

Divalent Cation Conduction in the Ryanodine Receptor Channel of Sheep Cardiac Muscle Sarcoplasmic Reticulum

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ABSTRACT The conduction properties of the alkaline earth divalent cations were determined in the purified sheep cardiac sarcoplasmic reticulum ryanodine receptor channel after reconstitution into planar phospholipid bilayers. Under bi-ionic conditions there was little difference in permeability among Ba^{2+} , Ca^{2+} , Sr^{2+} , and Mg^{2+} . However, there was a significant difference between the divalent cations and K^+ , with the divalent cations between 5.8- and 6.7-fold more permeant. Single-channel conductances were determined under symmetrical ionic conditions with 210 mM Ba^{2+} and Sr^{2+} and from the single-channel current–voltage relationship under bi-ionic conditions with 210 mM divalent cations and 210 mM K^+ . Single-channel conductance ranged from 202 pS for Ba^{2+} to 89 pS for Mg^{2+} and fell in the sequence $\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$. Near-maximal single-channel conductance is observed at concentrations as low as 2 mM Ba^{2+} . Single-channel conductance and current measurements in mixtures of Ba^{2+} - Mg^{2+} and Ba^{2+} - Ca^{2+} reveal no anomalous behavior as the mole fraction of the ions is varied. The Ca^{2+} - K^+ reversal potential determined under bi-ionic conditions was independent of the absolute value of the ion concentrations. The data are compatible with the ryanodine receptor channel acting as a high conductance channel displaying moderate discrimination between divalent and monovalent cations. The channel behaves as though ion translocation occurs in single file with at most one ion able to occupy the conduction pathway at a time.

INTRODUCTION

Calcium release from the sarcoplasmic reticulum (SR) plays a central role in excitation–contraction coupling in cardiac (Wier, 1990) and skeletal (Rios and Pizarro, 1991) muscle. Release occurs through ion channels located in the specialized junctional regions of the SR found in close apposition to the sarcolemma. Both the skeletal (Smith, Coronado, and Meissner, 1985) and cardiac (Rousseau, Smith, Henderson, and Meissner, 1986) Ca^{2+} release channels have been studied under voltage clamp conditions after the fusion of isolated junctional SR vesicles with planar

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phospholipid bilayers. These studies have yielded considerable information concerning the mechanisms of channel gating and its modulation by a variety of physiological (Smith, Coronado, and Meissner, 1986; Smith, Rousseau, and Meissner, 1989; Ashley and Williams, 1990) and pharmacological factors (Rousseau, Smith, and Meissner, 1987; Rousseau, LaDine, Liu, and Meissner, 1988; Holmberg and Williams, 1990, 1991; Sitsapesan and Williams, 1990; Williams and Holmberg, 1990).

The presence of K^+ (Miller and Racker, 1976; Tomlins, Williams, and Montgomery, 1984) and Cl^- channels (Rousseau, Roberson, and Meissner, 1988; Holmberg and Williams, 1989) in native SR membrane vesicles, which incorporate into bilayers along with the Ca^{2+} release channels, restricts the ionic conditions under which the Ca^{2+} release channel can be examined. As a result, only limited studies of ionic selectivity and conduction are possible using this approach.

These limitations have been overcome by the purification of the SR Ca^{2+} release channel protein and its consequent separation from other SR channel species. Purification has been aided by the specific, high affinity interaction of ryanodine with the SR Ca^{2+} release channel (Inui, Saito, and Fleischer, 1987*a, b*; Lai, Erickson, Rousseau, Liu, and Meissner, 1988*a, b*; Smith, Imagawa, Ma, Fill, Campbell, and Coronado, 1988; Anderson, Lai, Liu, Rousseau, Erickson, and Meissner, 1989). The purified ryanodine receptors of skeletal (Smith et al., 1988; Lai et al., 1988*b*) and cardiac (Lai et al., 1988*a*; Anderson et al., 1989) muscle function as ligand-regulated, cation-selective channels displaying a range of gating and conduction properties characteristic of the native SR Ca^{2+} release channel. The purification protocol developed in our laboratory (Lindsay and Williams, 1991) yields a ligand-regulated ion channel with a single conductance level after reconstitution into planar phospholipid bilayers. An earlier examination of monovalent cation conduction and selectivity properties led us to propose that the sheep cardiac SR receptor channel functions as a single-ion pore (Lindsay, Manning, and Williams, 1991). In this report we examine the conduction and selectivity properties of the receptor channel for the alkaline earth divalent cations. Our findings form the basis for the description of a possible overall mechanism for ion conduction and discrimination in the sheep cardiac SR ryanodine receptor channel.

