes a nearly complete inhibition of [^{3}H]ryanodine binding could be observed at micromolar [Ca^{2+}] (not shown). The binding data could be reasonably well fitted assuming that $Pipes^-$ inhibited [^{3}H]ryanodine binding by competing with Cl^- for an anion regulatory site. The derived Hill activation (for Cl^-) and inhibition (for $Pipes^-$) constants and coefficients are shown in Table IV. Qualitatively similar data were obtained with Mes^- as the competing buffer (not shown).

In Fig. 10, the effects of the buffers on [^{3}H]ryanodine binding were determined in 0.5 M choline-Cl media that contained different [Ca^{2+}] but a constant [Cl^-]. We found that Mes^- inhibited [^{3}H]ryanodine binding at submicromolar concentrations of free [Ca^{2+}]. No appreciable inhibition was observed at [Mes^-] $\leq$60 mM when the [Ca^{2+}] was raised to 134 μM. The inhibition pattern observed by Mes^- in the 0.5 M choline-Cl media was reminiscent of that by Mg^{2+} and the monovalent cations. In Fig. 10, *A* and *B*, the continuous lines were obtained

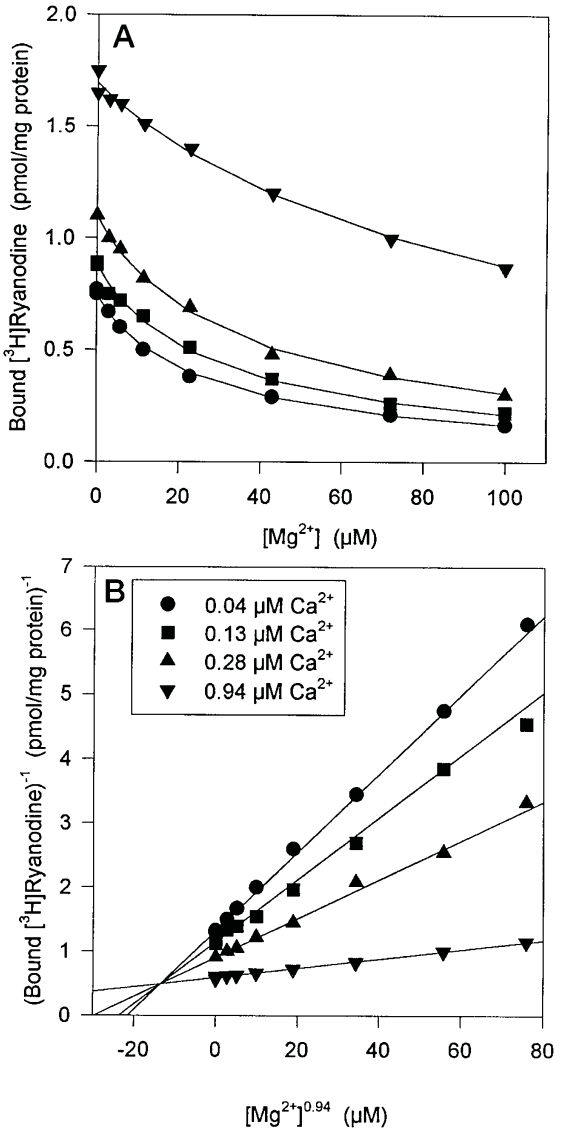

FIG. 6. **Inhibition of [^{3}H]ryanodine binding by Mg^{2+}.** Specific [^{3}H]ryanodine binding was determined in 0.5 M choline-Cl media containing the indicated concentrations of Mg^{2+} and free Ca^{2+}. In *A* and *B*, the continuous lines were obtained with Equations 3 and 4 under "Experimental Procedures," using a single set of parameters for all data. In *B*, data were plotted using derived Hill inactivation coefficient of 0.94. Averaged Hill constants and coefficients of four separate experiments are shown in Table IV.

assuming competitive inhibition, with Mes^- inhibiting Ca^{2+} binding to the Ca^{2+} activation site(s). A good fit was obtained at the elevated [Ca^{2+}], whereas the data at the lower [Ca^{2+}] deviated by a factor of up to 1.3 from the calculated values. Table IV summarizes the derived Hill constants and coefficients. No reasonable fits were obtained when it was assumed that Mes^- was a noncompetitive or uncompetitive inhibitor. In the presence of 5 mM AMP, a 1.8-fold decrease in the activation constant was obtained without a change in the Ca^{2+} inactivation constant (Table IV).

