Inositol (1,4,5)-trisphosphate ($InsP_3$)-gated Ca Channels from Cerebellum: Conduction Properties for Divalent Cations and Regulation by Intraluminal Calcium

ILYA BEZPROZVANNY and BARBARA E. EHRLICH

From the Laboratory of Molecular Hermeneutics, Departments of Medicine and Physiology, University of Connecticut, Farmington, Connecticut 06032-1305

ABSTRACT The conduction properties of inositol (1,4,5)-trisphosphate ($InsP_3$)-gated calcium (Ca) channels ($InsP_3R$) from canine cerebellum for divalent cations and the regulation of the channels by intraluminal Ca were studied using channels reconstituted into planar lipid bilayers. Analysis of single-channel recordings performed with different divalent cations present at 55 mM on the *trans* (intraluminal) side of the membrane revealed that the current amplitude at 0 mV and the single-channel slope conductance fell in the sequence: Ba (2.2 pA, 85 pS) > Sr (2.0 pA, 77 pS) > Ca (1.4 pA, 53 pS) > Mg (1.1 pA, 42 pS). The mean open time of the $InsP_3R$ recorded with Ca (2.9 ms) was significantly shorter than with other divalent cations (~5.5 ms). The "anomalous mole fraction effect" was not observed in mixtures of divalent cations (Mg and Ba), suggesting that these channels are single-ion pores. Measurements of $InsP_3R$ activity at different intraluminal Ca levels demonstrated that Ca in the submillimolar range did not potentiate channel activity, and that very high levels of intraluminal Ca ($\geq$10 mM) decreased channel open probability 5–10-fold. When $InsP_3R$ were measured with Ba as a current carrier in the presence of 110 mM *cis* potassium, a P_{Ba}/P_K of 6.3 was estimated from the extrapolated value for the reversal potential. When the unitary current through the $InsP_3R$ at 0 mV was measured as a function of the permeant ion (Ba) concentration, the half-maximal current occurred at 10 mM *trans* Ba.

The following conclusions are drawn from these data: (*a*) the conduction properties of $InsP_3R$ are similar to the properties of the ryanodine receptor, another intracellular Ca channel, and differ dramatically from the properties of voltage-gated Ca channels of the plasma membrane. (*b*) The estimated size of the Ca current through the $InsP_3R$ under physiological conditions is 0.5 pA, approximately

Dr. Ilya Bezprozvanny's present address is Department of Molecular and Cellular Physiology, Beckman Center, Stanford University Medical Center, Stanford, CA 94305-5426.

Address correspondence to Barbara E. Ehrlich, Division of Cardiology, Laboratory of Molecular Hermeneutics, University of Connecticut, 263 Farmington Avenue, Farmington, CT 06032-1305.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/94/11/0821/36 $2.00 821
Volume 104 November 1994 821–856

four times less than the Ca current through the ryanodine receptor. (*c*) The potentiation of $InsP_3R$ by intraluminal Ca in the submillimolar range remains controversial. (*d*) A quantitative model that explains the inhibitory effects of high *trans* Ca on $InsP_3R$ activity was developed and the kinetic parameters of $InsP_3R$ gating were determined.

INTRODUCTION

Inositol 1,4,5-trisphosphate ($InsP_3$) is a second messenger used by many cell types to stimulate release of intracellular calcium (Ca) from intracellular Ca stores into the cytoplasm (Berridge, 1993; Berridge and Irvine, 1989) due to activation of $InsP_3$-gated Ca channels (Bezprozvanny and Ehrlich, 1993*a;* Bezprozvanny, Watras, and Ehrlich, 1991*a;* Ehrlich and Watras, 1988; Ferris, Huganir, Supattapone, and Snyder, 1989; Maeda, Kawasaki, Nakade, Yokota, Taguchi, Kasai, and Mikoshiba, 1991; Watras, Bezprozvanny, and Ehrlich, 1991). $InsP_3$ receptors ($InsP_3R$) have been purified to homogeneity from several sources (Chadwick, Saito, and Fleischer, 1990; Maeda et al., 1991; Mourey, Verma, Supattapone, and Snyder, 1990; Supattapone, Worley, Baraban, and Snyder, 1988*b*) and multiple isoforms of $InsP_3R$ have been cloned (Blondel, Takeda, Janssen, Seino, and Bell, 1993; Furuichi, Yoshikawa, Miyawaki, Wada, Maeda, and Mikoshiba, 1989; Kume, Muto, Aruga, Nakagawa, Michikawa, Furuichi, Nakade, Okano, and Mikoshiba, 1993; Mignery, Newton, Archer, and Sudhof, 1990; Sudhof, Newton, Archer, Ushkaryov, and Mignery, 1991). Experimental results using Ca release assays and single-channel recordings have indicated that $InsP_3R$ are regulated by a variety of cofactors and cellular processes including cytosolic and intraluminal free Ca levels, phosphorylation of the $InsP_3R$, intracellular pH, and ATP levels (Bezprozvanny and Ehrlich, 1993*a;* Bezprozvanny et al., 1991*a;* Ferris, Cameron, Bredt, Huganir, and Snyder, 1991*a;* Ferris, Huganir, Bredt, Cameron, and Snyder, 1991*b;* Ferris, Huganir, and Snyder, 1990; Finch, Turner, and Goldin, 1991; Iino, 1990, 1991; Missiaen, De Smedt, Droogmans, and Casteels, 1992*a;* Nunn and Taylor, 1992; Supattapone, Danoff, Theibert, Joseph, Steiner, and Snyder, 1988*a;* Volpe and Alderson-Lang, 1990). Despite this wealth of information about $InsP_3R$ structure and regulation, very little is known about the conduction properties of $InsP_3$-gated Ca channels.

On the other hand, the permeability and selectivity properties of another type of intracellular Ca channel, the ryanodine receptor (RyR), was characterized in detail (Anderson, Lai, Liu, Rousseau, Erickson, and Meissner, 1989; Lai, Erickson, Rousseau, Liu, and Meissner, 1988; Lindsay, Manning, and Williams, 1991; Lindsay and Williams, 1991; Liu, Lai, Rousseau, Jones, and Meissner, 1989; Smith, Imagawa, Ma, Fill, Campbell, and Coronado, 1988; Tinker, Lindsay, and Williams, 1992, 1993; Tinker and Williams, 1992; Williams, 1992). In these reports, the activity of purified cardiac and skeletal RyR was recorded in planar lipid bilayers under a variety of ionic conditions and, based on these data, a quantitative model of ion conduction in the RyR was developed (Tinker et al., 1992) using Eyring rate theory (described in Hille [1992]). Tinker et al. (1992) concluded that permeation of divalent cations through the RyR occurs in a single file with, at most, one ion able to occupy the conduction pathway at any time (Tinker et al., 1992; Tinker and Williams, 1992). This result is distinct from a well-established model of ion permeation through voltage-gated Ca

channels of the plasma membrane (Almers and McClesky, 1984; Hess and Tsien, 1984; Tsien, Hess, McCleskey, and Rosenberg, 1987), where binding of at least two Ca ions in the pore is necessary for passage of the Ca current. Is the RyR an exception among Ca channels or do intracellular Ca channels operate in a manner fundamentally different from Ca channels of the plasma membrane? To answer this question and to obtain more information about the permeability and selectivity properties of $InsP_3R$, we studied the conduction of divalent cations through $InsP_3$-gated channels from canine cerebellum. Channels were reconstituted into planar lipid bilayers, as was done previously to characterize $InsP_3R$ regulation by cytosolic Ca (Bezprozvanny et al., 1991*a*) and ATP (Bezprozvanny and Ehrlich, 1993*a*).

In the first part of this report we describe the conduction properties of $InsP_3R$ for divalent cations. In the second part, we examine the controversial topic concerning the regulation of $InsP_3R$ by intraluminal Ca (compare Combettes, Claret, and Champeil [1992] and Shuttleworth [1992] with Missiaen et al. [1992*a*] and Missiaen, De Smedt, Droogmans, and Casteels [1992*b*]). In the last part of the paper we estimate the size of the Ca current through a single $InsP_3$-gated channel expected under physiological conditions.

Preliminary accounts of part of this work have been published in abstract form (Bezprozvanny and Ehrlich, 1993*b;* Bezprozvanny and Ehrlich, 1994).

METHODS

Preparation of Microsomes from Canine Cerebellum

Microsomal vesicles of canine cerebellum were obtained using gradient centrifugation procedures. Endoplasmic reticulum vesicles derived from canine cerebella were made as described previously (Watras et al., 1991). Briefly, cerebella, excised from anesthetized dogs, were minced, and then homogenized with a Brinkman Polytron in 4 vol of buffer A (5 mM NaN_3/100 μM EGTA/20 mM HEPES, pH 7.4). Another 4 vol of buffer A were added to the homogenate, and the suspension was centrifuged for 20 min at 4 k g_{max} (Beckman 35 Ti rotor, Beckman Instruments, Inc., Fullerton, CA). The supernatant fluid was filtered through two layers of cheese cloth, and the filtrate was centrifuged for 30 min at 90 k g_{max} (Beckman 60 Ti rotor, Beckman Instruments, Inc.). The pellet from the latter spin was resuspended in buffer B (0.6 M KCl/5 mM NaN_3/20 mM $Na_4P_2O_7$/10 mM HEPES, pH 7.2) to a final concentration of 4 ml/g cerebellum, and centrifuged for 20 min at 4 k g_{max} (Beckman 60 Ti rotor, Beckman Instruments, Inc.). The resulting supernatant fluid was centrifuged for 30 min at 63 k g_{max} (Beckman 60 Ti rotor, Beckman Instruments, Inc.). The pellet from this last spin was resuspended in buffer C (10% sucrose, 10 mM MOPS, pH 7.0) and either frozen in liquid nitrogen (and stored at −80°C), or used immediately for reconstitution experiments.

Planar Lipid Bilayers

Cerebellar microsomes were fused with planar lipid bilayers, made from phosphatidylethanolamine and phosphatidylserine (75:25%; Avanti Polar Lipids, Inc., Birmingham, AL). Bilayers were formed by painting a solution of lipid in decane across a 100-μM hole in a Teflon sheet that bisected a lucite chamber. The hole was prepainted with a phosphatidylcholine: phosphatidylserine mixture (75:25%) before formation of the membrane. Channel incorporation into the bilayer was followed as described previously (Bezprozvanny and Ehrlich, 1993*a*). Briefly, vesicles (~5 μg protein) were added to the *cis* chamber which contained 800 mM KCl, 250 mM

Tris-HEPES, 10 mM $CaCl_2$, pH 7.35. The *trans* chamber, which was held at virtual ground, contained 55 mM of the hydroxide of the divalent cation under investigation (Mg, Ca, Sr, Ba) dissolved in 250 mM HEPES, pH 7.35. Solutions in both chambers were stirred until the activity of several potassium (K) and/or chloride (Cl) channels appeared in the current recordings; this activity was used as an indication of fusion of vesicles to the bilayer. Sometimes the KCl concentration in the *cis* chamber was further increased by addition of an aliquot of 3 M KCl in order to stimulate fusion of the vesicles with the bilayer. In some experiments, the bilayer was broken and reformed in the presence of microsomes in order to facilitate fusion. After fusion of microsomes, the *cis* chamber was perfused with 10 vol of Tris-HEPES solution (250 mM HEPES/110 mM Tris, pH 7.35) leaving the divalent cation on the *trans* side of the bilayer as the only small ion present in the system. In the series of experiments where $InsP_3$-gated channels were recorded in the presence of cytoplasmic K, the *cis* chamber was perfused with 110 mM KOH dissolved in 250 mM HEPES, pH 7.35 instead of the Tris-HEPES solution.

The vesicles fused to the bilayer with an orientation such that the *cis* side corresponded to the cytoplasmic side of the reticular membrane and the *trans* side corresponded to the lumen of the reticulum. After perfusion of the *cis* chamber, $InsP_3$-gated channels were activated by addition of 2 μM $InsP_3$ to the *cis* side. In all experiments in this report, $InsP_3$-gated channels were recorded in the presence of 0.2 μM free Ca on the cytoplasmic side of the channel (1 mM EGTA and 0.7 mM $CaCl_2$ added to the *cis* chamber), which is in the range of free Ca concentrations found to maximize the activity of the $InsP_3R$ (Bezprozvanny et al., 1991*a*). ATP (0.5 mM) was added to the *cis* chamber in all experiments in order to maximize the activity of the channels (Bezprozvanny and Ehrlich, 1993*a*). Experiments were performed with five different cerebellar microsomal preparations. Data shown are examples selected from at least three similar experiments unless otherwise stated in the legend. Vesicle fusion occurred in virtually all attempts, but $InsP_3$-gated channels were observed only in 35% of the experiments.

Activity of $InsP_3$-gated channels was recorded using a bilayer clamp amplifier (BC-525A, Warner Instrument Corp., Hamden, CT) under voltage clamp conditions. The junction potential between the *cis* and *trans* chambers was measured using 3 M KCl 1% agarose bridge as the only electrical connection between *cis* and *trans* solutions. The junction potential was measured for each pair of solutions used and compensation for junction potential was done electronically during the experiments. For experiments with Tris (110 mM) in the *cis* chamber the junction potential was very close to 0 mV, and for experiments with K (110 mM) in the *cis* chamber it was −2 mV.

In some cases the concentration of ions in the *trans* chamber was manipulated during the experiment. Because perfusion of the *trans* chamber often caused breakage of the bilayer, we changed ion concentrations in the *trans* compartment by addition of aliquots of a concentrated stock solution with stirring for at least 1 min. A volume of the *trans* solution equal to the added volume was withdrawn after each addition in order to keep the volume of solution in the *trans* compartment constant. If this protocol is followed, then the concentration of each ion species in the *trans* chamber after k increment additions of volume v (typically in the range 75–150 μl in our experiments) to the chamber with total volume V (3 ml in our experiments) is equal to:

$$c_k = (c_0 - s)[V/(V + v)]^k + s \quad (1)$$

where c_k is the ion concentration in the *trans* compartment after k additions, c_0 is the concentration of the same ion in the beginning of the experiment, s is the concentration of this ion in the stock solution added. This equation was used for calculation of the *trans* ion concentration when necessary.