METHODS

Materials

Phosphatidylethanolamine was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and phosphatidylcholine from Sigma Ltd. (Dorset, UK). 3H Ryanodine was obtained from New England Nuclear (Boston, MA). Aqueous counting scintillant was purchased from Amersham International (Amersham, UK). All other chemicals of AnalaR grade or better were obtained from Aldrich Chemical Co. (Milwaukee, WI), BDH Ltd. (Essex, UK), or Sigma Ltd.

Preparation of Sheep Heavy Sarcoplasmic Reticulum Membrane Vesicles

The sheep hearts were transported from a local abattoir in ice-cold cardioplegic solution (Tomlins, Harding, Kirby, Poole-Wilson, and Williams, 1986). Homogenization of the septal and left ventricular muscle, followed by differential centrifugation, yielded a mixed membrane fraction. Further fractionation using discontinuous sucrose density gradient centrifugation

(Sitsapesan and Williams, 1990; Sitsapesan, Montgomery, MacLeod, and Williams, 1991) gave a sheep heavy sarcoplasmic reticulum (HSR) membrane vesicle fraction collecting at the 30/40% (wt/vol) interface. This fraction was resuspended in 0.4 M KCl and sedimented at 100,000 g_{av} for 1 h. The resulting pellet was resuspended in a solution containing 0.4 M sucrose, 5 mM HEPES titrated to pH 7.2 with Tris and snap-frozen in liquid nitrogen for storage overnight at -80°C .

Solubilization and Separation of the Ryanodine Receptor

The solubilization of the ryanodine receptor by the zwitterionic detergent 3-((3-cholamidopropyl)-diethylammonio)-1-propane sulfonate (CHAPS) was carried out as previously reported (Lindsay and Williams, 1991). The stored HSR vesicles were resuspended in 1 M NaCl, 0.1 mM EGTA, 0.15 mM CaCl_2 , and 25 mM PIPES-NaOH to pH 7.4 at a protein concentration of 1.5–2 mg protein/ml in the presence of 0.5% (wt/vol) CHAPS and 2.5 mg/ml L- α -phosphatidylcholine (PC). After 1 h of incubation on ice, unsolubilized material was sedimented by centrifugation for 45 min at 100,000 g_{av} .

Separation of the ryanodine binding protein from other solubilized membrane components was performed by sedimentation on 5–25% (wt/vol) continuous linear sucrose density gradients (overnight at 100,000 g_{av}). Gradient fractions were collected from the base of the tube and those containing the receptor were identified by comparison with an identical gradient whose membrane components were incubated in the presence of ^3H ryanodine during the solubilization period. The solubilized receptor was reconstituted into unilamellar liposomes as previously described (Lindsay and Williams, 1991) for incorporation into planar phospholipid bilayers.

Planar Lipid Bilayer Methods

Lipid bilayers, formed from suspensions of phosphatidylethanolamine in decane (35 mg/ml), were painted across a 200- μm -diam hole in a polystyrene partition which separated two chambers referred to as the *cis* (volume 0.5 ml) and *trans* (volume 1.5 ml) chambers. The *trans* chamber was held at virtual ground while the *cis* chamber could be clamped at various holding potentials relative to ground. Current flow across the bilayer was measured using an operational amplifier as a current-voltage converter as described by Miller (1982). Bilayers were usually formed in solutions of 200 mM KCl, 20 mM HEPES, and KOH to pH 7.4 resulting in a solution of 210 mM K^+ . An osmotic gradient was established by the addition of 50–100 μl of 3 M KCl to the *cis* chamber. Proteoliposomes were added to the *cis* chamber and stirred. To induce fusion of the vesicles with the bilayer a second small aliquot (50–100 μl) of 3 M KCl was added to the *cis* chamber. After incorporation, further fusion was prevented by perfusion of the *cis* chamber with 210 mM KCl or the desired test solution. Solutions contained 10 μM contaminant free Ca^{2+} which was sufficient for channel activation. Experiments were carried out at room temperature ($21 \pm 2^{\circ}\text{C}$).

All solutions were prepared with deionized water and subsequently filtered through Millipore HA 0.45- μm pore size filters. The chloride salts of both monovalent and divalent cations were used, buffered with 20 and 40 mM HEPES, respectively. To raise the pH of these solutions to 7.4 it was necessary to add 10 mM of the appropriate hydroxide. The only exception was in the preparation of low concentration Ba^{2+} solutions, which were prepared as follows: 2 mM Ba^{2+} (1.6 mM BaCl_2 , 0.4 mM $\text{Ba}(\text{OH})_2$, and 1 mM HEPES to pH 7.4 with concentrated HCl), 5 mM Ba^{2+} (4.0 mM BaCl_2 , 1.0 mM $\text{Ba}(\text{OH})_2$, and 2.5 mM HEPES to pH 7.4 with concentrated HCl), 10 mM Ba^{2+} (8.0 mM BaCl_2 , 2.0 mM $\text{Ba}(\text{OH})_2$, and 5 mM HEPES to pH 7.4 with concentrated HCl), and 50 mM Ba^{2+} (40 mM BaCl_2 , 10 mM $\text{Ba}(\text{OH})_2$, and 10 mM HEPES to pH 7.4 with concentrated HCl). The experimental method used to investigate conductance in these solutions was as follows. The channels were incorporated into the bilayer in 210 mM K^+ and