DISCUSSION

The goal of the present study was to characterize the action of monovalent cations and anions on the RyR/Ca^{2+} release channel of rabbit skeletal muscle. Among the various endogenous effectors of the RyR, Ca^{2+} is widely accepted to play a pivotal role. This study shows that inorganic monovalent cations affect RyR activity by competitive binding to the receptor's Ca^{2+} activation sites. Second, our results indicate that anion-

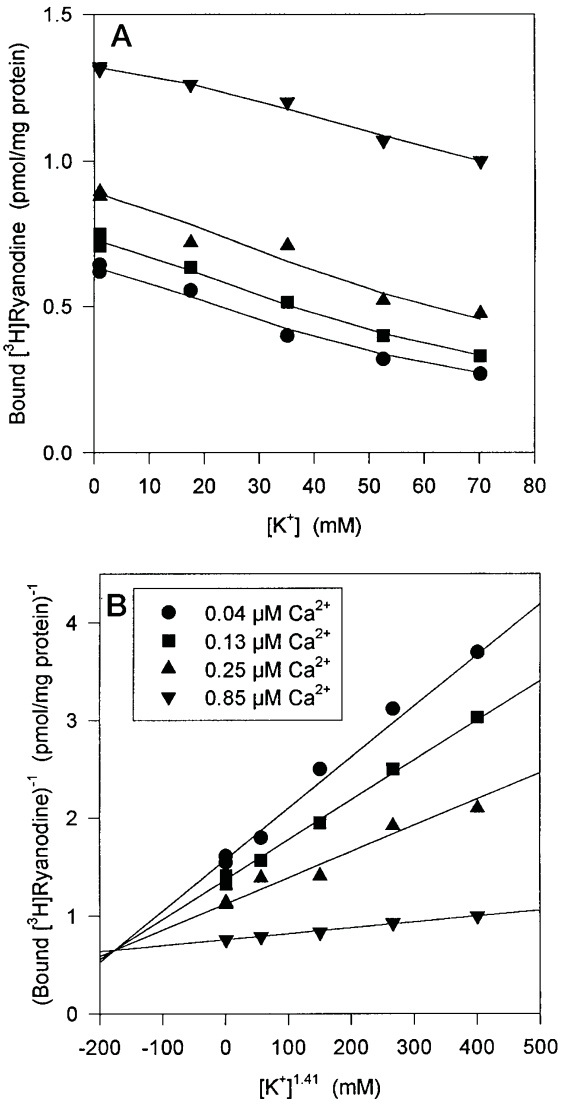

FIG. 7. **Inhibition of [^{3}H]ryanodine binding by K^+.** Specific [^{3}H]ryanodine binding was determined in 0.5 M choline-Cl media containing the indicated concentrations of K^+ and free Ca^{2+}. Total [Cl^-] was kept constant by adjusting [choline$^+$] so that [choline$^+$] + [K^+] = 0.5 M. In *A* and *B*, the continuous lines were obtained with Equations 3 and 4 under "Experimental Procedures," using a single set of parameters for all data. In *B*, data were plotted using derived Hill inactivation coefficients of 1.41. Averaged Hill constants and coefficients of five separate experiments are shown in Table IV.

specific binding sites play an important role in regulating RyR activity by modifying the apparent Ca^{2+} affinity of the receptor's Ca^{2+} regulatory sites.

The effects of ionic composition and ionic strength on skeletal muscle Ca^{2+} release channel activity were monitored with [^{3}H]ryanodine binding, SR vesicle-$^{45}Ca^{2+}$ flux, and single channel measurements. Although multiple ryanodine-binding sites and a complex interaction of ryanodine with these sites have been reported (17, 23–25), the binding kinetics are relatively straightforward when low ryanodine concentrations are used, concentrations that limit binding to a single high-affinity receptor site. Ryanodine is generally thought to preferentially bind to the open channel and, as observed in the present study, binding is thought to be affected by Ca^{2+} and other effectors similarly as SR Ca^{2+} release or single channel activities. However, it is unlikely that ryanodine binding and channel activity are regulated in exactly the same way, because of the different time scales on which the channel gates (μs to ms) and binds [^{3}H]ryanodine (minute to hour). Although [^{3}H]ryanodine binding provides less direct information on channel activity than single channel measurements, we chose to rely mostly on [^{3}H]ryanodine binding measurements because they allowed us to examine various ionic conditions.

