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Cations and Anions as Modifiers of Ryanodine Binding to the Skeletal Muscle Calcium Release Channel

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Abstract. Rate and equilibrium measurements of ryanodine binding to terminal cysternae fractions of heavy sarcoplasmic reticulum vesicles demonstrate that its activation by high concentrations of monovalent salts is based on neither elevated osmolarity nor ionic strength. The effect of the ions specifically depends on their chemical nature following the Hofmeister ion series for cations ($Li^+ < NH_4^+ < K^- \sim Cs^+ \leqslant Na^+$) and anions ($gluconate^- < Cl^- < NO_3^- \sim ClO_4^- \sim SCN^-$) respectively, indicating that both are involved in the formation of the salt-protein complex that can react with ryanodine. Activation by rising salt concentrations exhibits saturation kinetics with different dissociation constants (25–11 M) and different degrees of cooperativity ($n = 1.5$–4.0) for the respective salts. Maximal second order binding rates between 40,000 and 80,000 ($M^{-1} \cdot sec^{-1}$) were obtained for chlorides and nitrates of 1a group alkali ions with the exception of lithium supporting only rates of maximally 10,000 ($M^{-1} \cdot sec^{-1}$). The nitrogen bases, NH_4^+ and $Tris^+$, in combination with chloride or nitrate, behave divergently. High maximal binding rates were achieved only with NH_4NO_3. The dissociation constants for the ryanodine–protein complexes obtained by measurements at equilibrium proved to depend differently on salt concentration, yet, converging to 1–3 nM for the applied salts at saturating concentrations. The salts do not affect dissociation of the ryanodine protein complex proving that the effect of salts on the protein's affinity for ryanodine is determined by their effect on the on-rate of ryanodine binding. ATP and its analogues modify salt action resulting in elevated maximal binding rates and reduction or abolition of binding cooperativity. Linear relations have been obtained by comparing the rates of ryanodine binding at different salt concentrations with the rates or the initial amplitudes (15 sec) of salt induced calcium release from actively loaded heavy vesicles indicating that the various salts promote specifically and concentration dependently channel opening and its reaction with ryanodine.

Key words: Skeletal muscle — Ryanodine receptor — Ryanodine binding — Salt activation — Calcium release

Introduction

Preparations of skinned muscle fibers, isolated membrane fractions of the sarcoplasmic reticulum (SR) as well as the purified calcium release channel were preferentially used for studies intended to understand the mechanisms involved in excitation contraction coupling in skeletal and cardiac muscle (Ashley, Mulligan & Lea, 1991; Meissner, 1994; Melzer, Herrmann-Frank & Lüttgau, 1995). Skinned fibers and heavy membrane fractions of the SR allowed the exploration of processes connected with the initiation of calcium liberation from the terminal cisternae or their analogues by manipulating presumed electrical potentials across the sealed T-tubular membranes (Ikemoto et al., 1994; Anderson & Meissner, 1995; Yamaguchi, Igami & Kasai, 1997). The process of channel opening itself has been analyzed on partially or extensively purified membrane fractions enriched in channel protein after fusion with black lipid membranes (*cf.* Smith, Coronada & Meissner, 1986). The results obtained with the latter preparations, however, must be considered with care because the channel protein is deprived of its natural lipid as well as protein environment. Real or apparent contradictions might result when prepa-

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Abbreviations: SR, sarcoplasmic reticulum; $AMPP(CH_2)P$, β,γ-methylen-adenosinetriphosphate; AMPP(NH)P, β,γ-imido-adenosinetriphosphate; Chaps,3-(3-cholamidopropyl) dimethylamonio]-1-propanesulfonate

rations having different structural integrity are compared. This limitation does not apply when the action of potent effectors of calcium release like caffeine and or ryanodine are used to investigate the mechanism by which calcium is released from its intracellular stores in heavy membrane fractions containing mainly terminal cysternae with or without attached T-tubular membranes (Palade, 1987; Sumbilla & Inesi, 1987; Meissner & El-Hashem, 1992; Ma & Zhao, 1994). Prominent effects of caffeine exerted on glycerinated muscle fibers and isolated sarcoplasmic reticulum membranes were reported quite early (Weber, 1953; Weber, 1968). Studies with ryanodine, on the other hand, yielded only disappointing results for a long time, although the drug was well known as an effective muscle poison since 1961 (Haslett & Jenden, 1961; Jenden & Fairhurst, 1969; Fairhurst & Hasselbach, 1970; Jones & Cela, 1981). The reason for this became apparent after Pessah et al., (1986) showed that the binding of ryanodine to heavy SR-vesicles can be stimulated strongly by high concentrations of NaCl (1 M). In the following, ryanodine was used under these conditions as a label for the isolation of the calcium release channel protein residing in the terminal cisternae complex (Inui, Saito & Fleischer, 1987; Lai et al., 1988). Ionic strength of osmolarity effects were proposed as mechanisms of salt action (Ogawa & Harafuji, 1990; Ogawa, 1994). The conditions for ryanodine binding were analyzed and it was demonstrated that besides high salt concentrations, ATP and some of its congeners as well as low concentrations of ionized calcium could promote ryanodine binding while ionized magnesium, millimolar concentrations of calcium and some other positively charged agents like ruthenium red or tetracaine counteract its binding (Meissner, 1984, 1986; Smith et al., 1986; Palade, 1987). It was further shown that ryanodine could effect the opening of the calcium channel by arresting it in a low conductance state with slow open-close transitions (Xu, Jones & Meissner, 1993; Smith et al., 1986; Smith et al., 1988).

The observations showing that ryanodine binding was promoted by agents that can induce channel opening under various experimental conditions has led to the notion that channel opening is a prerequisite for ryanodine binding (Fleischer et al., 1985; Pessah, Stambuck & Casida, 1987; Chu et al., 1990; Ma et al., 1993). In a recent paper we reported that calcium release from and ryanodine binding to skeletal muscle heavy SR-vesicles are specifically affected by monovalent anions (Hasselbach & Migala, 1992). Subsequent studies confirmed and extended the occurrence of specific ion effects of calcium release and ryanodine binding of cardiac and skeletal muscle preparations (Sukhareva, Morissette & Coronado, 1994; Fruen et al., 1996; Meissner et al., 1997; Liu, Pasek & Meissner, 1998). In the present paper experimental evidence is presented indicating that channel opening and ryanodine binding are connected to the occupation of specific binding sites for monovalent cat- and anions by studying ryanodine binding kinetics and calcium release. The ionic specificity of this reaction is demonstrated and it is shown that quite a number of ions that often are used as charge carriers to study channel activity affects differently ryanodine binding.

The rather complete set of parameters furnished by the present analysis of ryanodine binding in the presence of various ionic activators allows to substantiate the relation between the functional state of the calcium release channel and its reactivity with ryanodine.

Materials and Methods

Materials

Ryanodine was purchased from Calbiochem, Frankfurt-Main (F.R.G.). ATP and AMP were obtained from Pharma-Waldhof, Mannheim (F.R.G.). All other reagents were analytical grade and bought from E. Merck, Darmstadt; Sigma Chemical, Deisenhofen and Serva, Heidelberg (F.R.G.). $^{45}CaCl_2$ and [^{3}H]ryanodine were supplied by Buchler & Co. Frankfurt-Main (F.R.G.).