further fusion was prevented by perfusion of the *cis* chamber with 210 mM K⁺. To study the intermediate Ba²⁺ concentrations (10 and 50 mM) the *cis* and *trans* chambers were perfused for 2 and 5 min, respectively (~15–20 changes of solution), with constant stirring of both chambers. To study the low concentrations, an intermediate concentration was established as above and the *cis* and *trans* were then once again perfused with the low concentration solutions. Ionic activities of the various solutions were calculated with activity coefficients obtained for Ba²⁺ and Ca²⁺ from Scatchard and Theft (1930) and for K⁺ from Hamer and Wu (1972) as given in Lobo (1989).

Single-Channel Data Acquisition and Analysis

Single-channel current fluctuations were displayed on an oscilloscope and stored on videotape. When analyzed, data were filtered using an 8-pole Bessel filter at 1.0 kHz and digitized at 4.0 kHz using an AT-based system (Intracel, Cambridge, UK). Single-channel current amplitudes were measured from digitized data using manually controlled cursors. The representative traces shown in the figures were displayed on a Hewlett Packard 7475A plotter after digitization using a PDP 11/73-based system (Indec Systems, Inc., Sunnyvale, CA).

Calculation of Permeability Ratios

The zero-current reversal potentials (E_{rev}) were obtained under bi-ionic conditions and represented the point at which no current fluctuation was visible. It was possible to determine the reversal potential to within ± 2 mV.

If ions X and Y are both either monovalent or divalent the permeability ratio (P_X/P_Y) is related to the reversal potential by the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$P_X P_Y = [Y^+]/[X^+] \exp(E_{rev}F/RT) \quad (1)$$

However, if ion Y is monovalent and ion X is divalent, a more complex expression results. We use the equation given by Fatt and Ginsborg (1958):

$$P_X P_Y = [Y^+]/4 \cdot [X^{2+}] \cdot \exp(E_{rev}F/RT) \cdot [\exp(E_{rev}F/RT) + 1] \quad (2)$$

where ion X is present in the *trans* chamber and ion Y in the *cis* chamber. R , T , and F have their usual meanings. The value of RT/F used was 25.2 mV at 20°C. $[Y^+]$, $[X^+]$, and $[X^{2+}]$ refer to the concentration of the ions and all permeability ratios given are calculated on this basis. Eq. 2 is a simplification of the much more general expression derived by Attwell and Jack (1978).

RESULTS

The purified sheep cardiac muscle SR ryanodine receptor functions as a ligand-regulated, cation-selective ion channel when reconstituted into planar phospholipid bilayers (Lindsay and Williams, 1991; Lindsay et al., 1991).

Divalent Cation Permeability

Channels were incorporated into planar phospholipid bilayers as described in Methods. After an initial fusion event the *cis* chamber was perfused with either 210 mM K⁺ or 210 mM Ba²⁺. The *trans* chamber was subsequently perfused with a 210-mM concentration of the divalent cation under investigation. Under these two sets of bi-ionic conditions it was possible to obtain single-channel current reversal potentials for K⁺ against the alkaline earth divalent cations and for Ba²⁺ against the

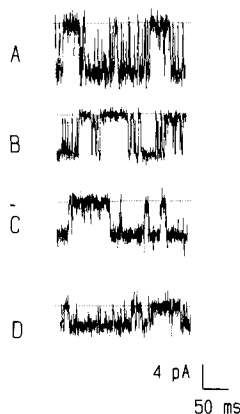


FIGURE 1. Representative single-channel current fluctuations at 0 mV holding potential with 210 mM K^+ in the *cis* chamber and 210 mM Ba^{2+} (A), 210 mM Sr^{2+} (B), 210 mM Ca^{2+} (C), and 210 mM Mg^{2+} (D), respectively, in the *trans* chamber. Open channel current levels are indicated by the dotted lines.

other divalent cations in the group. The zero-current potentials were corrected for junction potentials arising between the two solutions which were in the range of ± 5 mV. Representative single-channel traces are shown in Fig. 1 and current-voltage relationships are shown in Fig. 2. The permeability data are summarized in Table I. There is little permeability difference between the alkaline earth divalent cations, with perhaps Ba^{2+} marginally less permeable than Ca^{2+} , Mg^{2+} , and Sr^{2+} . However, there is a significant permeability difference between K^+ and the divalents as a group with $P_{X^{2+}}/P_{K^+}$ in the range of 5.8–6.7.