In some experiments the free Ca concentration in the *trans* chamber containing a divalent cation at 55 mM (Ba, Sr, Mg) dissolved in HEPES and a Ca-EGTA mixture, had to be

determined. First, the level of contaminated Ca in the divalent cation/HEPES solution was determined using atomic absorption spectroscopy (Galbraith Laboratories Inc., Knoxville, TN). Then, the free *trans* Ca concentration was estimated using a computer program developed by Fabiato (1988) based on the total *trans* Ca concentration (contaminated and added) and using apparent stability constants for divalent cation-EGTA complexes equal to $1.23*10^7$ M^{-1} for Ca-EGTA, $2.40*10^4$ M^{-1} for Ba-EGTA, $3.81*10^4$ M^{-1} for Sr-EGTA, and 70.8 M^{-1} for Mg-EGTA at pH 7.35 and 20°C (Fabiato, 1988).

Analysis

Data were stored with a tape recorder and analyzed using a personal computer. For computer analysis using pClamp 5.5, data were filtered to 800 Hz by an 8-pole Bessel filter and digitized at 2 kHz. Acquired data were filtered using a digital Gaussian filter (pClamp 5.5) with a cutoff frequency of 500 Hz and events longer than 2 ms were used for construction of the dwell time histograms; the threshold used for channel analysis was adjusted manually based on the size of the current. Events longer than 4 ms were chosen for construction of amplitude histograms. Because channel openings had a decreased duration in the experiments with Ca as a current carrier (see Results), the data from these experiments were digitized at 4 kHz and events longer than 1.5 ms were used in the construction of dwell time distributions. Under the experimental conditions used in this report, $InsP_3$-gated channels spent more than 90% of the open time in the main subconductance state (Watras et al., 1991); only openings to this level were considered in the analysis. Evidence for the presence of two to three functional channels in the bilayer was obtained in almost all experiments (e.g., Fig 1, traces *2* and *3*). The number of active channels in the bilayer was estimated as a maximal number of simultaneously active channels during the course of an experiment (Horn, 1991). The open probability for one channel was calculated using the binomial distribution for levels 0, 1, and 2, and assuming that the channels were identical and independent (Colquhoun and Hawkes, 1983).

In some experiments, the permeability ratio of divalent (D^{2+}, present in *trans* chamber) and monovalent (M^+, present in *cis* chamber) cations (P_D/P_M) was estimated based on the extrapolated value of the reversal potential (E_{rev}). In our calculations we used the following modification of Goldman-Hodgkin-Katz equation (Hille, 1992):

$$E_{rev} = \frac{RT}{2F} \ln \frac{4P_D[D^{2+}]}{P_M[M^+]} \tag{2}$$

where $[D^{2+}]$ and $[M^+]$ are the divalent and monovalent cation concentrations respectively. The symbols R, T, and F have their usual meaning. As all experiments were conducted at room temperature (20°C), a value of 25.3 mV was used for RT/F.

Where appropriate, average values are presented as mean ± SE (number of experiments).

RESULTS

$InsP_3$-gated Ca Channels from Canine Cerebellum in Planar Lipid Bilayers

After fusion of canine cerebellar endoplasmic reticulum vesicles with planar lipid bilayers and perfusion of the *cis* (cytoplasmic) chamber with Tris-HEPES solution (see Methods), channel openings were not observed in the absence of $InsP_3$ (Fig. 1, *top trace*). Addition of 2 μM $InsP_3$ to the *cis* side of the membrane induced channel openings (Fig. 1, *second and third traces*). Because the currents are shown on a compressed time scale in Fig. 1, most of the channel openings are not resolved. When the time scale of the current recording is expanded 15-fold (Fig. 1, *fourth trace*),

most of the openings are completely resolved. Usually several $InsP_3$-gated channels were present in the bilayer (at least two channels are active in the experiment shown in Fig. 1). The activity of $InsP_3$-gated channels in all experiments in this paper was recorded under "optimal" conditions, i.e., after addition of 2 μM $InsP_3$ in the presence of 0.2 μM free Ca (Bezprozvanny et al., 1991*a*; Finch et al., 1991; Iino, 1990) and 0.5 mM ATP (Bezprozvanny and Ehrlich, 1993*a*; Ferris et al., 1990; Iino, 1991) on the cytoplasmic side of the membrane. Because multiple channels often incorporated into the membrane, the exact value for the single-channel open probability could not be obtained, but it was estimated to be in the range of 5–12% under the optimal conditions used assuming that the channels in the bilayer were

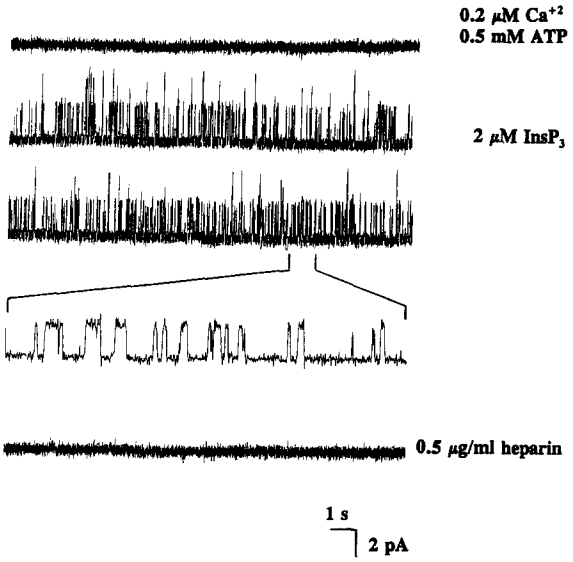

FIGURE 1. $InsP_3$-gated channels from endoplasmic reticulum of cerebellum in planar lipid bilayers. Calcium and ATP act as coagonists of the $InsP_3R$ (Bezprozvanny and Ehrlich, 1993*a*; Bezprozvanny et al., 1991*a*) but were not able to activate the channels without $InsP_3$ present (*first trace*). Addition of 2 μM $InsP_3$ to the *cis* (cytoplasmic) side opened at least two channels incorporated in the membrane (*second trace;* channel opening are upwards). Barium, present at 55 mM on the *trans* (intraluminal) side, was used as the current carrier. Channel activity was recorded under voltage clamp conditions with a transmembrane potential of 0 mV. $InsP_3$-gated channels remained active for extended periods of time without desensitization (second and third traces are separated by 5 min). Most of the channel openings are not resolved due to the compressed time scale used. When the time scale is expanded 15 times (*fourth trace*), most of the channel openings are resolved. Heparin completely and irreversibly inhibited the activity of the $InsP_3R$ (*last trace*). See text for details and the exact composition of experimental solutions. Experiment 20427IP3.

identical and independent (see Methods). In the experiment shown in Fig. 1, Ba was the current carrier (see below). Once activated by addition of $InsP_3$, channels maintained their activity for extended periods of time (from several minutes to 2 h in some experiments) with no signs of time-dependent channel inactivation (Fig. 1, second and third traces were separated by 5 min). Channel openings were completely abolished by the addition of heparin, a known inhibitor of $InsP_3$ binding and $InsP_3$-induced Ca release (Ghosh, Eis, Mullaney, Ebert, and Gill, 1988; Kobayashi, Somlyo, and Somlyo, 1988; Supattapone et al., 1988*b*), at concentrations as low as 0.5 μg/ml (Fig. 1, *bottom trace*). Ruthenium red (10 μM) or ryanodine (2 μM), which

are known to act on the RyR (Smith et al., 1988), had no effect on $InsP_3$-gated channel activity (data not shown).

Divalent Cation Permeation through $InsP_3$-gated Ca Channels

To study the permeation of $InsP_3$-gated Ca channels for the alkaline earth cations, a series of experiments was performed where the divalent cation under investigation was used as the permeant ion in the *trans* (intraluminal) chamber. The *cis* chamber in these experiments contained the same Tris-HEPES solution (see Methods) that was

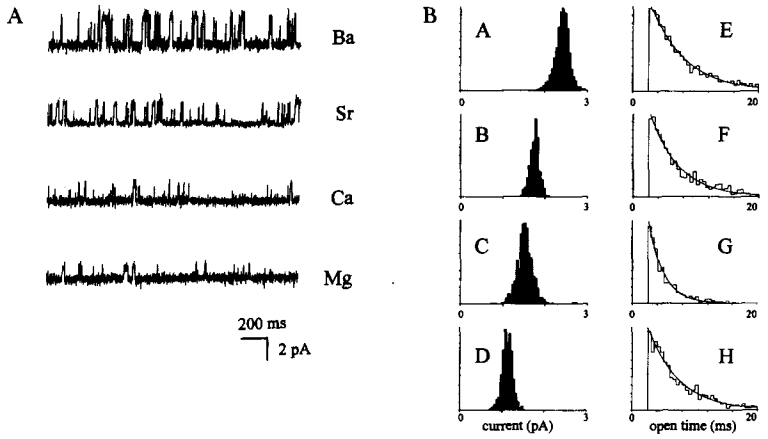

FIGURE 2. Comparison of $InsP_3$-gated channel recordings with different divalent cations (Ba, Sr, Ca, Mg) used as a current carrier (55 mM *trans*). (*A*) Representative single-channel current recordings at a transmembrane potential of 0 mV. The cation used in the experiment is indicated on the right side of each record. (*B*) Unitary current amplitude histograms and open time distributions obtained from analysis of $InsP_3$-gated channel activity with Ba (*A* and *E*, 3,502 events), Sr (*B* and *F*, 1,081 events), Ca (*C* and *G*, 769 events), and Mg (*D* and *H*, 777 events) as a current carrier. Events longer than 2 ms (1.5 ms in the experiments with Ca as a current carrier) were used for construction of open time distributions, events longer than 4 ms for amplitude histograms. Infrequent openings to subconductance levels were ignored in the analysis. The mean unitary current and mean open time were obtained assuming Gaussian distributions for current amplitudes and single-exponential distributions for channel open times. Data were taken from the experiments shown in *A*. Experiments 10923ATP, 30805Sri, 21002Cai, 30812Mgi.

used in previous reports (Bezprozvanny and Ehrlich, 1993*a*; Bezprozvanny et al., 1991*a*) and in the experiment shown in Fig. 1. In agreement with the results obtained for another intracellular Ca channel, the RyR (Ashley, 1989; Smith, Coronado, and Meissner, 1986; Tinker and Williams, 1992; Williams, 1992), all four alkaline earth cations tested (Ba, Sr, Ca, Mg) carried measurable current through $InsP_3$-gated channels incorporated in planar lipid bilayers at a transmembrane potential of 0 mV (Fig. 2 *A*). With all four cations used, channel currents were qualitatively similar, although some quantitative differences were observed. Single-channel data analyses revealed differences in the unitary current amplitude (*A–D* in Fig. 2 *B*) and in the

TABLE I

Divalent Cations Permeation through the $InsP_3$-gated Channels

Ion	Current	Mean open time	Conductance
	pA	*ms*	*pS*
Ba	2.2 ± 0.1 (n = 8)	6.4 ± 1.0 (n = 7)	85 ± 3 (n = 4)
Sr	2.0 ± 0.1 (n = 4)	5.9 ± 1.6 (n = 3)	77 ± 2 (n = 3)
Ca	1.4 ± 0.1 (n = 6)	2.9 ± 0.2 (n = 5)	53 ± 4 (n = 3)
Mg	1.1 ± 0.1 (n = 4)	4.3 ± 0.3 (n = 4)	42 ± 2 (n = 3)

mean open time of the channels (*E–H* in Fig. 2 *B*). The observed mean closed time of the channels varied dramatically from experiment to experiment, most probably reflecting the presence of a variable number of channels incorporated in the bilayer in different experiments. Therefore, a reliable estimate of the mean closed time and open probability variation as a function of the ion species used as a current carrier could not be obtained.

The properties of $InsP_3$-gated channels obtained in this series of experiments are summarized in Table I. At a transmembrane potential of 0 mV and with 55 mM divalent cation in the intraluminal (*trans*) chamber, the current amplitude ranged from 2.2 ± 0.1 pA (n = 8) with Ba as a current carrier to 1.1 ± 0.1 pA (n = 4) with Mg (Fig. 2, Table I) and fell in the sequence Ba > Sr > Ca > Mg. The mean open time of the channels was significantly smaller with Ca as a current carrier (2.9 ± 0.2 ms [n = 5]) than with other alkaline earths (~5.5 ms) (Fig. 2, Table I).

The single-channel conductance of the channels with different divalent cations was estimated from the slope of the current-voltage relationship between 0 and −40 mV. The planar lipid bilayers became unstable at transmembrane potentials below −40 mV and the current-voltage relationship was not linear at transmembrane potentials above 0 mV. It also was difficult to separate channel openings from the baseline noise at positive potentials, especially in experiments with Mg and Ca as a current carriers. Between 0 and −40 mV the current-voltage relationship was ohmic with all divalent cations tested (Fig. 3). The single-channel conductance (determined using least squares linear regression) ranged from 85 ± 3 pS (n = 4) for Ba to 42 ± 2 pS (n = 3)

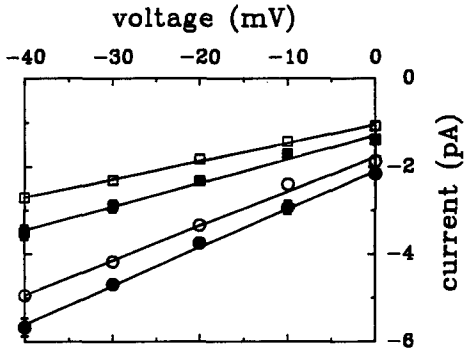

FIGURE 3. Single-channel current–voltage relationships of $InsP_3$-gated channels with Ba (*filled circles*, ●), Sr (*open circles*, ○), Ca (*filled squares*, ■), and Mg (*open squares*, □) as a current carrier obtained in the range of transmembrane potentials between 0 mV and −40 mV. The *trans* concentration of each ion was equal to 55 mM; the *cis* chamber contained Tris-HEPES solution. Individual points are the means ± SEM (n ≥ 3) of the current values obtained; if the SEM is not shown, it is smaller than the size of the symbol. Single-channel conductance of the $InsP_3R$ for each ion species was determined as the slope of the current-voltage relationship using linear regression (see Table 1).

for Mg and fell in the same sequence as the values of the current amplitude at 0 mV: Ba > Sr > Ca > Mg (Fig. 3, Table I).

In this series of experiments channels were recorded with Tris-HEPES solution on the cytoplasmic (*cis*) side of the membrane. No measurable currents in the opposite direction (carried by $Tris^+$ ions) through $InsP_3$-gated channels were observed even at potentials as high as +60 mV (data not shown). Therefore, a true value for the reversal potential could not be obtained from these experiments. A value for the reversal potential could not be obtained by extrapolating the linear segment of current-voltage relationship either because of the observed nonlinearity of the current-voltage relationship at positive potentials.