Preparation of SR Vesicles

Heavy sarcoplasmic reticulum vesicles were prepared from predominantly white rabbit hindleg muscles as described by Hasselbach and Migala, (1992) and modified according to Meissner (1984). 100 g minced muscle were homogenized in 400 ml of a solution containing 0, 1 M KCl, 5 mM potassium phosphate (pH = 7.0), 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM sodium azide in a mixer at 4°C. Myofibriles were removed from the suspension by centrifugation in a Sorval GS3 rotor at 6,000 rpm for 30 min. The heavy vesicles were separated from the supernatant by subsequent centrifugation in a Beckman type 19 rotor at 10,000 rpm for 30 min. The pellet was treated with ten volumes of 0.6 M KCl, 0.3 M sucrose, 3 mM ATP, 3 mM $MgCl_2$, 0.1 mM benzethonium-Cl, 0.1 mM benzamidine-HCl and 0.1 mM dithiothreitol for 20 min to remove contaminating contractile proteins. Subsequently, the heavy vesicles were sedimented by centrifugation in a Beckman rotor Ti 60 at 55,000 rpm for 50 min and resuspended in a solution containing 0.1 M KCl, 0.3 M sucrose and 0.1 mM dithiothreitol (30–50 mg protein $\cdot$ ml^{-1}).

[^{3}H]-Ryanodine Binding

Ryanodine binding to heavy vesicles was performed at 20°C in solutions containing 0.5–1.0 mg vesicular protein $\cdot$ ml^{-1}, 0.2 M sucrose, 50 mM Tris-Mops-Mes buffer pH = 7.4, 0.1 mM $CaCl_2$ and 0.1 μM [^{3}H] ryanodine; changes are mentioned in the legends, likewise the respective salt components and their concentrations. Activation of ryanodine binding by ATP or AMPP(CH_2)P was performed at 100 μM ionized calcium adjusted by using 0.5 mM as dissociation constant for CaATP and 1 mM for CaAMPP(CH_2)P, respectively. Free calcium was measured with a calcium sensitive electrode (Orion, Boston, MA) and used to calculate the dissociation constants in the respective salt solutions of pH = 7.4. The dissociation constant of the CaATP complex argees well with tabulated values considering high ionic strength in the present experiments (Sillén & Martell, 1971). ATP break down in the

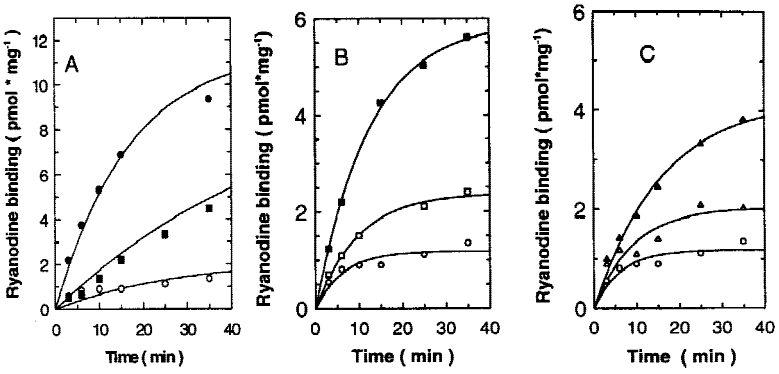

Fig. 1. Activation by sucrose of ryanodine binding of heavy SR-vesicles. The reaction media contained 100 nM ryanodine at pH = 7.0 and 20°C. (*A*) Reaction media were adjusted to equal osmolarity by 0.5 M NaCl (■), 0.5 M KCl (●) and 1 M sucrose (○); (*B*) Reaction media were supplemented with 0.2 M KCl (□), 0.2 M KCl + 1.0-M sucrose (■); (*C*) Reaction media additionally contained 0.2 M NaCl (△), 0.2 M NaCl + 1 M sucrose (▲) and 1 M sucrose alone (○) in *B* and *C*.

binding assays was monitored by measuring P_i production. ATP losses have been minimized by measuring initial binding rates (3–5 min) and by using low protein concentrations.

Ryanodine binding to heavy SR–vesicles was terminated by filtering aliquots containing 0.2–0.4 mg protein through Schleicher & Schuell nitrocellulose filters BA 0.45 μm, after appropriate reaction times. The filters were rinsed subsequently with 0.1 M NaCl, 0.05 M imidazole pH = 6.3. In some cases 0.1 mM cold ryanodine was added to the rinsing solution. Nonspecific binding was measured in media containing 2–5 mM EGTA (Hasselbach & Migala, 1992). Residual unspecific binding in the presence of EGTA can be neglected as long as the concentrations of ryanodine do not exceed 0.2 μM. In this study, all experiments were conducted at lower concentrations.

Calcium Loading

Actively loaded vesicles were used to study calcium release. The loading media contained 3 mM Mg-gluconate, 0.1 M K-gluconate, 50 mM Tris-Mops pH 7.0, 3 mM Li-K-acetylphosphate, 20 μM ^{45}calcium and 0.2 mg · ml^{-1} heavy vesicles. Loading was performed at 30°C for 30 min. Subsequently, the temperature was lowered to 20°C. A mean calcium load of 90 nmol · mg^{-1} was obtained.

Calcium Release

The salt-induced calcium release was studied under two conditions. Release was induced either by adding the respective concentrated salt solutions to the uptake medium containing 0.2 mg · mg^{-1} followed by filtration through nitrocellulose filters (Schleicher & Schuell, BA 0.45 μm) or by perfusing calcium loaded vesicles bound to nitrocellulose filters (Millipore, DA 0.6 μm) with the respective solutions, given in the legends, using the rapid filtration instrument of Biologic (Meylane, France). The first procedure allows to establish calcium release during an initial period of 15 sec, release burst, and the following slow calcium leakage for 5 min. The time resolution of rapid filtration is limited to 25 msec and can be reliably followed up to 500 msec. The data were documented and fitted by computer support using Graphit (Erithacus Software), Sigma Plot (Jandel Scientific) and Mathcad 5.0 (MathSoft) as software.