In addition, it is possible to calculate reversal potentials with 210 mM Ba^{2+} or Sr^{2+} in the *cis* chamber and 210 mM K^+ in the *trans* chamber. Under such conditions the reversal potentials (corrected for junctional potentials) are -38.5 ± 1.0 mV (\pm SD, $n = 3$) and -40.0 ± 1.0 mV (\pm SD, $n = 3$) for Ba^{2+} and Sr^{2+} vs. K^+ , respectively. These values agree closely with those determined with the monovalent cation in the *cis* chamber and the divalent cation in the *trans* chamber (Table I).

Divalent Cation Conductance

The conductance of Ba^{2+} and Sr^{2+} was studied in the ryanodine receptor channel under symmetrical conditions. With 210 mM Ba^{2+} or 210 mM Sr^{2+} in both *cis* and *trans* chambers the single-channel current-voltage relationships were symmetrical between -100 and 100 mV. There is some departure from ohmic behavior at high negative and positive holding potentials beyond ± 80 mV, but this effect is small (see Fig. 3). The mean single-channel conductance obtained from least-squares linear

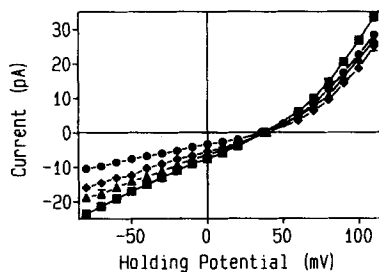


FIGURE 2. Single-channel current-voltage relationships with 210 mM K^+ in the *cis* chamber and 210 mM Ba^{2+} (■), 210 mM Sr^{2+} (▲), 210 mM Ca^{2+} (◆), and 210 mM Mg^{2+} (●) in the *trans* chamber. The individual points are the means of $n \geq 4$ bilayers and the SEM is included within the symbol or indicated by error bars if it is larger.

TABLE I
Summary of the Permeability Properties of the Divalent Cations

Ion	<i>n</i>	E_{rev} vs. K^+	$P_{\text{X}^{2+}}/P_{\text{K}^+}$	<i>n</i>	E_{rev} vs. Ba^{2+}	$P_{\text{X}^{2+}}/P_{\text{Ba}^{2+}}$
		mV			mV	
Ba^{2+}	7	37.1 ± 0.5	5.8	—	—	—
Sr^{2+}	5	39.0 ± 1.2	6.7	4	2.0 ± 0.4	1.1
Ca^{2+}	4	38.5 ± 0.6	6.5	4	1.5 ± 0.6	1.1
Mg^{2+}	4	37.3 ± 0.4	5.9	4	2.0	1.1

regression was 199 ± 7 pS (\pm SEM, $n = 5$) for Ba^{2+} and 183 ± 5 pS (\pm SEM, $n = 4$) for Sr^{2+} . Current–voltage relationships are shown in Fig. 3.

Channels incorporate into the phospholipid bilayers in a fixed orientation (Lindsay and Williams, 1991; Tinker, Lindsay, and Williams, 1992a) with the cytosolic side of the channel facing the *cis* chamber. The addition or substitution of millimolar concentrations of Mg^{2+} or high concentrations of Ca^{2+} (> 10 mM) into the *cis* chamber led to a decrease in single-channel open probability (P_o). This is comparable to observations in the native channel (Ashley and Williams, 1990; Sitsapesan, Boraso, and Williams, 1991). However, it is possible to obtain estimates of the single-channel conductance with these cations from experiments carried out under bi-ionic conditions with 210 mM divalent cation in the *trans* chamber and 210 mM K^+ in the *cis* chamber. The limb of negative current past the reversal potential will represent increasingly pure divalent current as the holding potential is made increasingly negative relative to the zero-current potential (Yue and Marban, 1990). The single-channel current–voltage relationship is largely ohmic in this region. Least-squares linear regression was performed on the single-channel current–voltage relationship between 0 and -80 mV (approximately -40 to -120 mV negative to the reversal potential) to obtain an estimate of single-channel conductance. The single-channel conductance ranged from 202 ± 3 pS (\pm SEM, $n = 7$) for Ba^{2+} to 89 ± 4 pS (\pm SEM, $n = 4$) for Mg^{2+} and fell in the sequence $\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$. The conduction data are summarized in Table II. The good agreement in values between single-channel conductances obtained for Ba^{2+} and Sr^{2+} under either symmetrical or bi-ionic conditions supports the contention that the conductance values obtained for divalent cations under bi-ionic conditions are valid.