The regulation of the skeletal muscle Ca^{2+} release channel was examined in the presence of nM to mM [Ca^{2+}] in media containing different mono- and divalent cations and anions. Assuming that the binding of Ca^{2+} to high affinity sites (K_a < 1 μM) activates the channel, while binding of Ca^{2+} to separate low affinity sites (K_i > 50 μM) inactivates it (10, 11, 13, 15, 18, 21, 22, this study), the results of our experiments can be described by expanding the scheme (Scheme 1) shown under "Experimental Procedures" as follows,

$$\mathrm{R} \underset{<1\ \mu\mathrm{M}\ Ca^{2+}}{\overset{(Mg^{2+},K^+)}{\rightleftharpoons}} \mathrm{A_{Ca}} \underset{}{\overset{\geq 50\ \mu\mathrm{M}\ X^{2+}}{\rightleftharpoons}} {}_{X^{2+}}\mathrm{I_{Ca}}$$

$$Y^- \downarrow\uparrow \mathrm{Cl}^-$$

$$\mathrm{R^*} \underset{<<1\ \mu\mathrm{M}\ Ca^{2+}}{\overset{(Mg^{2+},K^+)}{\rightleftharpoons}} \mathrm{A^*_{Ca}} \underset{}{\overset{>>50\ \mu\mathrm{M}\ X^{2+}}{\rightleftharpoons}} {}_{X^{2+}}\mathrm{I^*_{Ca}}$$

SCHEME 2

In the above scheme (Scheme 2), it is assumed that the RyR/Ca^{2+} release channel may be present in states of different Ca^{2+} binding affinities. At a low [Cl^-] or in the presence of a competing inhibitory anion (Y^-) Ca^{2+} binds at <1 μM free Ca^{2+} to the Ca^{2+} activation sites of a Ca^{2+}-free RyR (R) to yield a Ca^{2+}-activated receptor (A_{Ca}), and binds at >50 μM free Ca^{2+} to A_{Ca} to yield a Ca^{2+}-inactivated receptor ($_{X^{2+}}I^*_{Ca}$). An increase in [Cl^-] results in receptor forms (R^*, A^*_{Ca}, $_{X^{2+}}I^*_{Ca}$) that are characterized by an increased Ca^{2+} affinity of the Ca^{2+} activation sites and decreased Ca^{2+} affinity of the Ca^{2+} inactivation sites. Mg^{2+} and monovalent cations (in parentheses) are competitive inhibitors that inhibit the formation of the A_{Ca} and A^*_{Ca} receptor states by competing with Ca^{2+} for the Ca^{2+} activation sites. In the above scheme, in addition to Ca^{2+}, Mg^{2+} and other divalent cations (X^{2+}) inhibit the receptor by binding to the Ca^{2+} inactivation sites. The scheme further proposes that buffer anions (Mes^- and $Pipes^-$) deter the formation of the activated R^*, A^*_{Ca}, and $_{X^{2+}}I^*_{Ca}$ receptor forms by competing with Cl^- for anion regulatory site(s). As shown in this study, the Ca^{2+} release channel contains cooperatively interacting Ca^{2+} activation sites and Ca^{2+} inactivation sites. The above scheme has been simplified by showing only one Ca^{2+} activation and one Ca^{2+} inactivation site each.

The effects of monovalent cations on the Ca^{2+} dependence of [^{3}H]ryanodine binding were analyzed using the chloride salts of Li^+, Na^+, K^+, Cs^+, and choline$^+$. Ca^{2+} activated [^{3}H]ryanodine binding by a cooperative interaction with the highest apparent affinity in choline-Cl medium followed by CsCl, KCl, NaCl, and LiCl medium. The studies showed that choline$^+$ behaves like a weak Ca^{2+} agonist of the channel, and inorganic monovalent cations lower the apparent Ca^{2+} affinity by competitive binding to the Ca^{2+} activation sites. Hill coefficients greater than 1 (Tables II and IV) suggest that Ca^{2+} activates and inorganic monovalent ions inhibit the skeletal muscle Ca^{2+} release channel by cooperative interactions. Recently, the effects of [KCl] on the Ca^{2+} activation profile were also examined by determining the permeation of choline$^+$ in light scattering measurements with SR vesicles present in choline-Cl media (11). At variance with the present study, an increase in [KCl] from 0 to 1 M shifted the Ca^{2+} activation profile to higher Ca^{2+} concentrations. The decreases in the apparent Ca^{2+} affinities for both the Ca^{2+} activation and Ca^{2+} inactivation sites were