Results

Ion-Specific and Osmotic Effects on Ryanodine Binding

Ryanodine binding to isolated heavy SR-vesicles and isolated channel protein has preferentially been studied in solutions supplemented with 0.5–1.0 M NaCl or KCl [*cf.* Anderson, Cohn & Meissner, 1994; Tripathy et al., 1995; McGrew et al., 1989). More recently, however binding analysis was extended to salt concentrations in the more physiological range between 0.15 and 0.25 M (Fruen et al., 1996; Meissner et al., 1997; Liu et al., 1998). On the other hand, the interaction of ryanodine with the channel protein in black lipid membranes was analysed in the presence of quite different ionic constituents. K-Mops, Cs-gluconate or Cs-glutamate were used to avoid interference with chloride and or potassium channels present in the preparations (O'Brien, Valdiia & Block, 1995; Percival et al., 1994; Ma, 1995). The results depicted in Fig. 1 show that ryanodine reacts differently with its receptor in vesicular preparations depending on the kind of salt present in the solution. KCl proves superior to NaCl when the assays were supplemented with 0.5 M of the respective salts. The large difference between the binding rates observed in solution containing KCl or NaCl, respectively, cannot be due to the difference in the osmolarity of the reaction media (Fig. 1 *A*). The difference between the activity coefficients of NaCl and KCl are too small to account for osmotic effects. Figure 1, furthermore, demonstrates that one molar sucrose alone only weakly supports ryanodine binding. Consequently, osmolarity itself can only play a minor role in the observed difference in the activation of ryanodine binding by NaCl or KCl. Surprisingly, however, sucrose affects ryanodine binding in combination with ionic effectors. Figure 1 *B* and *C* indicate an intriguing relation between osmolarity and ionality. Addition of 1 M sucrose to 0.2 M NaCl or KCl containing assays considerably augments ryanodine binding. This is mainly due to the fact that in the presence of sucrose the binding capacity is preferentially increased while the rate constants of ryanodine binding are scarcely affected. They remain 10 times smaller than in experiments in which the salt concentrations were raised to the same osmolarity of 1.4 M (*cf.* Fig. 5) These results show that binding of ryanodine to its receptor is activated specifically by the cations present in the medium and their effect is modulated intensely by sucrose.

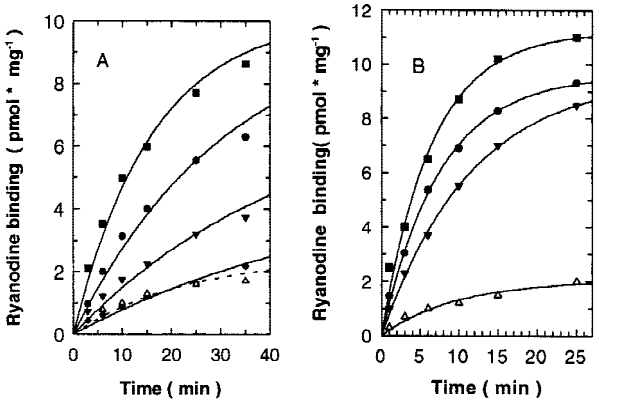

Fig. 2. Activation of ryanodine binding to heavy SR-vesicles by different monovalent cations. (*A*) The reaction was activated by 0.6 M of the chlorides of cesium (■), potassium (●), sodium (▼), lithium (△) and ammonium (♦); (*B*) Activation by the nitrates of Cs^+, K^+, Na^+ and Li^+; 100 nM ryanodine, pH = 7.0, T = 20°C.

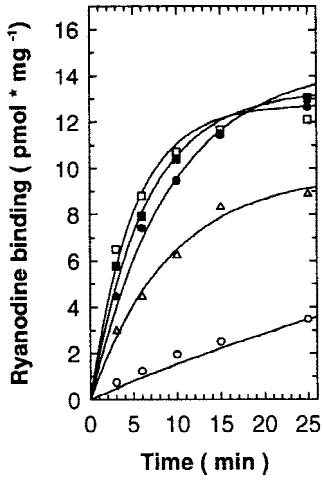

Fig. 3. Activation of ryanodine binding to heavy SR-vesicles by different monovalent anions. The reaction was activated by 0.4 M of the respective sodium salts: Chloride (○), trichloracetate (△), perchlorate (●), thiocyanate (■), nitrate (□); 100 nM ryanodine, pH = 7.0, T = 20°C.

The observed cation effect is in line with the finding of Buck et al. (1992) indicating that 0.5 M CsCl activates equilibrium binding of ryanodine more strongly than KCl.

Cations and Anions as Activators of Ryanodine Binding

The results depicted in Fig. 2 show that the rate of labeling increases according to the lyotropic series when supported by 0.6 M of the chlorides of group 1a monovalent cations. Under the prevailing conditions ryanodine binding proceeds 10 times faster with CsCl than with LiCl. The order of effectiveness does not depend on the accompanying anion. Yet, the reaction rates can be considerably enhanced or diminished when chloride is replaced by other anions. As shown in Fig. 2, nitrate is a considerably more effective activator than chloride. This is especially true in combination with the less effective cations lithium and sodium, while for potassium and cesium the activity increments achieved by nitrate is relatively small. The role of the anions in activating ryanodine binding was evaluated more completely by studying the effect of the anion series chloride, trichloracetate, perchlorate, thiocyanate and nitrate with sodium as counterion (Fig. 3). From the time course of ryanodine binding in the presence of 0.4 M salt and 0.2 μM ryanodine a series $Cl^- < CCl_3COO^- < NO_3^- \sim SCN^-, < ClO_4^-$ emerges corresponding likewise to the Hofmeister ion series. The corresponding second order rate constants range from 1400 (M^{-1} sec^{-1}) for NaCl to 16,000 (M^{-1} sec^{-1}) for $NaClO_4$. Binding saturation measured after 5 hr under these conditions reach from 12 to 15 pmol · mg^{-1} for all salts, except lithium chloride *cf.* Table 1. Evidently, binding capacity is not or only loosely related to binding reactivity, provided binding equilibrium has been reached and the salts do not cause irreversible inactivation during long incubation periods. Reaction rates slower than those observed for NaCl were found for sodium as well as potassium salts of gluconate, propionate, methylsulfonate, Mops or succinate, anions which are often used as substitutes for chloride (*not shown*). The fact that the rates with which ryanodine binds to its receptor in heavy SR-vesicles depends on the chemical character of both cations and anions strongly argues against the notion that activation of binding is based on reaction medium ionic strength or osmolarity. Our observations showing that activation exhibits a pronounced specificity concerning cations and anions rather indicate that both cations and anions are specifically involved in the formation of the protein complex which reacts with ryanodine. This concept could be substantiated by studying the kinetics of ryanodine binding over broad ranges of salt concentrations and demonstrating saturation kinetics with cooperativity for the applied ion combinations.

Kinetics of Ryanodine Binding as Affected by High Concentration of Activating Salts

Figure 4 demonstrates the time course of ryanodine binding when the concentrations of the activating salts, NaCl and CsCl as examples, are raised. The concentration ranges were extended to values higher than customarily applied in order to achieve, or at least to come near to saturation. In some cases it was necessary to dissolve solid salts in the assay media because even by addition of saturated solutions the intended concentrations could not be established. The graphs obtained by plotting the ob-

Table 1. Parameters of ryanodine binding as exhibited in media containing different monovalent salts

Salt	Max. binding	Max. rate constant	Dissociation constant	Cooperativity
M	pmol · mg^{-1}	M^{-1} sec^{-1}	M	*n*
NaCl	14.0 ± 0.4	70000 ± 23000	11 ± 3.0	1.7 ± 0.23
KCl	14.1 ± 0.5	37200 ± 3000	1.3 ± 0.3	2.0 ± 0.52
CsCl	12.9 ± 0.3	56000 ± 2300	0.37 ± 0.1	2.98 ± 0.15
NH_4Cl	13.1 ± 0.7	2400 ± 100	0.025 ± 0.012	3.1 ± 0.85
Tris-Cl	12.2 ± 0.26	12000 ± 2000	0.16 ± 0.03	2.8 ± 1.3
LiCl	4.6 ± 1.5			
$NaNO_3$	13.1 ± 0.3	81600 ± 4600	1.27 ± 0.1	1.28 ± 0.1
KNO_3	12.3 ± 0.3	77000 ± 17000	0.54 ± 0.18	1.55 ± 0.5
$CsNO_3$	13.7 ± 0.6	84000 ± 15000	0.24 ± 0.06	2.43 ± 0.4
NH_4NO_3	13.5 ± 0.6	40000 ± 600	0.09 ± 0.02	4.4 ± 0.3
Tris-NO_3	13.0 ± 0.5	13000 ± 2000	0.03 ± 0.01	2.1 ± 0.8
$LiNO_3$	13.1 ± 0.4	10100 ± 1700	0.1 ± 0.02	3.9 ± 2.4

The data were obtained from binding rate measurements at salt concentrations between 0.2 and 3.0 M and evaluated by assuming cooperative binding using the software of Grafit (Erithacus). The data were compiled from at least 3 sets of binding experiments performed with different preparations. Data are given as means ± SEM.