The Behavior of Single-Channel Conductance with Varying Ba^{2+} Activity

Single-channel current–voltage relationships were measured in a series of symmetrical Ba^{2+} concentrations ranging from 2 to 210 mM. The details of the solutions used

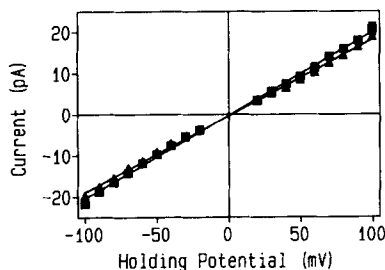


FIGURE 3. Single-channel current–voltage relationships in symmetrical 210 mM Ba^{2+} (■) and 210 mM Sr^{2+} (▲). The individual points are the means of $n \geq 4$ bilayers and the SEM is included within the symbol or indicated by error bars if it is larger.

TABLE II
Summary of Divalent Cation Conductance Properties

Ion	<i>n</i>	Conductance (bi-ionic)		Conductance (symmetrical)	
		<i>pS</i>		<i>pS</i>	
Ba ²⁺	7	202 ± 5	5	199 ± 7	
Sr ²⁺	5	166 ± 4	4	183 ± 5	
Ca ²⁺	4	135 ± 5	—	—	
Mg ²⁺	4	89 ± 4	—	—	

and experimental technique are given in Methods. Between 2 and 210 mM Ba²⁺ the single-channel conductance is relatively independent of concentration. For example, the conductance in symmetrical 2 mM Ba²⁺ is 155 ± 3 pS (*n* = 4, ±SEM), ~75% of the value obtained at 210 mM Ba²⁺. Representative single-channel traces are shown in Fig. 4.

We have fitted the conductance (the mean of four or more experiments) at five such concentrations to a Michaelis-Menten-type saturation curve so that

$$G = G_{\max}/(1 + K_D/[Ba^{2+}]) \quad (3)$$

where *G* is the single-channel conductance, *G*_{max} is the maximum conductance, and *K*_D is the activity at which half-maximal conductance occurs. To obtain these parameters, the data were transformed to an Eadie-Hofstee plot (data not shown). Such analysis gives a *K*_D of 406 μM and a *G*_{max} of 185 pS. We were unable to perform experiments at activities <1.7 mM due to a decline in single-channel *P*_o. At these activities *P*_o was unresponsive to the addition of secondary agonists such as caffeine. The absence of data points around the *K*_D means that the figure obtained ought to be viewed as an estimate and an upper limit for the point at which half-maximal conductance occurs.

Mole Fraction Dependence of Single-Channel Current and Conductance

A widely accepted method for assessing the degree of ion occupancy of a species of channel involves monitoring ion conduction with a varying permeant ion mole fraction (Hille and Schwarz, 1978; Eisenman, Latorre, and Miller, 1986). The ideal approach is to measure conductance in symmetrical solutions of varying mixtures of two conducting ions at a constant total ion concentration. In single-ion channels conductance or current at a particular voltage should vary monotonically with mole fraction. The presence of conductance minima or maxima with a varying mole fraction indicates multi-ion occupancy. Due to the reduced *P*_o values resulting from

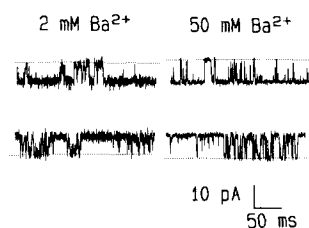


FIGURE 4. Representative single-channel current fluctuations at 60 mV (lower panel) and -60 mV (upper panel) holding potential in symmetrical Ba²⁺ concentrations as indicated in the figure. Open channel current levels are indicated by the dotted lines.

high concentrations of Ca^{2+} and Mg^{2+} at the cytosolic side of the receptor channel, it is not possible to examine symmetrical mixtures that include either of these ions.

An alternative approach was described by Yue and Marban (1990), who justified the use of part of the current–voltage relationship obtained with a mixture of divalent cations on one side of the membrane and a monovalent cation on the other, to perform mole fraction experiments on the L-type Ca^{2+} channel. We have examined the behavior of single-channel conductance in experiments of this type with mixtures of Ba^{2+} and Mg^{2+} and of Ba^{2+} and Ca^{2+} in the *trans* chamber with a constant total concentration of 210 mM. 210 mM K^{+} was present in the *cis* chamber throughout. Single-channel conductance was determined from least-squares linear regression of the points in the current–voltage relationship between -80 and 0 mV (-120 to -40 mV relative to the reversal potential). The relationship is illustrated in Fig. 5 *A* for