Mole Fraction Behavior of the Unitary Current through $InsP_3$-gated Channels

How many divalent cations can be present simultaneously in the permeation pathway of an $InsP_3R$? The "anomalous mole fraction effect" is one of the strongest pieces of evidence in favor of multiple occupancy of voltage-gated Ca channels (Almers and McClesky, 1984; Hess and Tsien, 1984; Tsien et al., 1987). To see if the anomalous mole fraction effect is observed for $InsP_3R$, we measured the activity of $InsP_3$-gated channels in planar lipid bilayers in the presence of varied molar ratios of Ba and Mg. Barium and Mg were chosen because the single-channel conductance and the unitary current amplitude at any given transmembrane potential differ most dramatically for this pair of ions (Figs. 2 and 3; Table I). To exclude potential masking effects of contaminating Ca, 1 mM EGTA was added to the *trans* chamber in these experiments. In these experiments, $InsP_3$-gated channels were first recorded in the presence of 55 mM Ba/HEPES and 1 mM EGTA in the *trans* chamber and then a series of additions of a solution of 55 mM Mg/HEPES and 1 mM EGTA to the *trans* chamber were made. After each addition, an equal volume of solution was withdrawn in order to keep the total volume in the *trans* chamber constant. The resulting *trans* concentrations of Ba and Mg were calculated as described in Methods using Eq. 1. This protocol allowed us to measure the current amplitude of the same $InsP_3$-gated channel at different molar ratios of Ba to Mg (the molar fraction for Mg ranged from 0 to 0.9) while the total concentration of divalent cations in the *trans* chamber was kept at 55 mM. In parallel experiments, channels were first recorded in the presence of 55 mM Mg/HEPES and 1 mM EGTA in the *trans* chamber and subsequent additions of 55 mM Ba/HEPES and 1 mM EGTA were performed, so that the molar fraction for Mg ranged from 1 to 0.1. The average sizes of the unitary currents at transmembrane potentials of 0 mV and −20 mV obtained in these experiments are plotted as functions of the Mg molar fraction in Fig. 4. The data were pooled from both series of experiments. The relatively small variation in the size of the current at any given Mg molar fraction provides evidence that Eq. 1 gives a reasonable estimation of the Mg and Ba concentrations during our experiments. It is apparent that both functions are monotonic; that is, the anomalous mole fraction effect was not observed for $InsP_3R$ at either 0 mV or −20 mV for mixtures of Ba and Mg. Linear regression provided satisfactory fits to the data (Fig. 4), indicating that the affinities of the divalent cation binding site in the $InsP_3R$ are similar for Ba and Mg. The

single-channel slope conductance can be estimated from the differences in the current amplitudes at 0 and −20 mV. From the data shown in Fig. 4, the slope conductance is a monotonic function of the Mg molar fraction; the slope conductance decreases from 73.5 to 38.5 pS as the Mg molar fraction increases from 0 to 1. These data are in good agreement with results obtained for the RyR from sheep cardiac muscle using a similar approach (Lindsay et al., 1991; Tinker and Williams, 1992).

The Effect of the Intraluminal Ca Level on the Activity of $InsP_3$-gated Channels

Regulation of the $InsP_3R$ by intraluminal Ca was studied in a series of experiments where the activity of $InsP_3$-gated channels was measured at different intraluminal (*trans*) Ca concentrations. Similar results were obtained using either Ba, Sr, or Mg as a current carrier. The use of divalent cations as current carriers in these experiments could compromise our results if these ions interact with intraluminal regulatory sites of the $InsP_3R$. Unfortunately, it was not possible to conduct these experiments using monovalent cations due to the presence of K channels in the microsomal membrane.

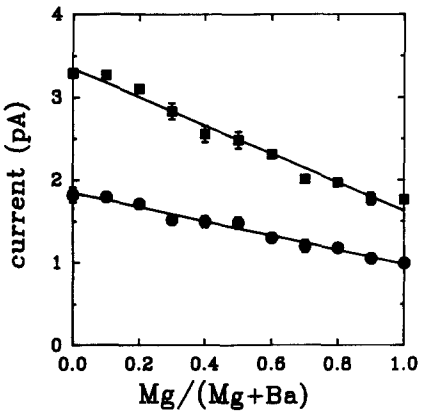

FIGURE 4. Test for the presence of the anomalous mole fraction effect. The dependence of the elementary current at 0 mV (●) and at −20 mV (■) through $InsP_3$-gated channels with a varying Mg to Ba molar ratio on the *trans* side of the membrane. The points are the means ± SEM of the current values obtained in three experiments in which the activity of the same $InsP_3R$ was recorded at different Mg to Ba molar ratios (two experiments where the Mg mole fraction was changed from 0 to 0.9, and one experiment where it was changed from 1 to 0.1). Mg and Ba concentrations were estimated using Eq. 1. See text for details.

Representative current traces from this series of experiments are shown on Fig. 5 *A* (Sr is the current carrier). In these experiments, channels were first recorded in the absence of Ca on the intraluminal side of the membrane (Fig. 5 A, *top trace*, 55 mM Sr/HEPES and 1 mM EGTA in the *trans* chamber, pCa of 5.5 based on the level of contaminated Ca in the Sr/HEPES solution) and then increasing amounts of Ca/HEPES solution were added to the *trans* chamber. The total concentrations of Ca, Sr, and EGTA were calculated using Eq. 1 (see Methods) after each addition of Ca/HEPES. Based on these values, the free Ca concentration in the *trans* compartment was estimated as described in Methods. This protocol allowed us to measure the activity of the same channel at different intraluminal free Ca concentrations from the micromolar range to several millimolar Ca (Fig. 5 *A*). Some decrease in single-channel current amplitude occurring in these experiments (Fig. 5 *A*, compare last two traces) could be attributed to the fact that at high *trans* Ca levels a substantial portion of the *trans* Sr was replaced by Ca ions. Because the conductance of the channels for Ca is less than that for Sr (Figs. 2 and 3), some reduction in current amplitude is

expected. During data analysis adjustments for these changes were accomplished by lowering the current threshold level.

The general result of these experiments is that the activity of $InsP_3$-gated channels was not potentiated by an increase in *trans* Ca levels in the expected physiological range of intraluminal free Ca concentrations, in disagreement with the previously proposed model (Irvine, 1990). As a matter of fact, $InsP_3$-gated channels were slightly *inhibited* by an increase in *trans* Ca (Fig. 5 *B*). On average, an elevation in the *trans* Ca level to 1 mM reduced the single-channel open probability to 67 ± 11% (n = 3) of its

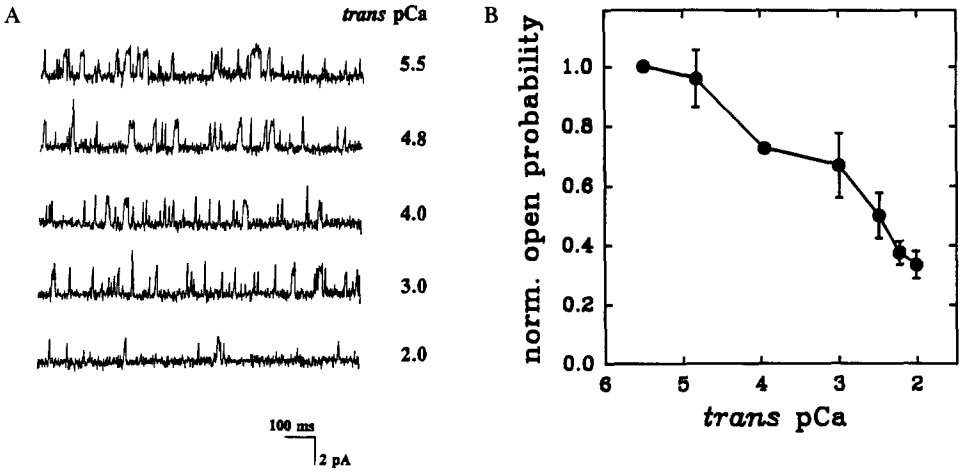

FIGURE 5. Regulation of $InsP_3$-gated channels by intraluminal Ca. (*A*) $InsP_3$-gated channels were recorded using Sr (55 mM *trans*) as a current carrier at different concentrations of *trans* Ca in the same experiment. The free *trans* Ca concentration, estimated as described in Methods based on total concentrations of Ca, EGTA, and Sr in the *trans* chamber after each addition of Ca/HEPES (see text for details), is indicated on the right side of each current record in log units. Experiment 30520Sri. (*B*) Normalized open-channel probability of $InsP_3R$ is plotted as a function of the free *trans* Ca concentration (log scale) from 3 μM (pCa 5.5) to 10 mM (pCa 2.0). To compare the results from different experiments the open-channel probability in each experiment was normalized to the open channel probability at pCa 5.5 (1 mM EGTA, no added Ca) obtained in the same experiment. After this normalization procedure the data from the three experiments performed under identical conditions were combined. The symbols represent the mean value ± SEM (n = 3). At least 100 s of continuous recording of $InsP_3R$ activity were used in the analysis at each *trans* Ca concentration.

control level (Fig. 5 *B*). Further increases in *trans* Ca caused additional decreases in the channel's activity. At 10 mM *trans* Ca, the single-channel open probability was equal to only 33 ± 5% (n = 3) of its control level (Fig. 5 *B*). It is notable that a similar result was previously obtained with another intracellular Ca release channel, RyR (Ma, Fill, Knudson, Campbell, and Coronado, 1988). In this section we focused on the effects of intraluminal Ca below 10 mM. A more detailed consideration of the inhibitory effects on $InsP_3$-gated channels caused by high intraluminal Ca levels ($\geq$ 10 mM) is presented in the next section of this paper.

Effect of High (≥ 10 mM) Intraluminal Ca on the Activity of $InsP_3$-gated Channels: a Kinetic Model of $InsP_3R$ Gating

To get a better understanding of the mechanism underlying the inhibitory effects of high (≥ 10 mM) intraluminal Ca on the gating of the $InsP_3R$, we conducted a series of experiments where channel activity was initially recorded in the absence of *trans* Ca (1 mM EGTA added) using 55 mM Sr as a current carrier. Then, *trans* Ca concentration was increased by serial addition of a solution containing 55 mM Ca/HEPES. After each addition, an equal volume of *trans* solution was withdrawn. By using this protocol the activity of the same channel was measured at various Ca to Sr molar ratios while the total concentration of divalent cations in the *trans* chamber was equal to 55 mM. The concentrations of Ca and Sr in the *trans* chamber were estimated using Eq. 1 (see Methods).

By using this method we were able to measure the activity of the same channel at Ca molar fractions in the *trans* chamber ranging from 0 to 0.8 (see Fig. 6 *A*). The single-channel open probability in this series of experiments decreased 5–10-fold by the increase in the Ca mole fraction. Single-channel data analysis revealed that high levels of intraluminal Ca inhibit $InsP_3$-gated channels by decreasing both the frequency of channel openings and the mean open time of the channels (Fig. 6 *B*). For the experiment shown on Fig. 6 *A*, the mean open time of the channels decreased from 4.73 to 2.86 ms when the Ca mole fraction in the *trans* chamber was increased from 0 to 0.8 (Fig. 6 *B, left*), approaching the values typically obtained in experiments where only Ca was the current carrier (Fig. 2, Table I). In the same experiment the observed mean closed time of the channels was simultaneously increased from 65 to 320 ms (Fig. 6 *B, middle,* two channels are active in the membrane). Single-channel current amplitude at 0 mV was only slightly affected in this experiment, changing from 1.65 pA to 1.49 pA (Fig. 6 *B, right*).

How is it possible to explain the inhibitory effects of high *trans* Ca on $InsP_3$-gated channels? We speculated that channel modulation could be explained as a result of an increase in the local Ca concentration in the vicinity of the channel pore on the cytoplasmic side of the membrane at every channel opening (Neher, 1986; Stern, 1992). This idea was inspired by a similar model that was recently suggested for the skeletal muscle RyR based on Ca-release measurements (Jong, Pape, Chandler, and Baylor, 1993). Because $InsP_3R$ are inactivated by cytoplasmic free Ca above 1 μM (Bezprozvanny et al., 1991*a;* Finch et al., 1991; Iino, 1990) this local elevation of Ca, that can reach 100 μM at macromolecular distances from the channel pore for 2 pA of Ca current (Stern, 1992), can inactivate the channels prematurely, which would then cause a decrease in the mean open time in the presence of high *trans* Ca. If it takes a long time for the bound Ca to dissociate from the inhibitory site when compared with the rate of channel openings, then a decrease in the frequency of channel openings would be also observed. Because Sr, Ba, or Mg in the *cis* solution at concentrations up to 1 mM did not inhibit the gating of the $InsP_3R$ (data not shown), these effects would be only observed in the presence of *trans* Ca. Indeed, when $InsP_3$-gated channels were recorded at different Mg to Ba molar ratios (see above), channel gating was not affected (data not shown). Therefore, the effects on open-channel probability and the channel's kinetic characteristics observed here are

specific for Ca. A direct test of the proposed model would have been to use a high concentration of BAPTA in order to buffer Ca in the vicinity of the channel pore (Stern, 1992). Unfortunately, BAPTA acts as potent inhibitor of $InsP_3$ binding to its receptor (Richardson and Taylor, 1993), a result we confirmed by finding that

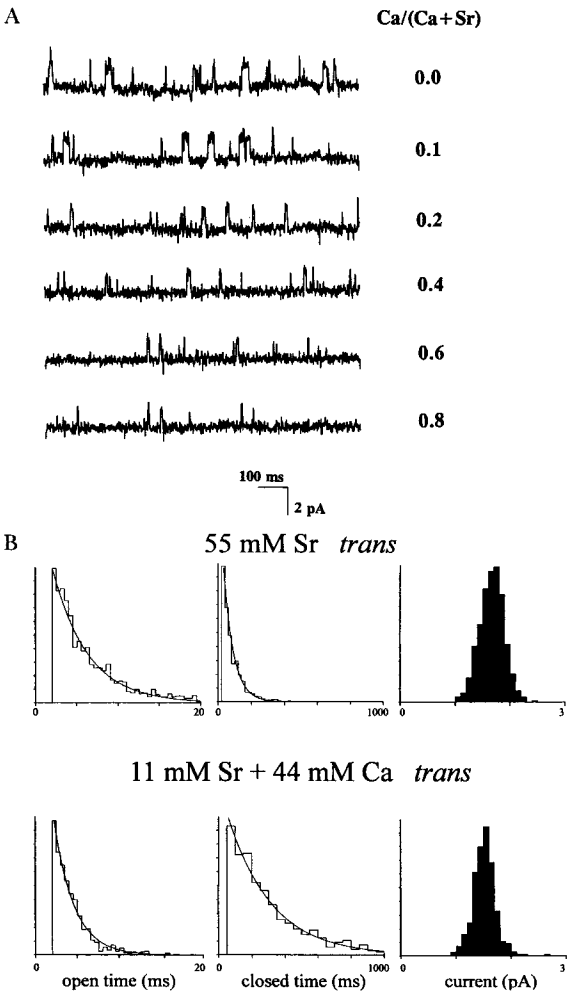

FIGURE 6. Effect of varying the *trans* Ca molar fraction on the gating of the $InsP_3R$. (*A*) $InsP_3$-gated channels were recorded at various Ca to Sr molar ratios in the *trans* chamber in the same experiment; the total concentration of divalent cations in the *trans* chamber was equal to 55 mM. The concentrations of Ca and Sr in the *trans* chamber were estimated using Eq. 1 (see Methods). The molar fraction of Ca in the *trans* chamber is indicated on the right side of each current record. (*B*) Open-time distribution (*left*), observed closed time distribution (*center*), and current amplitude histogram at 0 mV (*right*) are shown for the same $InsP_3R$ in the absence of added Ca in the *trans* chamber (*top*) and at a *trans* Ca molar fraction of 0.8 (*bottom*). Data for single-channel analysis are taken from the experiment shown in *A*. Histograms were constructed from 1,462 events in the absence of added *trans* Ca and 624 events at a *trans* Ca molar fraction of 0.8. Open and closed time distributions were fitted with single exponential functions. Experiment 40110Sr4.