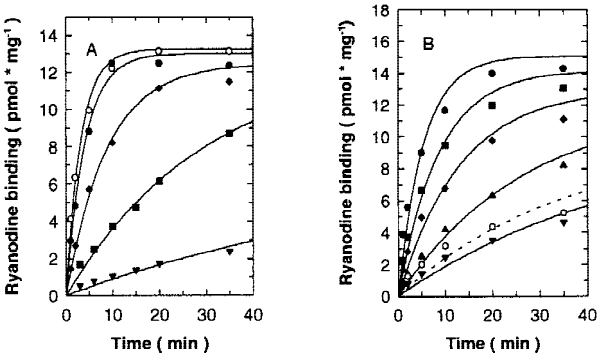

Fig. 4. Time course of ryanodine binding to heavy SR-vesicles activated by rising concentrations of sodium- and cesium-chloride. (*A*) Sodium chloride 0.6 M (▼), 1 M (■), 2 M (♦), 3 M (●), 4 M (○). (*B*) Cesium chloride 0.2 M (▼), 0.4 M (▲), 0.6 M (♦), 1 M (■), 1.5 M (●), 4 M (○). *A* and *B:* pH = 7.4, 100 nM ryanodine, T = 20°C.

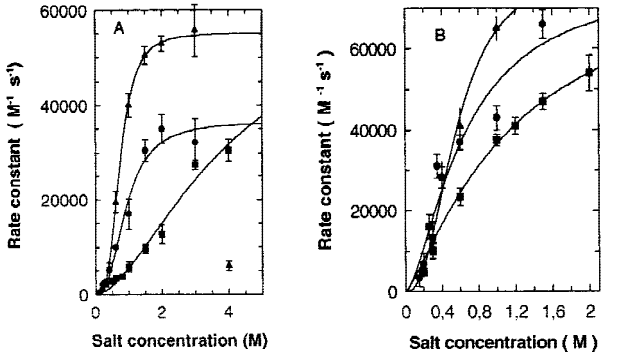

Fig. 5. Dependence of the rate of ryanodine binding to heavy SR-vesicles on the concentrations of sodium-, potassium- and cesium-chlorides (*A*) and the corresponding nitrates (*B*). The reaction media contained 100 nM ryanodine and the salt concentrations given on the abscissa. The rate constants plotted on the ordinate were calculated from the observed rate constants (*cf.* Fig. 4) assuming linear dependence on the concentration of ryanodine (31) (*A*) NaCl (■), KCl (●), Cs Cl (▲), (*B*) The corresponding nitrates. pH = 7.4, T = 20°C (*C*) Data were fitted assuming simple cooperativity, the Hill formalism, for the interaction of the salts with the ryanodine receptor: $V = V_{max} \cdot S^n/(K_d + S^n)$ where V is the observed binding rate, V_{max} is the maximal binding rate, K_d is the dissociation constant of the ryanodine receptor salt complex S is the salt concentration and n is the Hill coefficient.

served rates, transformed to second order rate constants, *vs.* the activating salt concentrations are characteristically S-shaped and tend to saturations at values between 40,000 and 60,000 M^{-1} sec^{-1} (Fig. 5). Generally, higher concentrations are needed for chlorides than for nitrates to reach or approach saturation. CsCl displays a special behavior. On applying high concentrations, exceeding 3 M, the reaction rate sharply drops to quite low values. A similar skewed bell-shaped activation profile has been observed for $LiNO_3$. The drop in activity at high salt concentrations is not an irreversible effect, since the reaction continues and maximum binding values are reached. Computer fitting assuming cooperative interactions as indicated by the S-shaped activation profiles yielded parameters which are summarized in Table 1. It also includes data for NH_4NO_3, Tris-NO_3, NH_4Cl and Tris-HCl. The maximal velocities obtained with the latter chloride salts remain far below the values reached with the chlorides of the 1a group alkali ions with the exception of lithium. This contrasts to the effect of ammonium nitrate supporting maximal reaction rates of 40,000 M^{-1} sec^{-1}. The dissociation constants of the complexes which the ryanodine receptor forms with the different alkali chlorides and nitrates vary over a wide range between 25 mM for NH_4Cl and 11 M for NaCl whereby

Table 2. Ryanodine binding parameters observed in salt media of different composition and concentrations

Concentration	KCl		KNO_3		$NaNO_3$	
M	Dissociation constant	Rate constant	Dissociation constant	Rate constant	Dissociation constant	Rate constant
	nM	M^{-1} sec^{-1}	nM	M^{-1} sec^{-1}	nM	M^{-1} sec^{-1}
1.0	1.0 ± 0.1	16000 ± 3000			2.5 ± 0.4	30000 ± 1500
0.6	3.6 ± 0.46	9000 ± 3000	1.34 ± 0.24	40000 ± 3500	3.8 ± 0.9	23000 ± 2500
0.4	7.8 ± 1.4	5000 ± 2000				
0.3	10.8 ± 2.2	2500 ± 500	3.9 ± 0.53	17000 ± 6000		
0.2	32 ± 11.5	1050 ± 200	8.0 ± 1.6	6300 ± 2000	9.2 ± 2.2	3000 ± 1000

The experiments were performed as described in Materials and Methods. Data from at least three preparations were compiled and evaluated assuming binding cooperativity. Data values are means ± SEM.

the dissociation constants for the nitrates are in general somewhat smaller than those for the chlorides. The high K_d value for NaCl, like the other parameters, must be considered with caution. Experimental data are available only up to 3 M. The applied fitting is justified with regard to the saturation behavior of the other salts, especially of $NaNO_3$. The data of Table 1 show that the Hill coefficients required to fit the S-shaped activity profiles can be subdivided into two groups. Values of two and smaller were found for chlorides and nitrates of sodium and potassium. Larger values are associated with cesium, ammonium and Tris.

In conclusion, the ions affect the binding of ryanodine to the native calcium release channel in a rather complex manner which is due to the different affinities of the salts to the receptor protein, on the one hand, and the different degree of cooperativity which characterizes receptor-ion interaction, on the other hand (*cf.* Discussion).