TABLE IV
Inhibition of [^{3}H]ryanodine binding by cations and anions

Derived Hill constants and coefficients were obtained as described in the legends to Figs. 6, 7, 9, and 10. Values are the mean ± S.D. of number of experiments shown.

Activating ion/ inactivating ion	Derived Hill constants and coefficients				Number of experiments
	K_a	n_a	K_i	n_i	
	μM		mM		
−AMP					
Ca^{2+}/Mg^{2+}	0.39 ± 0.17	1.9 ± 0.4	0.013 ± 0.004	1.1 ± 0.1	4
Ca^{2+}/Li^{+}	0.38 ± 0.09	2.7 ± 1.0	14 ± 3	1.6 ± 0.4	4
Ca^{2+}/Na^{+}	0.44 ± 0.15	3.4 ± 1.9	27 ± 7[a]	1.8 ± 0.2	4
Ca^{2+}/K^{+}	0.43 ± 0.10	2.5 ± 1.1	42 ± 14[a]	1.6 ± 0.4	5
Ca^{2+}/Cs^{+}	0.31 ± 0.05	3.3 ± 0.3	56 ± 37	1.4 ± 0.2	5
Cl^{-}/$Pipes^{-}$	251,000 ± 25,000	1.5 ± 0.1	45 ± 13	1.5 ± 0.3	4
Ca^{2+}/Mes^{-}	0.29 ± 0.15	1.8 ± 0.7	61 ± 47	1.0 ± 0.1	4
+ AMP					
Ca^{2+}/Mg^{2+}	0.18 ± 0.04	2.0 ± 0.2	0.018 ± 0.009	1.2 ± 0.3	4
Ca^{2+}/Li^{+}	0.34 ± 0.19	1.8 ± 0.4	9 ± 2	1.7 ± 0.1	3
Ca^{2+}/Na^{+}	0.23 ± 0.09	2.8 ± 1.3	24 ± 6[b]	1.7 ± 0.2	3
Ca^{2+}/K^{+}	0.27 ± 0.07	2.6 ± 0.8	46 ± 11[b,c]	1.8 ± 0.7	3
Ca^{2+}/Mes^{-}	0.16 ± 0.08	2.0 ± 0.6	62 ± 38	1.0 ± 0.3	5

[a] Significantly different from Li^{+} (−AMP).
[b] Significantly different from Li^{+} (+AMP).
[c] Significantly different from Na^{+} (+AMP).