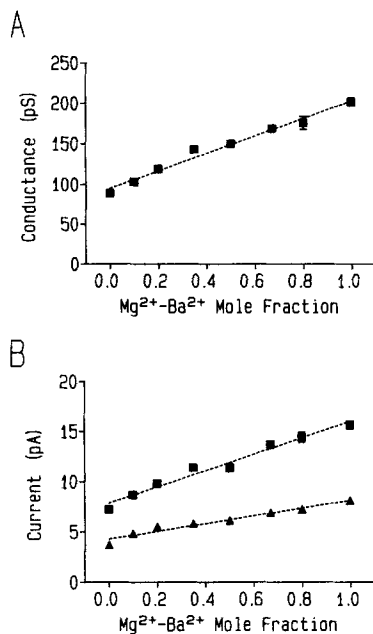


FIGURE 5. (*A*) The behavior of single-channel conductance, calculated as described in the text, with varying Mg^{2+} - Ba^{2+} mole fraction. The individual points are the means of $n \geq 4$ bilayers and the SEM is included within the symbol or indicated by error bars if it is larger. (*B*) The behavior of single-channel current at -40 mV (\blacktriangle) and -80 mV (\blacksquare) relative to the reversal potential with varying Mg^{2+} - Ba^{2+} mole fraction. The details of the ionic composition of the solutions in the *cis* and *trans* chambers is given in the text. The individual points are the means of $n \geq 4$ bilayers and the SEM is included within the symbol or indicated by error bars if it is larger.

mixtures of Ba^{2+} and Mg^{2+} and in Fig. 6 *A* for mixtures of Ba^{2+} and Ca^{2+} . It is apparent that there is no significant anomalous mole fraction behavior of single-channel conductance with these two mixtures of divalent cation.

There are two potential problems with using single-channel conductance as an indicator of mole fraction anomalies. The first is that varying degrees of hyperlinearity of the current–voltage relationship may mask mole fraction effects. The current–voltage relationship of the sheep cardiac ryanodine receptor channel, although largely ohmic under the above conditions, does show some deviation from linearity at higher voltages. Consequently, it is appropriate to look at the behavior of current at a fixed voltage relative to the reversal potential. Second, Campbell, Rasmusson, and Strauss (1988) demonstrated in a two-site multi-ion model of the L-type Ca^{2+} channel that in experiments performed as described by Yue and Marban (1990), mole

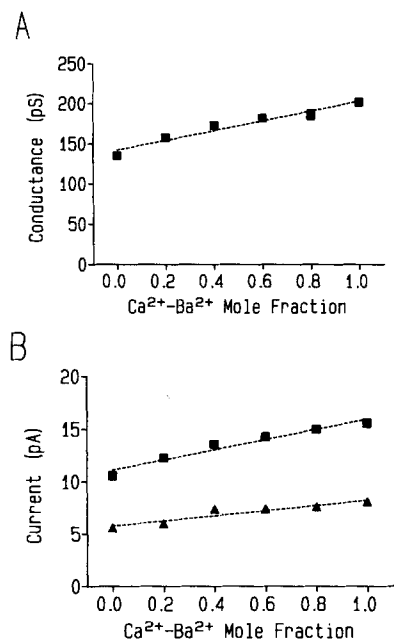


FIGURE 6. (A) The behavior of single-channel conductance, calculated as described in the text, with varying Ca²⁺-Ba²⁺ mole fraction. The individual points are the means of $n \geq 4$ bilayers and the SEM is included within the symbol or indicated by error bars if it is larger. (B) The behavior of single-channel current at -40 mV (▲) and -80 mV (■) relative to the reversal potential with varying Ca²⁺-Ba²⁺ mole fraction. The details of the ionic composition of the solutions in the *cis* and *trans* chambers is given in the text. The individual points are the means of $n \geq 4$ bilayers and the SEM is included within the symbol.

fraction anomalies may only be apparent at relatively low hyperpolarized potentials. Given these arguments, we measured the single-channel current (i.e., at ~ 0 and -40 mV holding potential) in mixtures of divalent cations (Ba²⁺-Mg²⁺ and Ba²⁺-Ca²⁺ in the *trans* chamber with a constant total concentration of 210 mM, and 210 mM K⁺ in the *cis* chamber). The resulting behavior of single-channel current with a varying mole fraction is illustrated in Fig. 5 B for mixtures of Ba²⁺ and Mg²⁺ and in Fig. 6 B for mixtures of Ba²⁺ and Ca²⁺. Once again there is no significant anomalous mole fraction effect with these two mixtures of ions.

In addition, the reversal potential measured under the various conditions described above varied little, ranging from 35 to 40 mV (data not shown). In some multi-ion occupancy models the reversal potential may show minima or maxima with varying mole fraction (Hille and Schwarz, 1978).

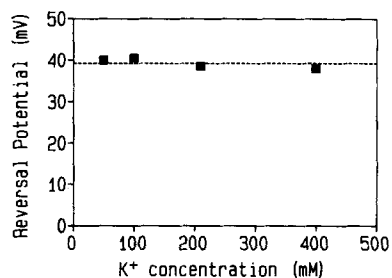


FIGURE 7. The concentration dependence of the K⁺-Ca²⁺ reversal potential. The individual points are the means of $n \geq 4$ bilayers and the SEM is included within the symbol. The solid line is drawn on the assumption that the reversal potential is concentration independent and is 39 mV. The experimental details are given in the text.