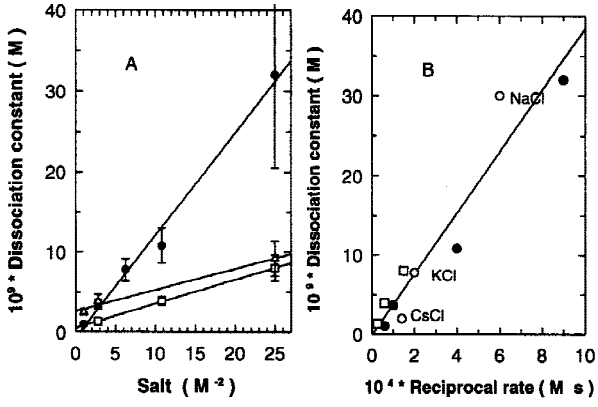

Fig. 6. Dependence of the dissociation constants of the ryanodine receptor complex in native membranes on the salt concentration (*A*) and on the reciprocal rate constant (*B*). (*A*) The dissociation constants are plotted vs. the second power of the reciprocal salt concentrations (M^{-2}) on the abscissa; KCl (●), KNO_3 (□) $NaNO_3$ (△). (*B*) The dissociation constants registered for different KCl (●) and KNO_3 (□) concentrations and for 0.4 M NaCl, KCl and CsCl (○) are plotted vs the reciprocal values of the corresponding rate constants. Ca^{2+} = 0.1 mM, pH = 7.4, T = 20°C.

Salt Effects on Ryanodine Equilibrium Binding

The interactions of the respective ions with the calcium channel in native heavy vesicles not only govern the on-rate of complex formation with ryanodine but appear also to determine binding equilibrium. Equilibrium for ryanodine binding was established at different salt concentrations for the respective salts by incubating the vesicles for 5 to 20 hr at 20°C with 2–50 nM ryanodine. Quite good linear relations are obtained when the dissociation constants obtained for different KCl, KNO_3 and $NaNO_3$ concentrations are plotted *vs.* the second power of their reciprocal values (Table 2 and Fig. 6 *A*). The second power necessary for linearization agrees approximately with the cooperativity factor found for salt activation in the previous section. Hence, linear relations are also obtained when the dissociation constants are plotted *vs.* the reciprocal values of the corresponding rate constants (k_{+1}) (Fig. 6 *B*). The constant relations between the dissociation constants and rate constant k_{+1} furthermore indicate that the off-rate for the dissociation of the receptor ryanodine complex, is neither significantly affected by the concentration of the salts nor by their ionic components. This could be confirmed by inducing complex dissociation by diluting 100-fold an aliquot of the binding assay. The dissociation rates are not significantly different ($2–4 \cdot 10^{-5}$ sec^{-1}) (Fig. 7). There are no significant differences in the magnitude of the difficult to interpret initial rapid ryanodine dissociation as described by Lai et al., (1989). Addition of high concentrations of cold ryanodine for replacing the labeled ryanodine in the complex strongly retarded dissociation which agrees with the result of previous studies (*cf.* McGrew et al., 1989).

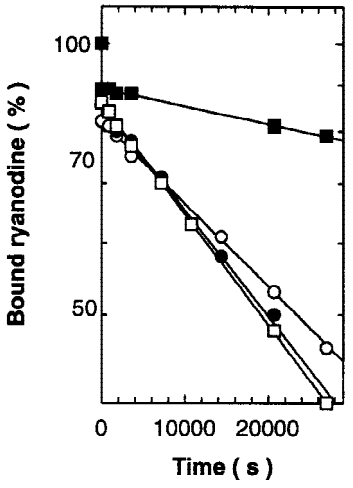

Fig. 7. Dissociation of the ryanodine receptor complex in heavy vesicles under different conditions. The complex was formed in the presence of 10 nm ^{3}H-ryanodine, pH = 7.4 and of 0.6 M of the respective salts KCl (○), NaCl (●) and KNO_3 (□). Dissociation was initiated by diluting an aliquot of the binding medium 100-fold in the corresponding salt solutions. The effect of 10 μM cold ryanodine on ^{3}H-ryanodine release was measured in presence of 0.6 M KCl (■).

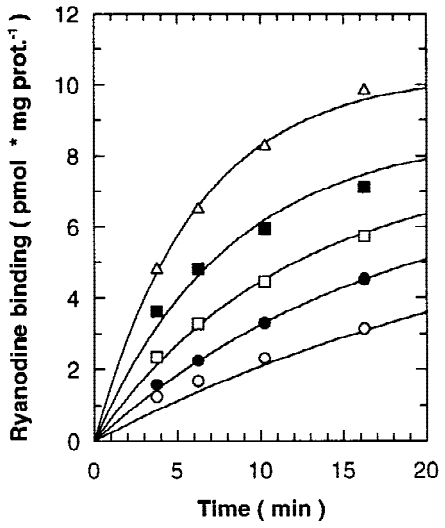

Fig. 8. Activation by ATP of ryanodine binding supported by 0.3 M KCl. No ATP (○), binding rate of ryanodine measured under the same ionic conditions. 0.1 mM ATP (●), 0.3 mM ATP (□), 0.5 mM ATP (■), 1.0 mM ATP (△). Ryanodine 0.2 μM, pH = 7.4, ionized calcium was kept constant at 0.1 mM. T = 20°C. At constant free calcium, CaATP changes in parallel with free ATP. In separate experiments keeping CaATP constant it could be excluded as binding activator. Under these conditions activation first increases as a consequence of rising ATP and then declines when free calcium falls below 5×10^{-5} M.

Modification of Ionic Activation by Adenine Nucleotides

The described protein-ion interactions are distinctly modified by ATP which is known as a very effective activator of calcium release and ryanodine binding (Smith et al., 1986; Meissner, 1984). Figure 8 demonstrates the effect of increasing concentrations of ATP on ryanodine binding in the presence of 0.3 M KCl. 0.3 M KCl was chosen because at that concentration, the effect of ATP can most clearly be seen. We showed in pilot experiments that an enhancement of the very low binding rate by ATP was difficult to realize at KCl concentrations below 0.1 M. Likewise it proves difficult to establish a further increase of the already high labeling rate at KCl concentrations $\geqslant$ 0.6 M. Results as depicted in Fig. 8 yield a dissociation constant of the ryanodine receptor with uncomplexed ATP of 0.4 ± 0.2 mM ($n = 4$) if ATP consumption (0.03 $\mu M \cdot mg^{-1} \cdot min^{-1}$) during the binding reaction is taken into account.