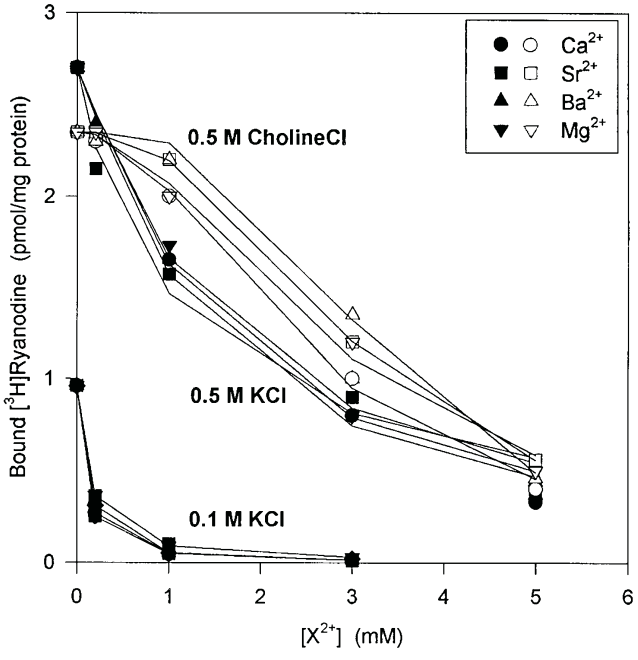

FIG. 8. **Inhibition of [^{3}H]ryanodine binding by millimolar concentrations of divalent cations.** Specific [^{3}H]ryanodine binding was determined in 0.1 M KCl, 0.5 M KCl, or 0.5 M choline-Cl media containing 2 nM [^{3}H]ryanodine, 5 mM AMP, 20 μM Ca^{2+} (0.1 M KCl), or 50 μM Ca^{2+} (0.5 M KCl and 0.5 M choline-Cl) plus the indicated concentrations of divalent cations (X^{2+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, or Ba^{2+}). Curves were obtained according to the equation: $B = B_o/(1+([X^{2+}]K_i^n)$ where B_o is the binding value in the absence of the indicated concentrations of the divalent cations and the other terms have their usual meaning.

explained by assuming competition between K^{+} and Ca^{2+} at the Ca^{2+} binding sites of the channel. The effects of [Cl^{-}] on channel activity were not considered by Kasai *et al.* (11). The present study shows that both the actions of the monovalent cations and anions need to be taken into account to understand the way in which the ionic milieu modulates activation and inactivation of the skeletal muscle Ca^{2+} release channel by Ca^{2+}.

In agreement with vesicle flux studies (18, 22), a competitive binding to the high-affinity Ca^{2+} activation sites was also observed for Mg^{2+}. The inhibition constant for Mg^{2+} was lower than those for the monovalent cations by more than 2 orders. For K^{+}, the major monovalent cation in muscle, the inhibition constant determined was 3000-fold higher than for Mg^{2+}. The intracellular free [K^{+}] in skeletal muscle is about 100 times higher than that of Mg^{2+}. Therefore, the channel's Ca^{2+} activation sites are likely occupied to a greater extent by Mg^{2+} than by K^{+} at rest. However, occupation of some sites by K^{+} may be of physiological importance because Ca^{2+} may bind faster to channel sites occupied by K^{+} than sites occupied by more tightly bound Mg^{2+}.

The interaction of di- and monovalent cations with the low-affinity channel inactivation sites was less amenable to analysis because of their concurrent interaction with the Ca^{2+} activation sites. The specificity of the inactivation sites with regard to Ca^{2+} and monovalent cations was determined in 0.25 M Cl^{-} media containing different monovalent cations (Fig. 2, *A* and *B*). Analysis of [^{3}H]ryanodine binding data suggests that Ca^{2+} binds with a higher apparent affinity to the inactivation sites in KCl and CsCl medium than in NaCl or choline-Cl medium. The Hill inactivation coefficients ranged from 1.3 in Na^{+} and K^{+} medium to 2.1 in choline-Cl medium (Table II), which suggests that the monovalent cations also affect the Ca^{2+} binding cooperativity to the Ca^{2+} inactivation sites. We conclude that monovalent cations affect by an as yet unidentified mechanism the interaction of the channel inactivation sites with Ca^{2+}.

The divalent cation specificity of the channel inactivation sites was tested in 0.1 M KCl, 0.5 M KCl, and 0.5 M choline-Cl media in the presence of 5 mM AMP and a relatively high [Ca^{2+}] to minimize interaction of the other divalent cations with the Ca^{2+} activation sites. In the three media, all four divalent cations tested (Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}) displayed a similar ability of inhibiting [^{3}H]ryanodine binding. These results are in agreement with vesicle-Ca^{2+} flux measurements which provided evidence of a similar affinity of the channel inactivation sites for Ca^{2+} and Mg^{2+} (18).

The effects of monovalent anions on channel activity were investigated by determining the Ca^{2+} dependence of [^{3}H]ryanodine binding in media of different anionic composition (Fig. 2*A*) and concentration (Fig. 4, *A* and *B*), and in competition studies at submicromolar [Ca^{2+}] (Fig. 10) or close to fully activating [Ca^{2+}] (Fig. 9). In agreement with previous studies (7–11), an increase in salt concentration (KCl and choline-Cl) greatly increased the levels of [^{3}H]ryanodine binding in the presence of 0.1 μM to 10 mM Ca^{2+}. Analysis of these data (Table