$InsP_3$-gated channels were inactivated in the presence of 10 mM BAPTA (data not shown).

A quantitative model developed based on the assumption that *trans* Ca modulated $InsP_3R$ activity due to the increase in the local Ca concentration on the cytoplasmic side and the kinetic parameters extracted from a fit to the experimental data are presented in the Appendix.

Measurements of $InsP_3$-gated Channels in the Presence of Cytoplasmic Potassium

One goal of our experiments is to estimate the size of the unitary current through the $InsP_3R$ under physiological conditions. In all experiments presented to this point, $InsP_3$-gated channels were measured in the presence of Tris-HEPES solution on the *cis* side of the membrane and divalent cations dissolved in HEPES on the *trans* side. It was necessary to use this ionic composition because cerebellar microsomes contain K and Cl channels, as well as $InsP_3R$, and therefore it was necessary to avoid K and Cl ions to measure solely the activity of the $InsP_3$-gated channel. However, during $InsP_3$-induced Ca release in the intact cell it is the selectivity of the channels for K ions, not for Tris, which contributes to the size of the Ca current. Therefore, it is important to measure the unitary current through $InsP_3$-gated channels in the presence of cytosolic K.

To perform these experiments, we decided to take advantage of the fact that K channels from the endoplasmic reticulum are inhibited by Ba. Unfortunately, the potency of Ba as an inhibitor of K channels from cerebellum is not as great as it is for K channels from skeletal muscle sarcoplasmic reticulum (Coronado, Rosenberg, and Miller, 1980). The inhibitory effect of Ba on the K channels from cerebellar microsomes is similar to its effect on the K channels from aortic sarcoplasmic reticulum (Bezprozvanny, Benevolensky, and Naumov, 1991*b*). However, the activity of K channels was greatly diminished by the presence of 55 mM Ba on the *trans* side, especially at negative potentials due to the voltage dependence of the Ba block (Bezprozvanny et al., 1991*b*).

A representative experiment from this series is shown in Fig. 7. In the first part of the experiment (Fig. 7 *A*) $InsP_3$-gated channels were recorded at 0 mV as usual, after addition of 2 μM $InsP_3$, with 55 mM Ba in the *trans* chamber as the current carrier, and the *cis* chamber filled with the Tris-HEPES solution supplemented with 0.2 μM free Ca and 0.5 mM ATP. The *cis* compartment was subsequently perfused with 20 vol of 110 mM K/HEPES solution (pH 7.35), a concentration of K chosen to emulate the K content in the cytoplasm. Change in the junction potential caused by the change in the ionic composition of *cis* solution was compensated electronically after perfusion (see Methods). After adjustment of the free Ca concentration in the cytosolic compartment to 0.2 μM and addition of 0.5 mM ATP and 2 μM $InsP_3$, $InsP_3$-gated channels were once again observed (Fig. 7 *B, first pair of traces*). Some K channel activity was not completely inhibited by Ba, but this did not create a problem in data analysis because these channels were easily distinguished from $InsP_3$-gated channels due to the marked differences in channel amplitude and kinetic characteristics (Fig. 7 *B, first pair of traces*). $InsP_3$-gated channels were also recognized by their sensitivity to heparin (Fig. 7 *B, second pair of traces*), whereas the activity of K channels was not affected by heparin (Fig. 7 *B, second pair of traces*).

It is clear from Fig. 7 that the unitary current through $InsP_3$-gated channels at 0 mV is substantially larger in the presence of 110 mM K on the *cis* side than in the presence of Tris in the same concentration. On average, the mean single-channel current amplitude in this series of experiments was equal to 3.0 ± 0.1 pA (n = 3) with 110 mM K in the cytoplasmic solution. This value is 1.36 times larger than the value obtained with Ba as a current carrier in the presence of Tris on the cytoplasmic side

(Fig. 2, Table I). The relatively small increase (8%) in channel slope conductance from 85 ± 3 pS (n = 4) for Ba vs Tris (see Fig. 3 and Table I) to 92 ± 3 pS (n = 3) for Ba vs K (Fig. 8) cannot account for the 36% difference in the single-channel amplitude at 0 mV. It should be pointed out that the current-voltage relationship is significantly nonlinear at positive potentials in the presence of Tris (see above) and that only the linear portion of the curve is shown in Fig. 3. Therefore, it is not correct to estimate the Ba to Tris permeability ratio (P_{Ba}/P_{Tris}) by extrapolating the linear portion of the current-voltage relationship. Although it appears that the current-

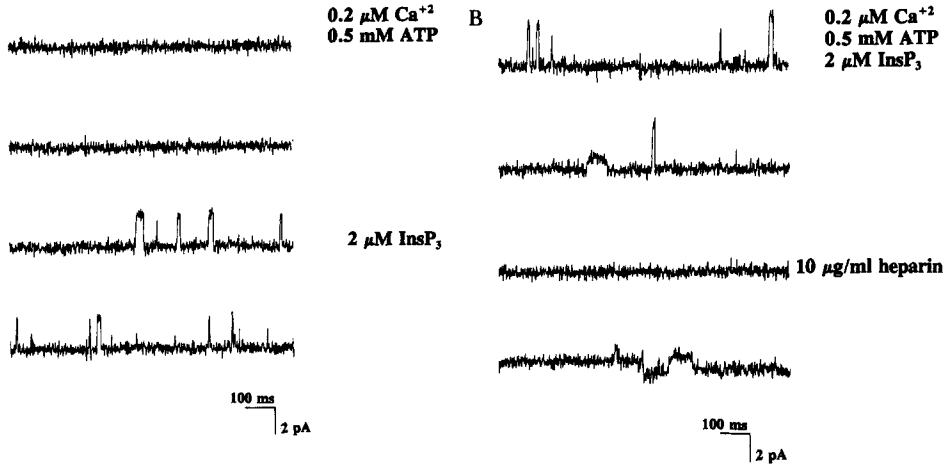

FIGURE 7. $InsP_3$-gated channels recorded in the presence of 110 mM *cis* K. In the first part of the experiment (*A*), the *cis* chamber was filled with Tris-HEPES solution supplemented with 0.2 μM Ca^{2+} and 0.5 mM ATP; the *trans* chamber contained 55 mM Ba/HEPES. In the absence of $InsP_3$, channel activity was not observed (*A, first pair of traces*). Addition of 2 μM $InsP_3$ induced channel activity (*A, second pair of traces*). After channel activation, the *cis* compartment was perfused with 20 vol of 110 mM K/HEPES solution. After adjustment of the free Ca concentration to 0.2 μM, addition of 0.5 mM ATP and 2 μM $InsP_3$, $InsP_3R$ were reactivated (*B, first pair of traces*). The single-channel current amplitude was 1.3 times larger under these conditions (Ba vs K) compared with the first part of the experiment (Ba vs Tris). A residual activity of K channels was sometimes observed (*B, first pair of traces*). Heparin inhibited $InsP_3$-gated channels (*B, second pair of traces*) but did not affect K channels (*last trace*). Current was recorded at 0 mV. Replacement of Tris-HEPES by K/HEPES caused a 2-mV change in the junction potential that was calibrated before the experiment and compensated electronically (see Methods). Experiment 30804BaK.

voltage relationship has shifted to the right with K, the mostly likely explanation is a decrease in the curvature of the current-voltage relationship in the presence of K, as it was reported in similar experiments for the RyR (Smith et al., 1988; Tinker and Williams, 1992).

The Ba to K permeability ratio (P_{Ba}/P_K) of the $InsP_3R$ could be estimated using Eq. 2 (see Methods) from the reversal potential of current through the channels under the biionic conditions used in this series of experiments. Unfortunately, K channels were no longer sufficiently inhibited by Ba at positive transmembrane potentials due

to the voltage-dependent nature of the inhibition (Bezprozvanny et al., 1991*b*). Therefore, the true value of the reversal potential for $InsP_3$-gated channels under biionic conditions (Ba vs K) could not be obtained. However, when the activity of the purified RyR was recorded in planar lipid bilayers under similar conditions (Smith et al., 1988; Tinker and Williams, 1992), the single-channel current–voltage relationship was composed of two linear segments with the inflection at the reversal potential. Because the permeation pathways of $InsP_3R$ and RyR seem to be constructed in a similar way (see Discussion), we assumed that the current-voltage relationship of the $InsP_3R$ under biionic conditions is also ohmic on both sides of the reversal potential. If this is the case, then a value for the reversal potential (E_{rev}) of +32 mV is obtained by extrapolating the current-voltage relationship until it intersects with the voltage axis (Fig. 8). From these data and the ionic conditions used in this series of experiments (55 mM Ba *trans* vs 110 mM K *cis*), P_{Ba}/P_K of 6.3 is estimated from Eq. 2 (see Methods). With the RyR a value for P_{Ba}/P_K of 6 was obtained (Smith et al., 1988; Tinker and Williams, 1992). Therefore, most likely both types of intracellular Ca

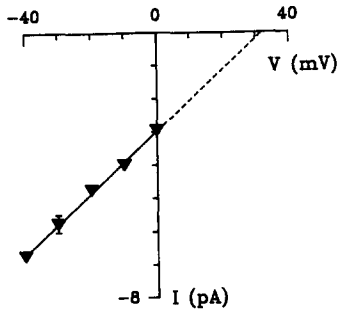

FIGURE 8. Single-channel current–voltage relationships for $InsP_3R$ obtained with Ba as a current carrier (55 mM Ba/HEPES *trans*) in the presence of cytosolic K (110 mM K/HEPES *cis*). The individual points are the means of the current values obtained in at least three experiments; if the SEM is not shown, it is smaller than the size of a symbol. At positive potentials, K channels were no longer inhibited by Ba and recordings of $InsP_3$-gated channels could not be analyzed. The single-channel conductance of the $InsP_3R$ (92 pS) was determined as the slope of the current-voltage relationship using linear regression; the extrapolated reversal potential (+32 mV) was determined as the intercept of the extrapolated current-voltage relationship with the voltage axis.

release channels have similar selectivity for divalent cations versus monovalent cations.

The Dependence of the Elementary Current on the Permeant Ion Concentration

In all experiments presented so far, the concentration of permeant ion in the *trans* chamber was fixed at 55 mM. However, it is generally believed that the free Ca concentration in the lumen of the reticulum does not exceed the level of several millimolar. To estimate the size of the current through $InsP_3R$ in the physiological situation we measured the amplitude of the divalent current through $InsP_3$-gated channels as a function of the permeant ion concentration. Fig. 9*A* shows the elementary currents recorded at a transmembrane potential of 0 mV at different *trans* Ba concentrations, indicated on the right side of each record in mM. In this series of experiments $InsP_3$-gated channels were first recorded in the presence of 5 mM Ba/HEPES on the *trans* side and then subsequent additions of aliquots of a 400 mM Ba/HEPES stock solution were made to the *trans* chamber. The Ba concentration in

the *trans* compartment after each addition was estimated as described in Methods. The *cis* chamber contained Tris-HEPES solution that was not changed during an experiment. Using this protocol we obtained recordings of the same channel at *trans* Ba concentrations ranging from 5 mM to 72 mM (Fig. 9 *A*) or up to 160 mM (data not shown).

The mean values of the elementary current obtained in this series of experiments are plotted as a function of the *trans* Ba concentration in Fig. 9 *B* (*filled circles*). The

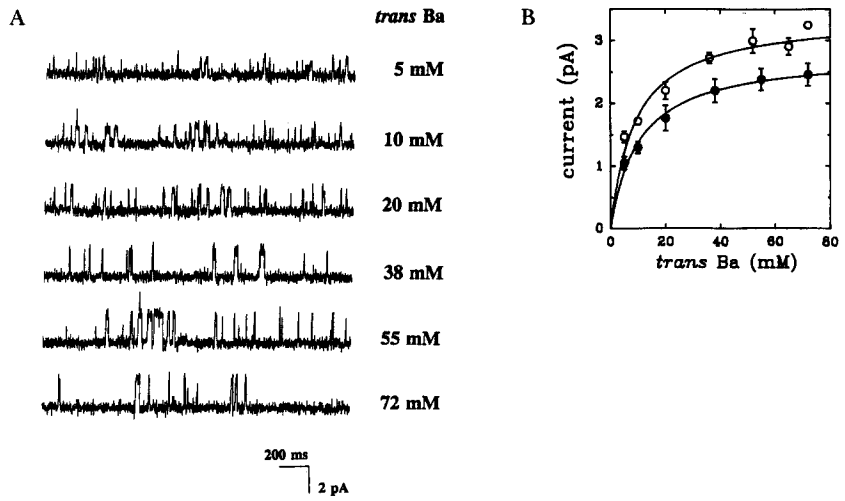

FIGURE 9. Dependence of the single-channel current amplitude at 0 mV on the Ba concentration in the *trans* chamber. (*A*) $InsP_3$-gated channels were recorded at different *trans* Ba concentrations from 5 to 72 mM in the same experiment. The *trans* Ba concentrations, calculated using Eq. 1 after each addition of Ba/HEPES (see text for details), are indicated in millimolar on the right side of each current record. The *cis* chamber contained the same Tris-HEPES solution throughout the experiment. Experiment 31231Bai. (*B*) The amplitude of elementary current through the $InsP_3R$ at 0 mV is plotted as a function of the *trans* Ba concentration for the experiments with Tris-HEPES on the *cis* side (●) and for the experiments with symmetrical 110 mM K/HEPES (○). The symbols represent the mean value ± SEM (n = 3). The smooth curve is the best fit to the equation $i = i_{max}/(1 + K_d/[Ba])$, where i is the elementary current, i_{max} is the saturating value of i, [Ba] is the *trans* Ba concentration, and K_d is the apparent dissociation constant. The best fit to the data (*smooth curves*) was obtained with i_{max} = 2.8 pA and K_d = 10.3 mM for the experiments with Tris-HEPES in the *cis* chamber and with i_{max} = 3.4 pA and K_d = 9.0 mM for the experiments with symmetrical 110 mM K/HEPES.

data from these experiments could be fit using the Michaelis-Menten equation with an apparent dissociation constant for Ba of 10.0 ± 0.2 mM (n = 3) and a maximal unitary current of 2.8 ± 0.2 pA (n = 3). The value of the apparent dissociation constant for Ba is close to the value for the K_d obtained in a similar series of experiments for cardiac L-type Ca channels (Hess, Lansman, and Tsien, 1986). Because Ca ions go through these channels under physiological conditions, we also measured the unitary current as a function of the *trans* Ca concentration (data not

shown). Unfortunately, it was not possible to analyze the data at *trans* Ca concentrations below 20 mM because channel openings are shorter in duration and the unitary current amplitude is smaller with Ca as a current carrier compared with Ba (Fig. 2, Table I). Because the size of the single-channel current at 20 mM *trans* Ca was equal to 85% of its maximal value, a reliable estimate of the apparent dissociation constant for Ca could not be obtained.