Concentration Dependence of the Reversal Potential

Further information concerning the degree of ion occupancy of the channel may be obtained from the determination of the reversal potential as the concentration of two permeant ions is varied. In single-ion models, under bi-ionic conditions there will be no change in the zero-current potential and thus no change in the permeability ratio if the ratio of the activities of the two permeant ions is kept constant as the total activity is varied (Lauger, 1973).¹ In contrast, if the reversal potential changes under these conditions, then this can only be reproduced by multi-ion models. Fig. 7 shows the effect on the reversal potential as the concentration of K^+ in the *cis* chamber is increased from 50 to 400 mM while matching this with the appropriate *trans* Ca^{2+} concentration so as to maintain the ratio of activities constant. The reversal potential remains close to 40 mV throughout, and within our experimental limits this behavior is compatible with single-ion occupancy.

DISCUSSION

The aim of this study was to characterize divalent cation conduction in the sheep cardiac muscle SR ryanodine receptor channel and to use this information in the development of a description of possible mechanisms of ion selectivity and translocation.

Ionic Selectivity in the Ryanodine Receptor Channel

Ionic selectivity in membrane channels is generally assessed by determining three major experimental parameters: permeability ratios, conductance ratios, and the ratios of binding affinities. The interrelationship of these factors is closely linked to the basic nature of ionic conduction in the channel protein.

The ryanodine receptor channel displays significant permeability differences between K^+ and the alkaline earth divalent cations. The $P_{X^{2+}}/P_{K^+}$ is ~ 6.0 when measured under bi-ionic conditions with 210 mM concentrations of the respective ions. However, there is little difference in permeability among the individual divalent cations, or for that matter among the group Ia monovalent cations (Lindsay et al., 1991; Williams, 1992). The permeability apparatus appears to be able to select divalent over monovalent cations but does not distinguish significantly between individuals within these groups. Mg^{2+} has a particularly high dehydration energy when compared with monovalent cations and the other alkaline earths (Hille, 1984).

¹ Lauger (1973) only analyzes monovalent interactions. However, it is possible to modify his Eqs. 40–43 to deal with current rather than flux (current = flux·valency·Faraday constant). Under bi-ionic conditions the reversal potential occurs when the net current, carried by monovalents in one direction and divalents in the other, across the membrane is zero. Thus, rewriting his Eq. 43 changes it in two ways. It introduces a factor of two into the expression for the divalent cation due to valency considerations. Second, it changes the absolute value of S_v if the ion is a divalent cation for the same concentration- and voltage-independent energy profile. However, the resulting equation still has the crucial property that it remains unchanged if all concentrations are multiplied by the same factor. In other words, if single-ion occupancy is obeyed for both mono- and divalent cations, the reversal potential determined under bi-ionic conditions ought to be independent of concentration, provided the ratio of activities of the monovalent and divalent ions are kept constant.

The fact that Mg^{2+} displays a similar permeability to the other divalents under comparable conditions suggests that ion dehydration does not play a significant role in the permeability process.

Despite there being only minor differences in permeability among the divalents, there are differences in conductance. The receptor channel displays high conductance with the four divalents studied, including Mg^{2+} . The conductance falls in the sequence $\text{Ba}^{2+} > \text{Sr}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$ and this corresponds to the order of free solution mobilities of these ions. It is also one of the seven sequences (I), determined by Sherry (1969), using equilibrium thermodynamic principles similar to those developed by Eisenman, Rudin, and Casby (1957) to describe ion exchange sequences for monovalent cations.

The receptor channel displays a high affinity for Ba^{2+} and, by inference, the other alkaline earth divalent cations. A figure of $\sim 400 \mu\text{M}$ was obtained for the K_D of Ba^{2+} . This figure should be interpreted with caution as even at a Ba^{2+} activity of 1.7 mM the conductance is $\sim 75\%$ of maximal conductance and it seems probable that this K_D should be viewed as an upper estimate. Given such high affinity for Ba^{2+} and probably the other alkaline earth divalent cations, it would be difficult to distinguish experimentally any differences in K_D between these ions on the basis of the conductance–activity relationship. Are there any other experimental indicators to possible differences? Any apparent curvature in the mole fraction relationship may give a clue (Hille, 1984). In the experiments described here a straight line was fitted to the data on the assumption that the affinities of the studied divalent ions were similar. Any other fit would probably not be justified for these data. A second pointer is the increase in single-channel current at holding potentials positive to the K^+ -divalent reversal potential (K^+ in the *cis* chamber and divalent in the *trans* chamber). In experiments of this kind, those involving divalents with a higher affinity ought to have a smaller current at a given potential relative to the reversal potential, reflecting divalent cation block of predominantly monovalent current. It does appear as if Ba^{2+} has a larger current than the other divalents. Further distinction between the remaining divalents is probably not justified based on the data presented in Fig. 2.