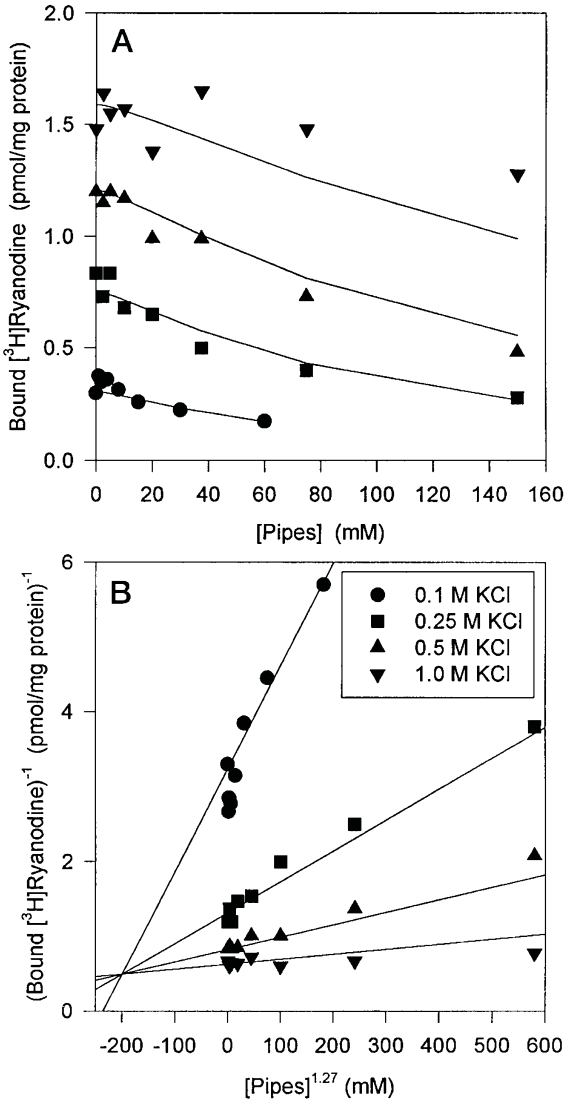

FIG. 9. **Inhibition of [^{3}H]ryanodine binding by $Pipes^-$ in media of different [KCl].** Specific [^{3}H]ryanodine binding was determined as described under "Experimental Procedures" in media containing 20 μM free Ca^{2+}, and the indicated concentrations of $Pipes^-$ (as the K^+ salt) and KCl. In *A* and *B*, the continuous lines were obtained with equations analogous to those of Equations 3 and 4 using a single set of parameters for all data. In the equations, the activating ion was Cl^- and the inhibitor was Pipes. In *B*, data were plotted with a derived Hill inactivation coefficient of 1.27. Averaged Hill constants and coefficients of four separate experiments are shown in Table IV.