It is notable that the increase in the size of the unitary current through the $InsP_3R$ is mainly due to changes in the driving force for Ba ions. The single-channel conductance at 5 mM *trans* Ba was equal to 71 ± 4 pS (n = 3, data not shown), 90% of its value at 55 mM intraluminal Ba. This result is in agreement with measurements of the single-channel conductance of sheep cardiac RyR, where the channel conductance in symmetrical 2 mM Ba was equal to 75% of its maximal value (Tinker and Williams, 1992). Therefore, both intracellular Ca channels are designed to have near maximal conductance at millimolar concentrations of divalent cations. Because the free Ca concentration in the lumen of the endoplasmic/sarcoplasmic reticulum is unlikely to exceed several millimolar, this feature optimizes the ability of these channels to pass ions in the physiological situation.

In the next series of experiments we made one more step towards measurements using physiological conditions. The activity of $InsP_3$-gated channels was recorded at 0 mV at different *trans* Ba concentrations in the presence of 110 mM symmetrical K. Because there is no driving force for K ions at 0 mV in these conditions, the recorded current is carried by divalent (Ba) ions. In the beginning of each experiment in this series the *cis* chamber contained 110 mM K/HEPES, pH 7.35 supplemented with 0.2 μM Ca^{2+} and 0.5 mM ATP; the *trans* chamber was filled with 5 mM Ba/HEPES and 110 mM K/HEPES, pH 7.35. After addition of 2 μM $InsP_3$ to the *cis* chamber Ba current through $InsP_3$-gated channels was recorded. The Ba concentration in the *trans* chamber was then increased by subsequent additions of aliquots of 200 mM Ba/HEPES stock solution dissolved in 110 mM K/HEPES, pH 7.35. The Ba concentration in the *trans* compartment after each addition was estimated as described in Methods.

Using this protocol we obtained recordings of the same channel at a transmembrane potential of 0 mV and at *trans* Ba concentrations ranging from 5 mM to 72 mM in the presence of 110 mM K on both sides of the membrane. Mean values of the single-channel current amplitude at every *trans* Ba concentration obtained in this series of experiments are plotted in Fig. 9 *B* (*open circles*). The data were fit using the Michaelis-Menten equation with an apparent dissociation constant for Ba equal to 9.5 ± 1.0 mM (n = 3) and a maximal unitary current equal to 3.5 ± 0.1 pA (n = 3) (smooth curve on Fig. 9 *B*). It is obvious from Fig. 9 *B* that the amplitude of the single-channel current at every *trans* Ba concentration in this series of experiments (Fig. 9 *B*, *open circles*) is 20–30% larger than the size of the current measured in the presence of Tris in the *cis* compartment in the previous series of experiments (Fig. 9 *B*, *filled circles*). These data are in agreement with the results obtained in the preceding section of the paper, where the activity of $InsP_3$-gated channels was measured in the presence of cytosolic K. Only measurements at 0 mV could be performed in the presence of symmetrical K because K channels were not sufficiently inhibited at low Ba concentrations.

DISCUSSION

This study focused on a characterization of the conduction properties of $InsP_3$-gated Ca channels from cerebellum. The main issues addressed are: (*a*) measurements of the single-channel conductance, the unitary current at 0 mV, and the mean open time of the $InsP_3R$ for different alkaline earth cations; (*b*) a test for the presence of the anomalous mole fraction effect in a mixture of divalent cations; (*c*) an evaluation of the effects of intraluminal Ca concentration on activity and kinetic characteristics of $InsP_3$-gated channels; (*d*) a description of how the unitary current varies with changes in the permeant ion (Ba) concentration; (*e*) measurements of $InsP_3$-gated channel activity in the presence of K. In the following discussion the results obtained will be evaluated and an effort to uncover some general themes will be made based on comparisons of our data with information available for another type of intracellular Ca channel, the RyR, and for the L-type Ca channel, the best studied example of voltage-gated Ca channels of the plasma membrane.

Conduction Properties of $InsP_3$ Receptor for Divalent Cations

The presence of Cl and K channels in the cerebellar microsomes used in our experiments imposed limits on the ion composition of the solutions that could be used in this study. For this reason we could not obtain as detailed and precise information about $InsP_3R$ selectivity and permeation as was recently done with another type of intracellular Ca channel, the RyR, purified from either cardiac or skeletal muscle (Lindsay et al., 1991; Lindsay and Williams, 1991; Smith et al., 1988; Tinker et al., 1992; Tinker and Williams, 1992). A detailed analysis of $InsP_3R$ selectivity properties must be postponed until reliable recordings from purified $InsP_3R$ incorporated in the bilayer are obtained. However, some generalizations about intracellular Ca channel selectivity and permeation properties can still be made based on the information obtained for $InsP_3R$ in the present study.

All four alkaline earth cations tested are able to pass through the $InsP_3R$ with single-channel conductances that fall in the sequence Ba > Sr > Ca > Mg (Figs. 2 and 3). The same order of conductances was reported for the RyR (Tinker and Williams, 1992) although the absolute values of the single-channel conductance are approximately twice as large for the RyR. Both channels have a similar selectivity for divalent cations: the P_{Ba}/P_K permeability ratio is 6.3 for $InsP_3R$ (see Results), whereas reported values of $P_{Divalent}/P_K$ permeability ratios for the RyR range between 4 and 8 (Lindsay and Williams, 1991; Liu et al., 1989; Smith et al., 1988; Tinker and Williams, 1992). When channel permeation was studied with mixtures of divalent cations, the anomalous mole fraction effect was not observed (Fig. 4 and Tinker and Williams, [1992]). It is premature to make a final conclusion that $InsP_3R$ and RyR are single-ion channels based solely on the absence of the anomalous mole fraction effect in our experiments and in the experiments of Tinker and Williams (1992) because in some recording conditions this effect is difficult to observe even for known multi-ion channels (Yue and Marban, 1990). Nevertheless, it seems reasonable to assume that both intracellular Ca channels act as single-ion pores until evidence for multiple occupancy are obtained. The similarity in the conduction properties of $InsP_3R$ and RyR is not surprising because the transmembrane domains of the $InsP_3R$ and the

RyR are 40% homologous and both channels are organized as tetramers (Anderson et al., 1989; Chadwick et al., 1990; Furuichi et al., 1989; Mignery, Sudhof, Takei, and De Camilli, 1989; Mignery et al., 1990; Otsu, Willard, Khanna, Zorzato, Green, and MacLennan, 1990; Takeshima, Nishimura, Matsumoto, Ishida, Kangawa, Minamino, Matsuo, Ueda, Hanaoka, Hirose, and Numa, 1989). Thus, it seems reasonable to assume that the permeation pathways of the $InsP_3$ and RyR are constructed in a similar manner.

It is notable that the order of conductances for divalent cations, selectivity against monovalent cations, and the mechanism of ion permeation for both intracellular Ca channels differ dramatically from those obtained in studies of L-type voltage-gated Ca channels of the plasma membrane. L-type Ca channels are extremely selective against monovalent cations ($P_{Divalent}/P_{Monovalent} > 1{,}000$ [Lee and Tsien, 1982, 1984]). To describe the function of the L-type channels a two-site model was proposed (Almers, McCleskey, and Palade, 1984; Hess and Tsien, 1984; Tsien et al., 1987) which explains Ca permeation through these channels as a result of electrical repulsion between Ca ions bound in the two intrapore binding sites. The strongest experimental evidence in support of this model is that with mixtures of cations the anomalous mole fraction effect is observed with the L-type Ca channel (Almers et al., 1984; Hess and Tsien, 1984). The sequence Ba > Ca = Sr ≫ Mg was reported for cardiac L-type channels (Hess et al., 1986) with Mg being practically impermeable. The ability of Mg ions to carry substantial currents through the RyR (Ashley, 1989; Smith et al., 1986, 1988; Tinker and Williams, 1992) and the $InsP_3R$ (Fig. 2 and 3, Table I) is especially striking when the very high hydration energy and extremely slow substitution rate of water molecules in the inner hydration shell of Mg ions (Hille, 1992) is taken into consideration. One possible explanation of this observation is that when Mg ions pass through the selectivity filters of both intracellular Ca channels they are able to keep their inner shell of water molecules. This suggestion implies that the narrowest portion of the channel pore is at least 10 $Å^2$ for both intracellular channels. An even larger estimate of the pore size (40 $Å^2$) was obtained for the RyR (Lindsay et al., 1991) based on the ability of large organic cations like Tris and TEA to permeate through these channels. The idea of a fairly wide pore fits with the observed order of conductances of these channels for divalent cations which corresponds to the order of mobilities of these ions in water.

From the data presented in this report and the studies of RyR permeation, both $InsP_3R$ and RyR act as rather nonspecific cation-selective channels, permeable to Ca, K, and Na. However, under physiological conditions, K and Na are equally distributed across the uncharged membrane of sarcoplasmic reticulum (Somlyo, Shuman, and Somlyo, 1977). Most likely there is no gradient for K and Na across the membrane of endoplasmic reticulum as well. Therefore, the main current through RyR and $InsP_3R$ is carried by Ca due to the large electrochemical gradient for Ca across the reticular membrane. The situation is quite different for voltage-gated Ca channels in the plasma membrane. In the operational voltage range of plasma membrane Ca channels, there is a large driving force for Na ions to enter the cell and for K ions to leave the cell due to concentration and electrical gradients across the plasma membrane. Therefore, voltage-gated Ca channels (for example, L-type) must

discriminate very well against Na and K ions in order to avoid large fluxes of monovalent cations at every opening of a Ca channel.

Are $InsP_3$ Receptors Potentiated by Submillimolar Intraluminal Ca?

Addition of submaximal $InsP_3$ concentrations causes partial discharge of Ca from the intracellular stores. This phenomenon is often called "quantal release" (Muallem, Pandol, and Beeker, 1989). Irvine (1990) suggested that regulation of the $InsP_3R$'s sensitivity to $InsP_3$ by intraluminal Ca concentration may be responsible for quantal release. In support of this hypothesis, a decrease in the rate of $InsP_3$-induced Ca release upon store depletion was demonstrated using permeabilized rat hepatocytes (Nunn and Taylor, 1992) and smooth muscle cells (Missiaen et al., 1992*a,b*). In contrast, no changes in the $InsP_3R$ sensitivity due to the depletion of Ca stores were observed by two other laboratories in experiments with either permeabilized rat hepatocytes (Combettes et al., 1992) or cells from the avian supraorbital nasal gland (Shuttleworth, 1992). The evaluation of these data is difficult, because the absolute levels of intraluminal free Ca concentration and their changes during the experiment are not measurable and can not be controlled. It is quite possible that different levels of intraluminal Ca concentrations were reached by different laboratories during the depletion process. Therefore, the experiments are difficult to compare and the data obtained are controversial. Hence, it is of interest to see whether the regulation of $InsP_3R$ by intraluminal Ca would be evident in planar lipid bilayer experiments, a situation in which the free Ca concentration on both cytoplasmic and intraluminal sides of the membrane can be easily manipulated.

In our experiments using either Sr, Ba or Mg as a current carrier, potentiation of $InsP_3$-gated channel activity by intraluminal Ca was not observed (Fig. 5). In fact, some *inhibitory* effect of intraluminal free Ca in the millimolar range on $InsP_3R$ activity was evident in our experiments (Fig. 5). This inhibitory action of elevated intraluminal Ca (≥ 1 mM; Fig. 5) is very similar to the inhibition of skeletal muscle RyR by intraluminal Ca (Ma et al., 1988). It seems that both intracellular Ca release channels are less likely to open when intraluminal Ca content is very high. Interestingly, a model with inhibition of $InsP_3R$ by high intraluminal Ca levels was recently proposed as a mechanism of Ca oscillations in gonadotrophs (Stojilkovic, Kukuljan, Tomic, Rojas, and Catt, 1993). Thus, the inhibitory effect of intraluminal Ca on $InsP_3R$ observed in our reconstitution experiments may be physiologically significant.