The modifying effect of ATP on the activation of ryanodine binding in the presence of the weak ionic activator, NaCl, and the much stronger activator, KNO_3, was studied in the presence of 1 mM ATP. The results are illustrated in Fig. 9 *A* and *B* and are compared to activation profiles taken from the experiments performed in the absence of ATP described above. ATP effects a distinct increase (30–50%) of the maximal binding rates whereby the affinities of the respective salts appear to be strongly affected: K_d for NaCl is reduced to 7 M, for KCl and KNO_3 to 0.77 M and 0.63 M, respectively. At the

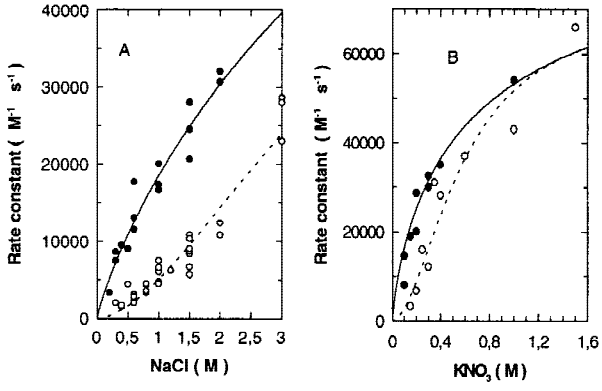

Fig. 9. Modulation by ATP of ryanodine binding supported by rising salt concentrations. (*A*) NaCl alone (○), NaCl + 1 mM ATP (●); (*B*) KNO_3 alone (○); KNO_3 + 1 mM ATP (●). Other conditions as in Fig. 8.

same time, the S-shaped activation profiles which are characteristic for the action of these salts are replaced by hyperbolic profiles characteristic for simple Michaelis-Menten saturation kinetics. Curve fitting reveals Hill coefficients somewhat smaller than one for KCl, KNO_3 and also for CsCl (*not shown*). A similar effect is exerted by AMP with a K_d value of 4 mM. The same modification can be accomplished with the ATP analogue, 2,3 methylene ATP, at concentrations of ~0.5 mM corresponding to the efficacy of ATP. Binding capacity is not affected

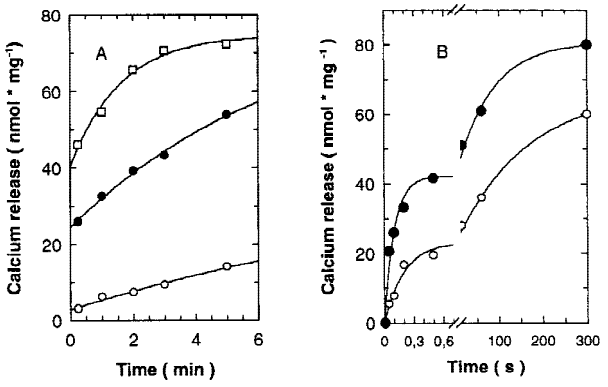

Fig. 10. Calcium release induced by high salt concentrations from actively loaded heavy vesicles. (*A*) 12 ml of the vesicular suspensions (0.2 mg · ml^{-1}) were supplemented with the respective salt solutions yielding a final concentration of 0.5 M Na-gluconate (○), NaCl (●) and $NaNO_3$ (□). Magnesium present in the uptake medium (3 mM) was complexed with EDTA added together with the sale solutions. Calcium release was followed for 15 sec–5 min by manual filtration. The extrapolations to the ordinate indicate the amplitudes of the initial rapid release phases. (*B*) Calcium release was followed for 25 msec–600 msec by rapid filtration. One ml of the uptake medium was placed on the filter in the rapid filtration instrument and subsequently perfused for the times given on the abscissa. The release media contained 50 mM Tris–Mops, pH 7.4, 0.1 mM EGTA, 0.05 mM $CaCl_2$ and 0.5 M NaCl (○) and 0.5M $NaNO_3$ (●). The abscissa is split at 600 msec. The data for the slow phases of release are taken from separate experiments run in parallel.

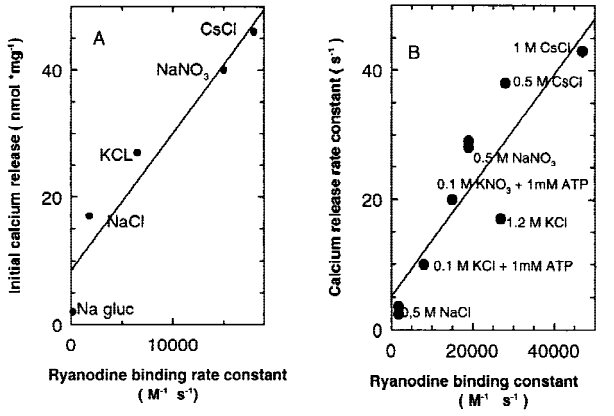

Fig. 11. Correlation between ryanodine binding and calcium release (*A*) The amount of calcium released during 15 sec (initial calcium release) is related to the binding rate of ryanodine observed under the same ionic conditions reported above with the exception of sodium gluconte which does not activate measurably ryanodine binding of native heavy vesicles. (*B*) The rate constants of calcium release measured by rapid filtration were likewise correlated to the respective ryanodine binding rates. The release media contained 50 mM Tris–Mops, pH 7.4, 0.1 mM EGTA, 50 μM $CaCl_2$ and were supplemented with 0.5 M of the respective salts shown in *A* or with the different salts at concentrations depicted in *B.*

by ATP or its analogues. The efficacy of the adenosine derivates having no transferable phosphate residues demonstrate that protein phosphorylation is not involved in the observed modulation of ryanodine binding supported by ionic reactants. Inorganic phosphate alone produces no observable change in ryanodine binding under comparable conditions (*not shown*).

Interrelation Between Ryanodine Binding and Ion Effected Calcium Release

The rather complete set of parameters furnished by the present analysis of ryanodine binding in the presence of various ionic activators allows a better substantiation of the relation between the functional state of the calcium release channel and its reactivity with ryanodine. The effect of the various salts on calcium release was studied on actively calcium-loaded vesicles under two conditions as described in Materials and Methods. Figure 10 *A* demonstrates that equal concentrations of sodium gluconate, -chloride and -nitrate (0.5 M) effect quite differently the initial release amplitudes (t ~15 sec), measured manually, and the following progressing calcium leakage: gluconate < chloride < nitrate. When calcium release is induced by equal concentrations of NaCl, KCl, CsCl (0.5 M) the pattern is similar, increasing in the order Na < K < Cs (*not shown*). Rutenium red (1 μM) or tetracaine (1 mM) reduce the initial burst by 60–70% and significantly diminish the slow calcium leakage (*not shown*). These findings exclude that the release is caused by the concomitant change in osmolarity. Change of a presumed electrical potential across the residues of the T-tubular fragments attached to the cysternal vesicles are also unlikely because the loading condition does not effect ion accumulation in the T-tubules. Time resolution of the initial burst obtained by rapid filtration yielded characteristically different rate parameters by double exponential fitting. They concern the amplitude of the first phase ranging between 10 and 40% of the releasable calcium fraction as well as the corresponding rate constants as shown for some examples in Fig. 10 *B.* Plots of the results of the described release experiments *vs.* the rate constants of ryanodine binding observed under identical conditions demonstrate that release amplitudes as well as initial release rates exhibit quite good linear correlations (Fig. 11 *A* and *B*). This finding proves that the distinct effects of the different salts cause in parallel opening of the calcium channel and its reaction with ryanodine.