The Nature of Ionic Conduction in the Ryanodine Receptor Channel

The “fingerprint” of ionic conduction in the ryanodine receptor channel differs considerably from that described for other well-characterized channels which allow the passage of both mono- and divalent cations, for example, the L-type Ca^{2+} channel and the endplate channel (Williams, 1992).

As in these channels, ionic conduction in the ryanodine receptor channel clearly does not follow the independence principle. The saturation of single-channel current with increasing divalent and monovalent cation activity, the large differences in single-channel conductance despite only small differences in permeability (for both group Ia cations and the alkaline earth divalent cations), and the blockade of K^+ conduction by small tetraalkyl ammonium cations (Tinker et al., 1992a) all argue for the existence of one or more saturable binding sites within the conduction pathway.

Having established that ionic conduction behaves as if occurring in single file, it is important to define the degree of ion occupancy of the channel, as this will have a

major influence on the interpretation of selectivity data. Models of ionic conduction where more than one ion is able to occupy the conduction pathway (multi-ion channels) can display properties that simple single-occupancy models are unable to reproduce (Hille and Schwarz, 1978). The classification of a channel as single- or multi-ion depends on the presence or absence of these distinguishing characteristics.

One of the most widely accepted lines of evidence supporting multi-ion occupancy of a given species of channel is the presence of an anomalous mole fraction effect. Work from our laboratory has previously demonstrated (Lindsay et al., 1991) that the relationship between single-channel conductance and Na^+/K^+ and Li^+/K^+ mole fraction is monotonic. From the data presented in this paper it is apparent that no anomalous mole fraction effect exists in single-channel conductance or current in mixtures of $\text{Ca}^{2+}/\text{Ba}^{2+}$ and $\text{Mg}^{2+}/\text{Ba}^{2+}$. However, this does not conclusively establish the receptor channel as a single-ion channel. Multi-ion channels may not necessarily display anomalous mole fraction effects (Hille and Schwarz, 1978) or may do so only under conditions that are difficult to measure experimentally (Campbell et al., 1988). Indeed, the single-channel studies of the L-type Ca^{2+} channel carried out by Yue and Marban (1990) revealed no evidence for an anomalous mole fraction effect in mixtures of Ba^{2+} and Ca^{2+} and yet these authors provide other evidence that clearly suggests multiple occupancy of this channel.

A conduction property that can only be explained by multi-ion occupancy models is the existence of concentration-dependent reversal potentials. The ryanodine receptor channel, under bi-ionic conditions, with Ca^{2+} in the *trans* chamber and K^+ in the *cis* chamber displays little variation in reversal potential over an eightfold concentration range.

Additional information on ion occupancy may be gained from the study of channel conductance at a range of ion activities. Single-ion channels ought to display simple saturation. Multi-ion channels may display multiple conductance maxima and minima in symmetrical ionic conditions (Hille and Schwarz, 1978). In agreement with the suggestion that the ryanodine receptor channel behaves as a single-ion channel, the conductance-activity relationships for the monovalent cations (Lindsay et al., 1991) and for Ba^{2+} do not display significant maxima or minima. However, the absence of such phenomena does not necessarily imply single-ion occupancy as multi-ion models can produce simple saturation. Other interpretations of the conductance-activity relationship are conceivable. One possibility is the presence of significant negative surface charge present in the mouth of the receptor channel, which could concentrate ions at low ionic activity and thus elevate conductance (Green and Andersen, 1991). An experimental test of this possibility would involve varying permeant ion concentration while maintaining total ionic strength constant with an inert electrolyte. Unfortunately, all the organic and inorganic cations tested so far either permeate or block the receptor channel. Similarly, we cannot exclude the possibility of some multi-ion occupancy at high divalent cation concentration to explain the slight increase in conductance above the apparent saturating value.

A final piece of evidence in support of our contention that the ryanodine receptor channel functions as a single-ion channel is our earlier demonstration that the smaller tetraalkyl ammonium cations act as voltage-dependent blockers with an effective valence of block of less than one (Lindsay et al., 1991; Tinker et al., 1992a).

Thus there is no direct evidence to suggest multi-ion conduction in the ryanodine receptor channel, and at the present it seems more economical to propose single-ion occupancy with both monovalent and divalent cations. This proposition can be further tested by attempting to model ionic conduction in the receptor channel and this is the subject of the accompanying paper (Tinker, Lindsay, and Williams, 1992b).

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