It follows from our experiments (Fig. 5) that the activity of the $InsP_3R$ is not potentiated by intraluminal Ca. If this is the case, then how can the results of Missiaen et al. (1992*a, b*) obtained using permeabilized cells be explained? One possible explanation is that when intracellular stores are depleted in the experiments with permeabilized cells and the free intraluminal Ca concentration falls, the unitary Ca current through $InsP_3R$ decreases below its normal value (Fig. 9) and the rate of $InsP_3$-induced Ca flux is diminished. Because of the high value for the apparent dissociation constant of the divalent cation binding site in the channel pore, equal to 10 mM for Ba (Fig. 9 *B*), one can conclude that in the physiological range of intraluminal free Ca concentrations the elementary current through $InsP_3$-gated channels is a linear function of the free Ca concentration in the lumen of the reticulum. Therefore, the observed decrease in the rate of $InsP_3$-induced Ca release

upon store depletion could be caused not by inhibition of $InsP_3R$ but rather, by a decrease in the size of the unitary Ca current through $InsP_3R$ due to a diminished driving force for Ca ions across the reticular membrane. The best way to describe the ability of the $InsP_3R$ to release Ca as a function of the intraluminal Ca will be to calculate the product of the curves shown on Figs. 5 *B* and 9 *B*. When this is done, an "optimal" intraluminal Ca concentration of ~10 mM corresponding to the maximal rate of $InsP_3$-induced Ca release is expected. Unfortunately, the free intraluminal Ca concentration is not known precisely and it is difficult to estimate changes in the degree of store depletion in the experiments with permeabilized cells. The lack of information regarding the intraluminal Ca concentration makes it difficult to predict $InsP_3R$ behavior in physiological experiments.

Although this explanation seems to be very plausible, other potential explanations for the observed discrepancy between our data and the results reported using permeabilized cells (Missiaen et al., 1992*a*,*b*; Nunn and Taylor, 1992) may include:

(*a*) There is a hypothetical "intraluminal regulatory site" that will bind other divalent cations. Because 55 mM Ba, Sr, or Mg must be present in the intraluminal compartment in order to measure channel currents in planar lipid bilayer experiments, the $InsP_3R$ could be maximally activated at any intraluminal Ca concentration due to the binding of other divalent cation to this site.

(*b*) Some accessory protein that is necessary for regulation of $InsP_3R$ by intraluminal Ca was lost at some point during the isolation and reconstitution of the $InsP_3R$ in the bilayer.

(*c*) Different isoforms of $InsP_3R$ may vary in their regulation by intraluminal Ca. Unfortunately, functional expression of different isoforms of the $InsP_3R$ has not been achieved to date and the distinctive functional characteristics of the $InsP_3R$ isoforms are not yet known. Thus, it is not possible to precisely identify the isoform used in our experiments. It is likely that cultured smooth muscle cells and hepatocytes may primarily express isoforms of the $InsP_3R$ that differ from the type I $InsP_3R$ which is the most abundant isoform found in cerebellum, the tissue used in our study.

It seems that additional experiments performed using a variety of methods will be necessary in order to clarify the issue of $InsP_3R$ regulation by intraluminal Ca. At this point, however, the mechanism for potentiation of $InsP_3R$ activity by increased intraluminal Ca remains controversial.

Kinetic Model of $InsP_3$ Receptor Gating

When $InsP_3$-gated channels were recorded using 55 mM Ca on the *trans* side as a current carrier, the mean open time of the channels was significantly shorter than with other divalent cations (Fig. 2, Table I). High Ca concentration on the *trans* side inhibited the activity of $InsP_3R$ by decreasing both the frequency of channel opening and the mean open time of the channels (Fig. 6). It is possible that these effects of high *trans* Ca concentration on $InsP_3R$ gating indicate the presence of an intraluminal inhibitory Ca binding site of $InsP_3R$ complex. However, we suggested an alternative explanation to the observed phenomenon (see Results and Appendix). We speculated that channel modulation by intraluminal Ca could be explained as a result of an increase in the local Ca concentration in the vicinity of the channel pore on the cytoplasmic side of the membrane every time a channel opens and passes Ca current

(Neher, 1986; Stern, 1992). The local elevation of Ca on the cytoplasmic side of the membrane near the $InsP_3R$ causes channel inactivation due to the bell-shaped dependence of $InsP_3R$ activity on the free Ca level in the cytosol (Bezprozvanny et al., 1991*a*; Finch et al., 1991; Iino, 1990). An advantage of this model is that it is based on the known properties of $InsP_3R$ and it is not necessary to introduce any new functional characteristics of $InsP_3R$, such as a hypothetical intraluminal Ca binding inhibitory site. Notably, a similar model was suggested recently for the skeletal muscle RyR based on Ca-release measurements (Jong et al., 1993).

Based on this hypothesis, we were able to develop the model of $InsP_3R$ gating that was in quantitative agreement with the observed effects of intraluminal Ca on $InsP_3R$ functional properties (see Results and Appendix). The four state model of $InsP_3R$ gating that was used in our calculations is shown in Fig. 10. Although this model is simplified, it adequately reflects the essence of $InsP_3R$ regulation by cytosolic Ca (Bezprozvanny et al., 1991*a*; Finch et al., 1991; Iino, 1990). Inactivation of an open

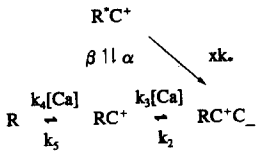

FIGURE 10. Simplified four-state model of $InsP_3R$ regulation by cytosolic Ca (Bezprozvanny, 1994). State R is the $InsP_3R$ without Ca bound, state RC^+ is the $InsP_3R$ with Ca bound to the activating site, state RC^+C_- is the $InsP_3R$ with Ca bound to both activating and inhibitory sites and R^*C^+ is the only open state of the channel. It is assumed that the channel can open only when Ca is bound to the activating site and cannot open when Ca is bound to the inhibitory site. $InsP_3$ is bound to $InsP_3R$ in all four states of the model. The model is based on the bell-shaped Ca dependence of the $InsP_3R$ (Bezprozvanny et al., 1991*a*). The model is simplified because most probably multiple activating and inhibitory sites are present in the $InsP_3R$ complex (Bezprozvanny et al., 1991*a*). However, this model adequately reflects the essence of $InsP_3R$ regulation by cytosolic Ca (Bezprozvanny et al., 1991*a*; Finch et al., 1991; Iino, 1990). Inactivation of an open channel by an increase in the local Ca concentration in the vicinity of an open-channel pore is included in the model by introducing the direct transition from R^*C^+ (*open*) state to RC^+C_- (closed and inactivated) state with a rate constant proportional to the local Ca concentration on the cytoplasmic side (see Eqs. A9 and A10).

channel by an increase in the local Ca concentration in the vicinity of an open-channel pore is included in the model by introducing the direct transition from R^*C^+ (open) to RC^+C_- (closed and inactivated) with a rate constant proportional to the local Ca concentration. The following estimates for the kinetic parameters of $InsP_3R$ gating were obtained from the fit to the data (see Appendix):

$$\alpha = 224\ s^{-1};\ \beta = 25\ s^{-1};\ k_2 = 0.8\ s^{-1};\ k_3 = 4\ \mu M^{-1}s^{-1};\ k_* = 183\ s^{-1} \qquad (3)$$

Thus, not only the effects of intraluminal Ca on $InsP_3R$ function can be explained by immediate negative feedback of elevated cytosolic Ca in the vicinity of a channel pore, but this hypothesis also allowed us to estimate the kinetic parameters of $InsP_3R$ gating. Some predictions about the expected functional properties of the $InsP_3R$ can be made based on the model of $InsP_3R$ gating shown in Fig. 10 with the values of the rate constants from Eq. 3.

It is expected from the model of $InsP_3R$ gating shown in Fig. 10, that if cytosolic Ca concentration is suddenly elevated to a value equal to c ($c \gg k_d^+, k_d^-$), then the $InsP_3R$ will be converted into the inactivated state RC^+C_- with a rate constant approximately equal to $ck_3/(1 + \beta/\alpha)$ (binding of Ca to the activating site is assumed to be much faster than to the inhibitory site). The time course of Ca-induced inactivation of the $InsP_3R$ was studied in experiments with brain microsomes using a rapid superfusion system (Finch et al., 1991) and with permeabilized smooth muscle cells using caged Ca (Iino and Endo, 1992). In the experiments with brain microsomes (Finch et al., 1991) a conditioning pulse of 10 μM Ca^{2+} was given for varying durations and the microsomes were challenged with 1 μM $InsP_3$. The characteristic time for $InsP_3R$ inactivation by Ca under these conditions was 580 ms (Finch et al., 1991). In the experiments with permeabilized smooth muscle cells (Iino and Endo, 1992) the cytosolic Ca concentration was suddenly elevated by flash photolysis of DM-nitrophen from ~270 nM to ~850 nM in the presence of 3 μM $InsP_3$ and immediate slowing of the rate of $InsP_3$-induced Ca release was observed. Unfortunately, it is not possible to estimate exactly the time constant of $InsP_3R$ inactivation from the data of Iino and Endo (1992). Nonetheless, it is obvious that divergent values for the rate of $InsP_3R$ inactivation by Ca have been obtained using different methods. Based on the rate constants from Eq. 3, the characteristic time of $InsP_3R$ conversion to the inactivated state estimated for experimental conditions similar to those used with the brain microsomes should be 25 ms, and for those used with permeabilized smooth muscle cells should be 320 ms. Additional measurements of the rate of Ca-induced inactivation of the $InsP_3R$ as a function of the Ca concentration with high temporal resolution are necessary to test the model.

The value of k_2 equal to only 0.8 s^{-1} means that recovery of the $InsP_3R$ from Ca-induced inactivation should take on average 1.25 s. Moreover, if the presence of several inhibitory sites in the $InsP_3R$ complex is taken into consideration and it is assumed that binding of a Ca ion to at least one of these sites is sufficient to prevent channel opening, then this time will be even longer. For example, it is easily shown that for four inhibitory sites the average time of recovery is 2.6 s. Indeed, when the time course of $InsP_3R$ recovery from Ca-induced inactivation was studied in *Xenopus* oocytes (Parker and Ivorra, 1990) or in brain synaptosomes (Finch et al., 1991), a characteristic time of recovery in the range of several seconds was measured, in agreement with our estimations.

The open-channel probability of the $InsP_3R$ in planar lipid bilayer experiments is in the range 5–12% under optimal conditions (Fig. 1; Bezprozvanny and Ehrlich, 1993*a*; Bezprozvanny et al., 1991a; Watras et al., 1991). This number has to be viewed as an upper estimate because of the uncertainty about the number of active channels in the bilayer. It follows that $InsP_3$ is a partial agonist of the $InsP_3R$ which means that $InsP_3$ has a low *intrinsic efficacy* (Bezprozvanny and Ehrlich, 1993*a*; Pallotta, 1991). One possible explanation for this fact comes from the model of $InsP_3R$ gating shown in Fig. 10. Indeed, even at an optimal cytosolic Ca concentration, the $InsP_3R$ spends just a fraction of its time in the RC^+ state, the only state from which the channel can open. Therefore, the intrinsic efficacy of $InsP_3$ is decreased. The intrinsic efficacy of $InsP_3$ is also affected by ATP (Bezprozvanny and Ehrlich, 1993*a*), an allosteric regulator of the $InsP_3R$. Although a high concentration of $InsP_3$

(2 μM) was used in our experiments, this concentration may not suffice to saturate $InsP_3$ binding sites completely (Bezprozvanny, Bezprozvannaya, and Ehrlich, 1994; Watras et al., 1991). In our model (Fig. 10) the effects of submaximal ATP and $InsP_3$ concentrations on channel gating are built into the rate constants α and β. All these factors influence the open probability of $InsP_3R$ observed in our experiments. From the model of $InsP_3R$ gating considered in this report (Fig. 10) a maximal open channel probability of only 3.6% (at 0.2 μM Ca^{2+}) is calculated based on the rate constant estimations from Eq. 3, in agreement with the observed values.

The mean open time of the $InsP_3R$ under physiological conditions can be estimated from the model shown in Fig. 10. The rate constant of the direct transition from R^*C^+ to RC^+C_- (Fig. 10) is proportional to the local Ca concentration in the vicinity of a channel pore $[Ca]^*$; the local Ca concentration in the vicinity of the pore is, in turn, proportional to the size of Ca current through $InsP_3R$ (Eqs. A1 and A2). The size of the unitary Ca current through $InsP_3R$ in the physiological situation is estimated to be ~0.5 pA (see below), four times less ($x = 0.25$) than the size of the single-channel current in the series of experiments used to estimate $InsP_3R$ kinetic parameters (Fig. 6 *B*). According to the model shown in Fig. 10 the mean open time of the $InsP_3R$ under physiological conditions is (Eq. A11) $\tau_0 = 1/(\alpha + xk_*) = 3.7$ ms, if numerical values for α and $k_*{}^\circ$ are taken from Eq. 3. Note that only one out of five channel closings occurs by direct transition from the open to the inactivated state because $xk_*/(\alpha + xk_*) \sim 0.2$ under physiological conditions. Thus, in the first approximation, the simplified model of channel gating that does not take this direct transition into account can be used for the analysis of $InsP_3R$ behavior under physiological conditions as it was recently done in a paper devoted to the theoretical analysis of Ca wave propagation (Bezprozvanny, 1994).

Physiological Size of Ca Current through $InsP_3$ Receptors

To estimate the size of the unitary current through the RyR under physiological conditions, Tinker et al. (Tinker et al., 1992, 1993) developed a quantitative model for ion conduction through the RyR with the use of Eyring rate theory (Hille, 1992). This model allowed them to estimate the size of the unitary Ca current through the RyR to be ~2 pA at a transmembrane potential of 0 mV under physiological conditions (Tinker et al., 1992, 1993). Unfortunately, we were more limited in our study of $InsP_3R$ permeation due to the presence of K and Cl channels in the membrane. Because we did not have the luxury of being able to create a mathematical model of the energetic profile of $InsP_3R$ permeation pathway, we had to resort to approximations of the unitary Ca current through the $InsP_3R$ with the ionic conditions expected in the physiological situation. Although the intraluminal free Ca concentration in the endoplasmic and sarcoplasmic reticulum is not precisely known, it is believed to be somewhere in the millimolar range. To compare $InsP_3R$ and RyR properties we will estimate the size of the unitary Ca current through $InsP_3R$ assuming that the intraluminal free Ca concentration is 2.5 mM and that 110 mM K is present on both sides of the reticular membrane; these are the same conditions used in the calculations of the Ca current through the RyR (Tinker et al., 1992, 1993). Because K, Na, and Cl ions are evenly distributed across the membrane of sarcoplasmic reticulum (Somlyo et al., 1977) there is no voltage across this mem-

brane. Most likely endoplasmic reticulum membrane is also uncharged and therefore, it is assumed in the following estimates that the transmembrane voltage is 0 mV.

In our experiments, the amplitude of the unitary current through $InsP_3$-gated channels was measured at a transmembrane potential of 0 mV and at different intraluminal Ba concentrations (5–72 mM) in the presence of 110 mM symmetrical K (Fig. 9 *B, open circles*). Because there was no driving force for K ions at 0 mV under these conditions, the recorded current was carried by divalent (Ba) ions. The data were fit using the Michaelis-Menten equation with an apparent dissociation constant for Ba of 9.0 mM and a maximal unitary current of 3.4 pA (Fig. 9 *B, smooth curve*). If these parameter values are used, then the unitary Ba current through $InsP_3R$ at 2.5 mM intraluminal Ba and 110 mM symmetrical K is equal to 0.74 pA.