Discussion

The presented results indicate that the activation of ryanodine binding to native heavy SR-vesicles effected by elevated concentrations of different monovalent salts is

based on specific interactions of the respective cations as well as anions with the ryanodine receptor. The salt effects were most clearly revealed when moderate concentration, 0.4–0.6 M, were applied. They concern mainly the rates of binding and to a much lesser extent the maximum amounts bound at equilibrium. Under these conditions the efficacy of the cationic constituents increases in the order $Li^+ < NH_4^+ < Na^+ < K^+ < Cs^+$ and that of the anionic components in the order gluconate < Cl < NO_3^- ~ ClO_4^-. These findings largely exclude that activation of ryanodine binding by high concentrations of salts is brought about by either high ionic strength or elevated osmolarity. This also applies to the release of calcium from actively loaded heavy vesicles induced under these conditions. As to the action of high osmotic pressure, addition of one molar sucrose scarcely activated the rate of ryanodine binding. However, sucrose was shown to enhance ryanodine binding activated suboptimally by low concentrations, 0.1–0.2 M of KCl. This effect of sucrose also exhibited ion specificity. Sucrose proved only a little effective when instead of KCl, NaCl was present in the reaction medium. The discrepancy between these results and Ogawa's conclusion that high osmolarity promotes ryanodine binding can be attributed to the fact that neither binding kinetics nor true binding equilibria were determined (Ogawa & Harafuji, 1990). In the following, we will discuss our observations in terms of binding capacities, dissociation constants and reaction rates.

Binding Capacity

Ryanodine binding capacity of heavy SR-vesicles represents the number of ryanodine binding sites saturable in the applied concentration range of ryanodine during incubation periods of 5–24 hr. Two classes of binding sites have been described to be present in heavy SR-vesicles isolated from rabbit skeletal muscle (Pessah et al., 1986; Meissner & El-Hashem, 1992; McGrew et al., 1989). Saturation and separation of these binding sites is, however, not easy to accomplish which makes it difficult to determine unambiguously the number of maximal binding sites. Under the conditions applied in this study, pH 7.0–7.4, Ca^{2+} = 10–100 μM, T = 20°C and the presence of 0.3 to 1–3 M chloride or nitrates of first group cations, maximal binding in the presence of 30–100 nM ryanodine reached 12–18 pmol · mg^{-1} after 5–18 hr incubation depending on the preparation's quality. These numbers agree quite well with most published values obtained under similar conditions. While ryanodine binding remained quite small when supported by lithium chloride, in line with Meissner et al. (1997), binding is elevated to maximal values when lithium nitrate is applied. Binding numbers as observed for the metal ions were also reached with ammonium or tris(hydroxymethyl)aminomethan (Tris) as cationic components stressing the modifying effect of the cations. In the presence of gluconate as anionic component as well as in nearly complete absence of ionic activators, ryanodine binding remained marginal (Fruen et al., 1996; Meissner et al., 1997; Hasselbach & Migala, 1992). In the absence of ionic activators binding could not be enhanced by ATP. Yet, it proved to be very effective in promoting the effect of lithium chloride and of potassium gluconate that were used in this study as weak activators. In the presence of 1 mM ATP the same high ryanodine binding values were reached as observed for the most effective salts, like KNO_3. The finding that the enhancement of ryanodine binding by ATP depends on the presence of ionic activators is in line with recent findings reported by Fruen et al. (1996). This effect of ATP cannot be related to phosphoryl transfer reactions since the same effect could be achieved with AMP or AMPP(CH_2)P. The observation that irrespective of the nature of the activating salts the same maximal binding capacity was reached shows that it is an intrinsic property of the ryanodine receptor in the membranes and can only be used with caution as a measure of the functional state of the ryanodine receptor under the respective experimental conditions. The competition between calcium at suboptimal or overoptimal concentrations and the respective cations in long time binding experiments is subjected to the same limitation (Meissner et al., 1997). However, the present results obtained at optimal concentrations of calcium (0.1 mM) cannot contribute to the proposed reciprocal interaction of the ryanodine receptor with calcium, magnesium, monovalent cations and anions (*cf.* Meissner et al., 1997). Yet, when ryanodine binding is measured at equilibrium at low ryanodine concentrations, the increasing binding with rising KCl concentrations show the same progress as observed for the on-rate of ryanodine binding. This holds also for media containing different cations of the Hofmeister series (Meissner et al., 1997; Liu et al., 1998).

Dissociation Constant for Binding of Ryanodine and Activating Salts

The affinity of native membranes to ryanodine, respectively, the dissociation constant of the ryanodine complex in the membranes, proved to depend significantly on the nature and the concentration of the salts used for activation at concentrations in the range of 0.3 to 1.0 M (*cf.* Table 1 and 2). Yet, at higher salt concentrations these differences largely disappear and the dissociation constants of the ryanodine complex formed in the presence of the different salts converged to 1–3 nM at infinite salt concentrations as could be shown by reciprocal extrapolation. This range well agrees with the data reported by Chu et al. (1990) observed in the presence of

0.3 M KCl and 1 mM AMPP(CH_2)P or AMPP(NH)P. The fact that this limiting value does not depend on the nature of the salt is in line with the observation that the rates, with which the ryanodine membrane complex dissociates, are practically identical in the presence of KCl and $NaNO_3$, respectively. On account of the measured reaction rates (Table 1 and 2) we could exclude that binding equilibrium has not been reached at low salt and low ryanodine concentrations during reaction times of 18–20 hr which also could pretend to a convergence of the K_d values with increasing salt concentrations. It can be calculated from the rate constants given above that the binding reaction reaches 95% completion even in the presence of only 0.2 M KCl and 2 nM ryanodine at 20°C, which were the most unfavorable conditions applied in this study. The discussed interference might, however, apply to the very differing values of the dissociation constants that have been reported in the literature (Pessah et al., 1986; Meissner et al., 1994; Inui et al., 1987). In most of these experiments KCl or NaCl were used as activating salts and the attainment of binding equilibrium was not always rigorously controlled.

In good agreement with all previous studies ryanodine binding up to 50 nM free ryanodine in the assay could be described assuming a single binding site in the membrane integrated receptor. This result also relies on the attainment of binding equilibrium at low ryanodine concentrations.

In contrast to the binding of ryanodine, the parameters describing the interaction of the activating ions with the ryanodine receptor could only be derived indirectly from their effects on the rate of ryanodine binding.