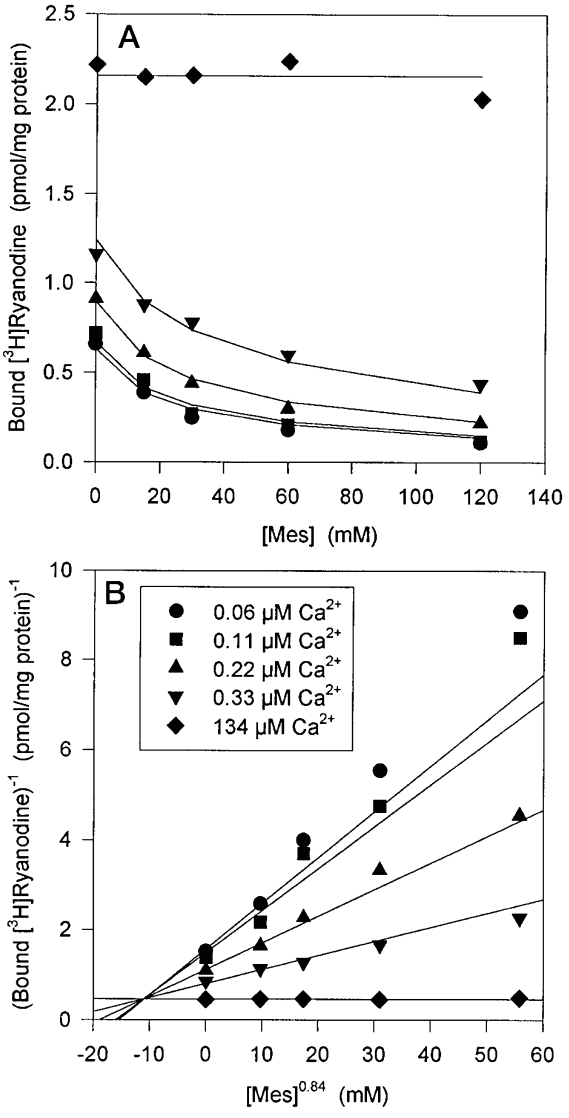

FIG. 10. **Inhibition of [^{3}H]ryanodine binding by Mes^- in media of different [Ca^{2+}].** Specific [^{3}H]ryanodine binding was determined as described under "Experimental Procedures" in 0.5 M choline-Cl media containing the indicated concentrations of Mes^- and free Ca^{2+}. In *A* and *B*, the continuous lines were obtained with Equations 3 and 4, using a single set of parameters for all data. In the equations, the activating ion was Ca^{2+}, and the inhibitor was Mes^-. In *B*, data were plotted using a derived Hill inactivation coefficient of 0.84. Averaged Hill constants and coefficients of four separate experiments are shown in Table IV.

II) suggests that an increase in [Cl^-] was responsible for elevating the [^{3}H]ryanodine binding levels by increasing the apparent Ca^{2+} affinity of the Ca^{2+} activation sites and decreasing the apparent Ca^{2+} binding affinity of the channel inactivation sites. In favor of this suggestion is the observation that replacement of Cl^- with Mes^- in 0.25 M $choline^+$ media lowered [^{3}H]ryanodine binding (Fig. 2*A*). Accordingly, Cl^- appears to widen the "Ca^{2+} window" of receptor activation, that is to allow a more complete occupation of the Ca^{2+} activation sites by Ca^{2+} before substantial Ca^{2+} binding to the Ca^{2+} inactivation sites occurs. A consequence of a widened Ca^{2+} window was that increased affinities (Table III) and levels (Fig. 2*A*) of [^{3}H]ryanodine binding could be observed in Cl^- media. The initial decrease in apparent Ca^{2+} affinity as the [KCl] was raised from 0.1 to 0.25 M can be explained assuming that in this concentration range K^+ competes more strongly with Ca^{2+} for the activation sites than Cl^- increases the Ca^{2+} affinity of the Ca^{2+} activation sites.

A decrease in the vesicle Ca^{2+} efflux rates and [^{3}H]ryanodine binding was seen when Cl^- was replaced by Mes^- or $Pipes^-$. Substitution of Cl^- by Mes^- or $Pipes^-$ in $choline^+$ medium resulted in ~2-fold decrease in the maximum level of [^{3}H]ryanodine binding. [^{3}H]Ryanodine binding decreased close to background levels when these experiments were done in K^+ media. In these cases, a decrease in the apparent Ca^{2+} affinity of the Ca^{2+} activation sites and increase in the apparent Ca^{2+} affinity of the inactivation sites resulted in a narrowing of the Ca^{2+} window of receptor activation. In the above scheme these observations are taken into account by proposing that the binding of Cl^- to anion regulatory sites mediates the transition of the RyR channel to a state of greater susceptibility to activation by Ca^{2+}. The presence of anion regulatory sites was verified by showing a competitive inhibition of the Cl^--activated receptor by $Pipes^-$. In these studies a free [Ca^{2+}] of 20 μM was used to maintain the receptor in its different Ca^{2+}-activated A_{Ca} and A^*_{Ca} states.

A strong functional interaction between the Ca^{2+} activation sites and anion regulatory sites was observed in choline-Cl media in the presence of submicromolar [Ca^{2+}] and using Mes^- as the competing ion. To our surprise, we found that to a first approximation our data could be described by a competitive inhibition mechanism, with Mes^- competing with Ca^{2+} for the Ca^{2+} activation sites. We consider it unlikely that Mes^- competed with Ca^{2+} by direct binding to the Ca^{2+} activation sites. Two other more likely mechanisms would be an occlusion of the Ca^{2+} activation site by the bulky Mes^- or a protein conformational change that is caused by binding of the anion to a specific site and distorts the Ca^{2+} activation site. Additional experiments will be required to characterize more fully the functional interaction between the anion regulatory and Ca^{2+} activation channel sites.