In the series of experiments discussed above, Ba was the current carrier. However, Ca ions go through $InsP_3R$ in cells. When $InsP_3$-gated channels were recorded under standard conditions, that is, in the presence of 55 mM divalent cation in the *trans* chamber and Tris-HEPES solution in the *cis* chamber, the size of the Ca unitary current was equal to 64% of the unitary Ba current. After introducing this correction, the size of the unitary Ca current through the $InsP_3R$ at a transmembrane potential of 0 mV under physiological conditions (2.5 mM intraluminal free Ca; 110 mM symmetrical K) is equal to 0.47 pA, fourfold less than the Ca current through the cardiac RyR under exactly the same conditions (Tinker et al., 1992, 1993). As it was pointed out already, because of the high apparent k_d for divalent cations, the elementary Ca current through the $InsP_3R$ should be a linear function of the intraluminal free Ca concentration in the physiological range. Of course, the numbers obtained must be viewed as estimations until detailed studies of permeation through the purified $InsP_3R$ for monovalent and divalent cations are conducted, the intraluminal free Ca concentration is known, and the voltage across the membrane of endoplasmic reticulum is measured.

How many Ca ions are released into the cytosol every time an $InsP_3R$ opens? The mean open time of the $InsP_3$-gated channels under physiological conditions was estimated to be 3.7 ms (see preceding section). Taking this number and the size of the Ca current of 0.47 pA estimated above, a value of 5,400 Ca ions/channel opening is obtained. This number and estimates of $InsP_3R$ density should be used when modeling spatiotemporal aspects of Ca signaling. It is notable that not only is the size of the Ca current through the RyR fourfold higher, but the mean open time of RyR type channels measured under similar conditions is ~20 ms (Smith et al., 1986), five times longer than for the $InsP_3R$. As a result, at every channel opening ~20 times more Ca ions are released through the RyR than through the $InsP_3R$. One can speculate that the coexistence of two intracellular Ca channel types activated via different mechanisms allows the cell to choose between two modes of Ca release: rapid dumping of accumulated Ca through the RyR channel or slow leakage through the $InsP_3R$. This suggestion is also supported by the observation that the RyR can be activated to an open probability of virtually 100% (Smith et al., 1986), whereas the $InsP_3R$ has never been observed with an open probability greater than 10% (Fig. 1; Bezprozvanny and Ehrlich, 1993*a*; Bezprozvanny et al., 1991*a*; Watras et al., 1991). In specialized cells such as cardiac and skeletal muscle cells that need very rapid Ca release to initiate contraction in milliseconds, $InsP_3R$ are essentially absent, whereas

RyR are abundant (Anderson et al., 1989; Imagawa, Smith, Coronado, and Campbell, 1987; Lai et al., 1988). In contrast, smooth muscle cells, which control the steady level of tension by changing the rate of Ca efflux into the cytoplasm in minutes, employ predominantly $InsP_3R$ for Ca transport (Chadwick et al., 1990; Walker, Somlyo, Goldman, Somlyo, and Trentham, 1987; Watras and Benevolensky, 1987). It is interesting to note that both receptor types are found in the brain (Bezprozvanny et al., 1991*a;* Ellisman, Deernick, Ouyang, Beck, Tanksley, Walton, Airey, and Sutko, 1990; McPherson, Kim, Valdivia, Knudson, Takekura, Franzini-Armstrong, Coronado, and Campbell, 1991; Ross, Meldolesi, Milner, Satoh, Supattapone, and Snyder, 1989; Supattapone et al., 1988b), suggesting additional complexity in neuronal Ca signaling.

APPENDIX

Quantitative Model of $InsP_3R$ Modulation by Intraluminal Ca

In the results section the inhibitory effects of high intraluminal Ca levels on $InsP_3R$ gating are described. A model was suggested explaining this effect as a result of an increase in the local Ca concentration in the vicinity of the pore on the cytoplasmic side of the membrane. In this Appendix, the predictions of the model are compared with the experimental data and kinetic parameters of $InsP_3R$ gating are extracted from fits of the model to the data. In the vicinity of an open channel the free Ca concentration on the cytoplasmic side ([Ca]*) is equal to (Stern, 1992; Jong et al., 1993; Neher, 1986):

$$[Ca]^* = \phi/2\pi Dr + [Ca] \tag{A1}$$

where ϕ is the flux of Ca ions through the open channel, D is the diffusion constant for Ca ions in the *cis* solution, r is the distance from the mouth of a channel, and [Ca] is the free Ca concentration in the *cis* chamber which is equal to 0.2 μM in our experiments. The value of [Ca]* is not affected by the presence of 1 mM EGTA in the *cis* chamber (Stern, 1992). The increase of Ca level near the mouth of channel to [Ca]* happens within a few microseconds after opening of a channel (Jong et al., 1993).

The flux of Ca ions through the open channel is equal to:

$$\phi = i_{Ca}/2F \tag{A2}$$

where i_{Ca} is the Ca current through the channel and F is the Faraday constant. Let the parameter x be equal to the Ca molar fraction in the *trans* chamber:

$$x = [Ca]_{trans}/([Ca]_{trans} + [Sr]_{trans}). \tag{A3}$$

Because $InsP_3$-gated channels seem to function as single ion pores, the affinity of the binding site for different divalent cations are similar (see discussion of Fig. 4), and the value of the single-channel current in our experiments did not change significantly with an increase in Ca *trans* molar fraction (Fig. 6 *B, right*):

$$i_{Ca} = xi \tag{A4}$$

where i is the observed value of single-channel current through the $InsP_3R$. From Eqs. A1, A2, and A4:

$$[Ca]^* = xi/4\pi FDr + [Ca]. \tag{A5}$$

At macromolecular distances (r = 20 nm) for 2 pA of current (typical value in our experiments):

$$i/4\pi FDr = 100\ \mu M \gg 0.2\ \mu M = [Ca]. \tag{A6}$$

Therefore, in the first approximation:

$$\begin{aligned} [Ca]^* &= xc \\ c &= i/4\pi FDr \end{aligned} \tag{A7}$$

where parameter c does not change with an increase in the *trans* Ca mole fraction in our experiments. The conclusion from Eq. A7 is that in our experiments the free Ca concentration in the vicinity of an open channel on the *cis* side is directly proportional to the Ca mole fraction on the *trans* side.

In modeling $InsP_3R$ regulation by Ca, it is necessary to consider at least four distinct kinetic states of the $InsP_3R$ because of the bell-shaped Ca dependence of the channels (Bezprozvanny et al., 1991*a;* Finch et al., 1991; Iino, 1990). A simplified model of $InsP_3$ channel regulation by Ca with one activating and one inhibitory site is (Bezprozvanny, 1994):

$$\begin{array}{ccccc} & & R^*C^+ & & \\ & & \beta \uparrow\downarrow \alpha & & \\ R & \underset{k_5}{\overset{k_4[Ca]}{\rightleftharpoons}} & RC^+ & \underset{k_2}{\overset{k_3[Ca]}{\rightleftharpoons}} & RC^+C_- \end{array} \tag{A8}$$

where R is the $InsP_3R$, RC^+ is the $InsP_3R$ with Ca bound at the activating site, RC^+C_- is the $InsP_3R$ with Ca bound at both activating and inhibitory sites and R^*C^+ is the only open state of the channel. The main focus of this model presented here is to describe the regulation of $InsP_3R$ by Ca. Because our experiments were conducted in the presence of high concentrations of $InsP_3$ (2 μM) and ATP (0.5 mM) it is assumed that both agonists (Bezprozvanny and Ehrlich, 1993*a;* Watras et al., 1991) are bound to the receptor in all four states of the model.

Inactivation of an open channel by an increase in the local Ca concentration in the vicinity of a channel pore is included in the model of channel gating by introducing the direct transition from the R^*C^+ state to the RC^+C_- state with the rate constant proportional to [Ca]*:

$$\begin{array}{ccccc} & & R^*C^+ & & \\ & & \beta \uparrow\downarrow \alpha \quad \searrow^{k_1[Ca]^*} & & \\ R & \underset{k_5}{\overset{k_4[Ca]}{\rightleftharpoons}} & RC^+ & \underset{k_2}{\overset{k_3[Ca]}{\rightleftharpoons}} & RC^+C_- \end{array} \tag{A9}$$

It is assumed that a channel in the RC^+C_- state can not open until Ca is dissociated

from inhibitory site; that is, the transition from R^*C^+ to RC^+C_- is irreversible. The proposed mechanism of $InsP_3$-gated channel inactivation by an increase in the local Ca concentration is identical to the mechanism suggested previously for the skeletal muscle RyR (Jong et al., 1993).

From Eq. A7:

$$k_1[\mathrm{Ca}]^* = k_1 xc = xk.$$
$$k_* = k_1 c. \tag{A10}$$

Therefore, the rate of transition from R^*C^+ to RC^+C_- is directly proportional to the Ca mole fraction on the *trans* side in our experiments. This result is valid only if intraluminal Ca does not have other effects on $InsP_3R$ gating besides those caused by alterations in the local cytosolic Ca concentration.

Using the method of Colquhoun and Hawkes (Colquhoun and Hawkes, 1982) it can be shown that for the model (Eq. A9) the mean open time of the channels and the observed mean closed time of the channels (the mean of all shut times) are equal to (taking Eq. A10 into consideration):

$$\tau_o = 1/(\alpha + xk_*) \tag{A11}$$

$$\tau_c = (1 + k_3[\mathrm{Ca}]/k_2 + k_5/k_4[\mathrm{Ca}])/\beta + xk_*/[(\alpha + xk_*)k_2] \tag{A12}$$

Let us introduce the parameter β':

$$\beta' = \beta/(1 + k_3[\mathrm{Ca}]/k_2 + k_5/k_4[\mathrm{Ca}]) \tag{A13}$$

Then, Eq. A12 becomes:

$$\tau_c = 1/\beta' + xk_*/[(\alpha + xk_*)k_2] \tag{A14}$$

As a result, both the mean open time of the channels (Eq. A11) and the observed mean closed time of the channels (Eq. A14) are now expressed as simple functions of the Ca mole fraction on the *trans* side (x). From Eq. A11, the mean rate of channel closing should be a linear function of Ca *trans* mole fraction:

$$\text{mean rate of channel closing} = 1/\tau_o = \alpha + xk_* \tag{A15}$$

A reasonable fit to the experimental data (Fig. 11 *A*) is provided by Eq. A15 assuming $\alpha = 224\ s^{-1}$, $k_* = 183\ s^{-1}$.

The mean duration of channel shut times as a function of the Ca *trans* mole fraction was measured in the same experiment. According to the model (Eq. A9) the distribution of all shut times must be fit by the sum of three exponential functions. Therefore, it is not correct to use a single exponential fit to the distribution of the observed closed times in this context. We estimated the mean shut time by calculating the ratio of total shut time of the channels and the number of openings detected in the same segment of data. The value obtained was multiplied by the number of channels active in the membrane (equal to 2 in the experiment under consideration) in order to obtain a value for the mean shut time of a single channel. Because the single-channel open probability was low (<10%) in our experiments, simultaneous openings of several channels were very infrequent and only a small error was introduced by applying this procedure to multichannel recordings. Eq. A14 provides

an adequate fit to the data (Fig. 11 *B*) if $\beta' = 8.4\ s^{-1}$, $k_2 = 0.8\ s^{-1}$ and the parameters α and k_* are taken from the fit to the mean open time (Fig. 11 *A*).

From the bell-shaped Ca dependence of $InsP_3$-gated channels, we previously estimated (Bezprozvanny et al., 1991*a*) that

$$k_5/k_4 = k_d^+ = k_2/k_3 = k_d^- = 0.2\ \mu M = [Ca] \quad (A16)$$

where k_d^+ and k_d^- are the affinities of the activating and inhibitory sites for Ca, respectively.

From Eqs. A16 and A13 and the estimation of $\beta' = 8.4$, s^{-1} obtained above:

$$\beta = (1 + k_3[Ca]/k_2 + k_5/k_4[Ca])\beta' = 3\beta' = 25.2\ s^{-1} \quad (A17)$$

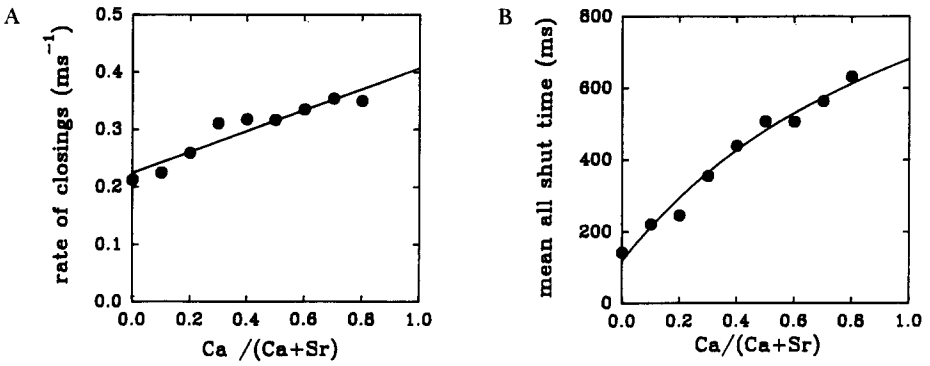

FIGURE 11. Dependence of $InsP_3R$ gating kinetic parameters on the *trans* Ca molar fraction. Data were fit using the model of $InsP_3R$ gating described in the text (Eq. A9). (*A*) Dependence of the mean rate of channel closing ($1/\tau_0$) on the *trans* Ca molar fraction (x) was fit with a linear regression according to Eq. A15. The values of the mean open-time constants (τ_0) were obtained at every *trans* Ca molar fraction from a single exponential fit to the open time distributions as shown on Fig. 6 *B*. The best fit to the data was obtained assuming $\alpha = 224\ s^{-1}$, $k_* = 183\ s^{-1}$. (*B*) Dependence of the mean of all shut times (τ_c) on the *trans* Ca molar fraction (x) was fit using Eq. A14. The value of the mean of all shut times at every *trans* Ca molar fraction was determined as described in the text. The best fit to the data was obtained assuming $\beta' = 8.4\ s^{-1}$, $k_2 = 0.8\ s^{-1}$ and the parameters α and k_* taken from the fit to the rate of channel closing shown in *A*. The data are from the experiment shown in Fig. 6.