We assumed that the salts interact as a chemical entity in the activation process and evaluated the data supposing cooperative binding. Thus, two nearly identical sequences, following closely the Hofmeister series, were obtained for the K_d values of the chlorides $Na^+ > K^+ > Cs^+ > Tris^+ > NH_4^+$ and of the nitrates $Na^+ > K^+ > Cs^+ > Li^+ \sim NH_4^+ > Tris^+$, respectively. Li^+ which is missing in the chloride series supports ryanodine binding so weakly that binding rates cannot reliably be measured *cf.* (Meissner et al., 1997). The nitrate component tends to diminish the K_d values most markedly for sodium nitrate. For all applied salts the description of the activation profiles requires to assume cooperative binding. The Hill coefficients were found in all cases greater than one. Computation was performed under the assumption that the salts were bound as chemical entities. Hill-coefficients near two or even higher support the concept that the binding of both cat – and anions is essential for ryanodine binding, since the binding of two reactants mostly yields S-shaped activation profiles. The question remains undecided whether we are dealing with simultaneous or sequential binding. High Hill coefficients also might result from salt-induced dissociation of the ryanodine receptor complex — of several (four) elementary units — as a prerequisite for ryanodine binding (Anderson et al., 1989; Hasselbach & Migala, 1992; Meissner, 1994; Rademacher et al., 1994; Orlova et al., 1997). This notion has to take into account that the values of all Hill coefficients are reduced to unity in the presence of ATP or AMP. The decline of the Hill values to near one, in the presence of ATP, correspond to a decrease of the K_d value for NaCl and a considerable increase for KCl and KNO_3. When the K_d values found for the noncooperative receptor were potentiated by the Hill coefficients found for the cooperative complex its K_d values listed in Table 1 were obtained. This reciprocal relationship strongly supports the idea that ATP and the salts modify the cooperative behavior of the ryanodine receptor. The effect of the salts on the channel's cooperative properties might be related to their property to interfere with the protein's hydration shell according to their charge density following the Hofmeister series for anions and cations. By this mechanism, the Hofmeister ions not only affect proteins but also DNA and lipids (*cf.* Yu & Inesi, 1993; Baldwin, 1996; Collins, 1997). An interference of the salts with the calcium channel's hydration shell is also supported by the observed, strong modifying effect of sucrose as well as of other polyols which are known to unspecifically affect the interaction of water with protein surfaces. (Murayama, Kurebayashi & Ogawa, 1998). The shifts in the affinities of the activating and inhibiting calcium binding sites by anions and cations reported by Meissner et al. (1997) and Liu et al. (1998) also follow the Hofmeister series. Yet, it remains undecided if these effects are mediated by global hydration changes of the protein alone or if a more specific competition for the protein's ion binding sites takes place. The latter notion gains some support from the observations that activation by chloride is pronouncedly inhibited by anions like Pipes or Mes and activation by choline is suppressed by potassium.

Reaction Rates of Ryanodine Binding and Calcium Release

The second order rate constants that we have determined in this study under various conditions arise from complex interactions of ryanodine with the receptor in its membranous surrounding. An essential prerequisite for the evaluation of the present results was our previous observation demonstrating that the second order rate constant (k_{+1}) remained invariant in the presence of 0.6 M KCl when the ryanodine concentrations were varied (Hasselbach & Migala, 1992). When k_{+1} was determined from the plot of the observed rate constant *vs.* the applied ryanodine concentrations (0.2–1.0 μM) the intersection of the straight line with the ordinate giving k_{+1} could just be detected ($<10^{-4} \cdot sec^{-1}$). It can be seen

more clearly when the rates are determined between 3 and 20 nM. The independence of the second order rate constant on the concentration of ryanodine could be reconfirmed. The maximal second order rate constants for ryanodine binding activated by the different monovalent salts at saturating concentrations cover a quite large range from 10,000–80,000 M^{-1} sec^{-1}. It is evident from the data collected in Table 1 that the activating effect of nitrate is superior to the effect of chloride irrespective of the accompanying cation. The difference of the activating effect between nitrate and chloride becomes most prominent if they were used in combination with NH_4^+, $Tris^+$ and Li^+. With chloride as anion, ryanodine binding reached significant rates only with $Tris^+$. Nitrate, on the other hand, promoted ryanodine binding appreciably even when combined with lithium which, together with chloride, did not measurably support activation. The cations of the first group metals Na^+, $K,^+$ Cs^+ cannot be arranged in a detailed order of effectiveness in combination either with chloride or with nitrate, when the velocity of the binding reaction approaches saturation at high salt concentrations. This apparently contradicts the effect of these cationic activators at nonsaturating concentrations where their effectiveness follows the Hofmeister series. The graduation results from the combined effect of the differing dissociation constants and the Hill coefficients. In contrast to Na^+, K^+, and Cs^+, the weak cationic activators $Tris^+$, NH_4^+ and Li^+ exhibit a clear order of decreasing maximum reaction rates in combination with chloride as well as nitrate.

Our finding concerning the maximum second order rate constants of 40–80,000 M^{-1} sec^{-1} for the activation of ryanodine binding by the group 1a metal ions chlorides and nitrates needs additional comments. The low value of the maximal rate constant which we found when binding was supported by KCl appears difficult to comprehend. Two effects might contribute to this limitation. The solubility of KCl confines the applicable concentrations to 3 M while the effects of NaCl and CsCl can be studied up to 4 M. Furthermore, one has to consider that various ionic effects pass through distinct optima followed by a decline in activity at further rising concentrations. With CsCl as activators such an effect has been observed to occur above 3 M that led to a steep decline from 58,000 to 7,000 M^{-1} sec^{-1}. Hence, we must consider if the reaction we are dealing with displays true saturation or approaches and passes through an optimum. The alternative does not necessarily require different reaction mechanisms of ion-protein interaction. The simpler assumptions, used to describe saturation kinetics, can be extended by assuming that higher salt concentrations might effect inhibition (*cf.* Meissner et al., 1997). If the binding constant for inhibition is sufficiently distant from that for activation, the maximum reaction rate is not affected by the inhibiting mechanism. This applies evidently to the reaction profiles for activation by CsCl and perhaps to a lesser extent to KCl. Similar considerations might apply to NH_4Cl, NH_4NO_3, $TrisNO_3$ and $LiNO_3$ (Table 1). Inhibition mechanisms can be disregarded for the activation by NaCl displaying only slight saturation tendencies above 3 M. A further general question concerns the maximal second order rate constant which limits the rate by which ryanodine can be bound to its receptor. it is at least by a factor of 1,000 smaller than the theoretical rate obtained for diffusion controlled binding reactions. This suggests the formation of an initial complex in agreement with the observation that no indication of saturation for the rate of ryanodine binding could be observed when the rate of binding was measured up to 10 μM ryanodine. The second class of low affinity binding sites which saturates above 1 μM in the heavy membrane fraction, resulting in channel closing, might perhaps be identical with the sites that are involved in the formation of the initial complex. Yet, direct titration of these sites proved difficult because of the presence of quite a large number of unspecific sites. As an alternative explanation for the extended concentration range in which the rate of ryanodine binding increases Chu et al. (1990) had proposed that ryanodine has to diffuse to relatively inaccessible binding sites. In this case one had to assume that these sites should get more accessible in the presence of high salt concentrations.

The quite good linear correlation between the rate of ryanodine binding to native heavy vesicles and the rate of calcium release (Fig. 11) both measured under the same ionic conditions must be considered as the most direct support for the concept that ryanodine only reacts with the channel in its open configuration (*cf.* Ma et al., 1993). On the other hand, this finding is a strong argument against the idea that channels other than the ryanodine receptor may control calcium permeability in the sarcoplasmic reticulum and that the effect of cationic components can largely by disregarded (Sukhareva et al., 1994). The respective salts effect opening of the channel allowing calcium efflux and concomitantly ryanodine binding. On the other hand, when high salt concentrations (4 M CsCl) suppress calcium efflux ryanodine binding is strongly inhibited. The maximal rate constants for salt-induced calcium release come near to the values reported for calcium release optimally activated by calcium, ATP and caffeine (Meissner, 1994; Sumbilla & Inesi, 1987; Hasselbach & Migala, 1992). The close correspondence between salt-induced calcium efflux and ryanodine binding is of great interest but needs further exploration especially with respect to the question of how channel properties observed in black lipid preparations are related to the observed ion effects.

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