An alternative explanation for the anion-sensitivity of the SR Ca^{2+} permeability has been given by Sukhareva *et al.* (26) who identified a nonselective Cl^- and Ca^{2+} conducting channel activity that displayed a pharmacology different in several respects from that of the skeletal muscle RyR. Replacement of methanesulfonate$^-$ by Cl^- caused an increase in SR Ca^{2+} permeability but not single Ca^{2+} release channel open probability. These observations led Sukhareva *et al.* (26) to suggest that a separate, nonselective Cl^- channel mediates the Cl^--dependent Ca^{2+} release. Our results suggest that single Ca^{2+} release channel and ryanodine binding activities are strongly dependent on Cl^- concentration. Thus, it is possible to explain our SR permeability studies (Fig. 1, Table I) with the existence of one Cl^--dependent Ca^{2+} release pathway in the SR membrane.

Our [^{3}H]ryanodine binding measurements confirm previous SR vesicle-ion flux, single channel and [^{3}H]ryanodine binding measurements, which showed that adenine nucleotides and caffeine activate the skeletal muscle Ca^{2+} release channel (4, 5). As observed in the present study, caffeine primarily activated the channel by increasing the apparent affinity of the Ca^{2+} activation sites for Ca^{2+}. In most previous studies, ATP or a nonhydrolyzable ATP analog (AMP-PCP or AMP-PNP) were used to study the effects of adenine nucleotides. In the present study, we limited the number of potential channel effector species by using AMP because this compound, in contrast to ATP and the ATP analogs, binds Ca^{2+} with only a negligible affinity. We found that the apparent affinity of the Ca^{2+} inactivation sites for Ca^{2+} was lowered by greater than 3-fold by AMP, whereas only a modest increase (<2-fold) in the Ca^{2+} affinity of the channel activation sites was observed (Tables II and IV). Interestingly, AMP did not substantially increase the affinity of the Ca^{2+} activation sites for the competing cations (Mg^{2+}, monovalent cations) (Table IV). Taken together, our results suggest that caffeine primarily activated the skeletal muscle Ca^{2+} release channel by increasing the affinity of the channel's high-affinity Ca^{2+} activation sites for Ca^{2+}, whereas the primary effect of AMP was to decrease the Ca^{2+} affinity of the low-affinity channel inactivation sites.

Identification of Ca^{2+}-binding sites has been handicapped by the absence of clearly identifiable Ca^{2+} binding motifs in the primary amino acid sequence of the rabbit skeletal muscle Ca^{2+} release channel (5). However, some experimental evidence for the involvement of several channel protein regions in regulating Ca^{2+}-dependent channel activity has been obtained. In malignant hyperthermia-susceptible pigs, the channel contains an arginine residue at position 615, which when mutated to cysteine, altered the Ca^{2+} and caffeine sensitivity of the channel (27). Evidence for several Ca^{2+}-sensitive regions has been obtained in $^{45}Ca^{2+}$ and ruthenium red overlay studies with trpE fusion proteins (28). An antibody directed against one of these peptides (amino acid residues 4478–4512) increased the Ca^{2+} sensitivity of Ca^{2+} release channels incorporated into planar lipid bilayers without affecting single channel conductance. However, it is unlikely that the antibody bound directly to a critical Ca^{2+} activation site, because Ca^{2+} was still able to activate the antibody-Ca^{2+} release channel complex. Our characterization of the cation specificity of the Ca^{2+} activation and Ca^{2+} inactivation sites should help to identify these sites in future studies.

In this study, we chose nonphysiological concentrations to identify the principal mechanisms by which monovalent cations and anions regulate the channel. In resting frog skeletal muscle, the concentrations (all in mM) of the principal ionic species have been reported to be K^+ (141), phosphocreatine (50), carnosine (19), amino acids (12), Na^+ (9), $MgATP^{2-}$ (6), Cl^- (2), and Mg^{2+} (0.8) (29). How these ionic species separately and in combination affect the function of the skeletal muscle Ca^{2+} release channel remains to be explored in future studies. Moreover, all our experiments were done under steady-state conditions and therefore did not address the possibility that the rate of Ca^{2+} application may influence the affinity constants (30).

In conclusion, the results of this study show that monovalent ions profoundly affect the regulation of the skeletal muscle Ca^{2+} release channel by Ca^{2+}, in a manner that can be accounted for as changes of the Ca^{2+} binding affinities of the activation and inactivation channel sites.

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