From Eq. A16 and the estimation of $k_2 = 0.8\ s^{-1}$,

$$k_3 = k_2/k_d^- = 4\ \mu M^{-1}\ s^{-1} \quad (A18)$$

It seems reasonable to assume that $k_3 = k_1$, because both constants represent the rates of Ca binding to the same inhibitory site of the $InsP_3R$ (assuming that the channel closes immediately after Ca is bound in the inhibitory site). If this is true, then for maximal i_{Ca} current ($x = 1$; $i_{Ca} = i = 1.5$ pA) from our estimation of $k_* = 183\ s^{-1}$:

$$[Ca]^* = k_*/k_1 = 46\ \mu M. \quad (A19)$$

This value is in the range of local Ca concentrations expected for the macromo-

lecular distances from the mouth of an open channel passing 1.5 pA Ca current (Stern, 1992). Therefore, the model of channel inactivation by an increase in local Ca concentration described above makes physical sense.

Because the activity of the $InsP_3R$ in our experiments was measured at optimal cytoplasmic Ca levels (0.2 μM Ca), additional increases in local Ca levels could only inhibit $InsP_3R$ channel activity. Therefore, Ca-induced activation of the $InsP_3R$ (Bezprozvanny et al., 1991*a*; Finch et al., 1991; Iino, 1990) was not a factor in the experiments testing the effects of intraluminal Ca on channel gating and no estimations of k_4 and k_5 can be obtained from the experimental data in this paper.

Finally, the following kinetic diagram of $InsP_3R$ gating that is in agreement with our data can be drawn using the values of the rate constants obtained above assuming [Ca] = 0.2 μM, as it was in our experiments:

$$\begin{array}{ccccc} & & R^*C^+ & & \\ & & 25.2 \uparrow\downarrow 224 & \searrow^{183x} & \\ R & \underset{k_5}{\overset{k_4[Ca]}{\rightleftharpoons}} & RC^+ & \underset{0.8}{\overset{0.8}{\rightleftharpoons}} & RC^+C_- \end{array} \qquad \text{(A20)}$$

The estimates obtained for the rate constants are based on the assumption that the only mechanism responsible for the effects of *trans* Ca on the $InsP_3R$ gating is an immediate negative feedback caused by an increase in the local Ca concentration in the vicinity of a channel pore. However, additional effects of intraluminal Ca on the gating of the channels can not be completely ruled out. It is necessary to note that several inhibitory and activating Ca binding sites are actually present in the channel complex (Bezprozvanny et al., 1991*a*). Therefore, a more complicated kinetic model may need to be considered for an exact determination of the rate constants. It was also assumed in our calculations that $InsP_3$ and ATP were bound to the receptor continuously, which probably is an oversimplification. However, the simplified model presented above adequately reflects the essence of $InsP_3R$ regulation by cytoplasmic Ca and can explain the effects of intraluminal Ca on channel gating observed in our experiments.

This paper is dedicated to the late A. P. Naumov who initiated I. Bezprozvanny's work on $InsP_3$-gated channels and was a constant source of encouragement and inspiration. The authors thank Drs. Larry Cohen, Knox Chandler, and Laurinda Jaffe for comments on the manuscript and Dr. Knox Chandler for detailed discussions of the model of $InsP_3R$ gating. We are especially grateful for the expert technical assistance from Svetlana Bezprozvannaya.

This work was supported by NIH grants HL 33026 and GM 39029, and a grant-in-aid from the Patrick and Catherine Weldon Donaghue Medical Research Foundation.

Original version received 28 February 1994 and accepted version received 23 May 1994.

REFERENCES

Almers, W., E. W. McCleskey, and P. T. Palade. 1984. A non-selective cation conductance in frog muscle membrane blocked by micromolar external calcium ions. *Journal of Physiology.* 353:565–583.

Almers, W., and E. W. McClesky. 1984. Non-selective conductance in calcium channels in frog muscle: calcium selectivity in a single-file pore. *Journal of Physiology.* 353:585–608.

Anderson, K., F. A. Lai, Q.-Y. Liu, E. Rousseau, H. P. Erickson, and G. Meissner. 1989. Structural and functional characterization of the purified cardiac ryanodine receptor-Ca^{2+} release channel complex. *Journal of Biological Chemistry.* 264:1329–1335.

Ashley, R. H. 1989. Activation and conductance properties of ryanodine-sensitive calcium channels from brain microsomal membranes incorporated into planar lipid bilayers. *Journal of Membrane Biology.* 111:179–189.

Berridge, M. J. 1993. Inositol trisphosphate and calcium signalling. *Nature.* 361:315–325.

Berridge, M. J., and R. F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature.* 341:197–205.

Bezprozvanny, I. 1994. Theoretical analysis of calcium wave propagation based on inositol (1,4,5)-trisphosphate ($InsP_3$) receptor functional properties. *Cell Calcium.* 16:151–166.

Bezprozvanny, I., S. G. Bezprozvannaya, and B. E. Ehrlich. 1994. Caffeine-induced inhibition of inositol (1,4,5)-trisphosphate-gated calcium channels from cerebellum. *Molecular Biology of the Cell.* 5:97–103.

Bezprozvanny, I., and B. E. Ehrlich. 1993*a*. ATP modulates the function of inositol 1,4,5-trisphosphate-gated channels at two sites. *Neuron.* 10:1175–1184.

Bezprozvanny, I., and B. E. Ehrlich. 1993*b*. Divalent cation conduction of the inositol 1,4,5-trisphosphate gated calcium channels of canine cerebellum. *Biophysical Journal.* 64:A328. (Abstr.)

Bezprozvanny, I., and B. E. Ehrlich. 1994. How small is the "quantum" of inositol 1,4,5-trisphosphate induced Ca release? *Biophysical Journal.* 66:A146. (Abstr.)

Bezprozvanny, I., J. Watras, and B. E. Ehrlich. 1991*a*. Bell-shaped calcium-response curves of $Ins(1,4,5)P_3$- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature.* 351:751–754.

Bezprozvanny, I. B., D. S. Benevolensky, and A. P. Naumov. 1991*b*. Potassium channels in aortic microsomes: conductance, selectivity, barium-induced blockage and subconductance states. *Biochimica et Biophysica Acta.* 1064:75–80.

Blondel, O., J. Takeda, H. Janssen, S. Seino, and G. I. Bell. 1993. Sequence and functional characterization of a third inositol trisphosphate receptor subtype, IP_3R-3, expressed in pancreatic islets, gastrointestinal tract, and other tissues. *Journal of Biological Chemistry.* 268:11356–11363.

Chadwick, C. C., A. Saito, and S. Fleischer. 1990. Isolation and characterization of the inositol trisphosphate receptor from smooth muscle. *Proceedings of the National Academy of Sciences, USA.* 87:2132–2136.

Colquhoun, D., and A. G. Hawkes. 1982. On the stochastic properties of bursts of single ion channel openings and of clusters of bursts. *Proceedings of the Royal Society (London) B.* 300:1–59.

Colquhoun, D., and A. G. Hawkes. 1983. The principles of stochastic interpretation of ion-channel mechanisms. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., NY. 135–174.

Combettes, L., M. Claret, and P. Champeil. 1992. Do submaximal $InsP_3$ concentrations only induce the partial discharge of permeabilized hepatocyte calcium pools because of the concomitant reduction of intraluminal Ca^{2+} concentration? *FEBS Letters.* 301:287–290.

Coronado, R., R. L. Rosenberg, and C. Miller. 1980. Ionic selectivity, saturation, and block in a K^+-selective channel from sarcoplasmic reticulum. *Journal of General Physiology.* 76:425–446.

Ehrlich, B. E., and J. Watras. 1988. Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature.* 336:583–586.

Ellisman, M. H., T. J. Deerinck, Y. Ouyang, C. F. Beck, S. J. Tanksley, P. D. Walton, J. A. Airey, and J. L. Sutko. 1990. Identification and localization of ryanodine binding proteins in the avian central nervous system. *Neuron.* 5:135–146.

Fabiato, A. 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods in Enzymology.* 157:378–417.

Ferris, C. D., A. M. Cameron, D. S. Bredt, R. L. Huganir, and S. S. Snyder. 1991*a*. Inositol 1,4,5-trisphosphate receptor is phosphorylated by cyclic AMP-dependent protein kinase at serines 1755 and 1589. *Biochemical and Biophysical Research Communications.* 175:192–198.

Ferris, C. D., R. L. Huganir, D. S. Bredt, A. M. Cameron, and S. H. Snyder. 1991*b*. Inositol trisphosphate receptor-phosphorylation by protein kinase-C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles. *Proceedings of the National Academy of Sciences, USA.* 88:2232–2235.

Ferris, C. D., R. L. Huganir, and S. H. Snyder. 1990. Calcium flux mediated by purified inositol 1,4,5-trisphosphate receptor in reconstituted lipid vesicles is allosterically regulated by adenine nucleotides. *Proceedings of the National Academy of Sciences, USA.* 87:2147–2151.

Ferris, C. D., R. L. Huganir, S. Supattapone, and S. H. Snyder. 1989. Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. *Nature.* 342:87–89.

Finch, E. A., T. J. Turner, and S. M. Goldin. 1991. Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science.* 252:443–446.

Furuichi, T., S. Yoshikawa, A. Miyawaki, K. Wada, N. Maeda, and K. Mikoshiba. 1989. Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P_{400}. *Nature.* 342:32–38.

Ghosh, T. K., P. S. Eis, J. M. Mullaney, C. L. Ebert, and D. L. Gill. 1988. Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. *Journal of Biological Chemistry.* 263:11075–11079.

Hess, P., J. B. Lansman, and R. W. Tsien. 1986. Calcium channel selectivity for divalent and monovalent cations. *Journal of General Physiology.* 88:293–319.

Hess, P., and R. W. Tsien. 1984. Mechanism of ion permeation through calcium channels. *Nature.* 309:453–456.

Hille, B. 1992. Ionic Channels of Excitable Membranes. Sinauer Associates, Inc., Sunderland, MA. 607 pp.

Horn, R. 1991. Estimating the number of channels in patch recordings. *Biophysical Journal.* 60:433–439.

Iino, M. 1990. Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig *taenia caeci*. *Journal of General Physiology.* 95:1103–1122.

Iino, M. 1991. Effects of adenine nucleotides on inositol 1,4,5-trisphosphate-induced calcium release in vascular smooth muscle cells. *Journal of General Physiology.* 98:681–698.

Iino, M., and M. Endo. 1992. Calcium-dependent immediate feedback control of inositol 1,4,5-trisphosphate-induced Ca release. *Nature.* 360:76–78.

Imagawa, T., J. Smith, R. Coronado, and K. Campbell. 1987. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca-permeable pore of the calcium release channel. *Journal of Biological Chemistry.* 262:16636–16643.

Irvine, R. F. 1990. "Quantal" Ca release and the control of Ca entry by inositol phosphates—a possible mechanism. *FEBS Letters.* 263:5–9.

Jong, D.-S., P. C. Pape, W. K. Chandler, and S. M. Baylor. 1993. Reduction of calcium inactivation of sarcoplasmic reticulum calcium release by Fura-2 in voltage-clamped cut twitch fibers from frog muscle. *Journal of General Physiology.* 102:333–370.

Kobayashi, S., A. V. Somlyo, and A. P. Somlyo. 1988. Heparin inhibits the inositol 1,4,5-triphosphate-dependent, but not the independent, calcium release induced by guanine nucleotide in vascular smooth muscle. *Biochemical and Biophysical Research Communications.* 153:625–631.

Kume, S., A. Muto, J. Aruga, T. Nakagawa, T. Michikawa, T. Furuichi, S. Nakade, H. Okano, and K. Mikoshiba. 1993. The *Xenopus* IP_3 receptor: structure, function, and localization in oocytes and eggs. *Cell.* 73:555–570.

Lai, F. A., H. P. Erickson, E. Rousseau, Q.-Y. Liu, and G. Meissner. 1988. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature.* 331:315–319.

Lansman, J. B., P. Hess, and R. W. Tsien. 1986. Blockage of current through single calcium channels by Cd^{2+}, Mg^{2+}, and Ca^{2+}. *Journal of General Physiology.* 88:321–347.

Lee, K. S., and R. W. Tsien. 1982. Reversal of current through calcium channels in dialysed single heart cells. *Nature.* 297:498–501.

Lee, K. S., and R. W. Tsien. 1984. High selectivity of calcium channels in single dialysed heart cells of the guinea-pig. *Journal of Physiology.* 354:253–272.

Lindsay, A. R. G., S. D. Manning, and A. J. Williams. 1991. Monovalent cation conductance in the ryanodine receptor-channel of sheep cardiac muscle sarcoplasmic reticulum. *Journal of Physiology.* 439:463–480.

Lindsay, A. R. G., and A. J. Williams. 1991. Functional characterization of the ryanodine receptor purified from sheep cardiac muscle sarcoplasmic reticulum. *Biochimica et Biophysica Acta.* 1064:89–102.

Liu, Q.-Y., F. A. Lai, E. Rousseau, R. V. Jones, and G. Meissner. 1989. Multiple conductance states of the purified calcium release channel complex from skeletal sarcoplasmic reticulum. *Biophysical Journal.* 55:415–424.

Ma, J., M. Fill, C. M. Knudson, K. P. Campbell, and R. Coronado. 1988. Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science.* 242:99–102.

Maeda, N., T. Kawasaki, S. Nakade, N. Yokota, T. Taguchi, M. Kasai, and K. Mikoshiba. 1991. Structural and functional characterization of inositol 1,4,5-trisphosphate receptor channel from mouse cerebellum. *Journal of Biological Chemistry.* 266:1109–1116.

McPherson, P. S., Y. K. Kim, H. Valdivia, C. M. Knudson, H. Takekura, C. Franzini-Armstrong, R. Coronado, and K. P. Campbell. 1991. The brain ryanodine receptor—a caffeine-sensitive calcium release channel. *Neuron.* 7:17–25.

Mignery, G., T. C. Sudhof, K. Takei, and P. De Camilli. 1989. Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. *Nature.* 342:192–195.

Mignery, G. A., C. L. Newton, B. T. Archer, and T. C. Sudhof. 1990. Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. *Journal of Biological Chemistry.* 265:12679–12685.

Missiaen, L., H. De Smedt, G. Droogmans, and R. Casteels. 1992*a*. Ca release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca in permeabilized cells. *Nature.* 357:599–602.

Missiaen, L., H. De Smedt, G. Droogmans, and R. Casteels. 1992*b*. Luminal Ca controls the activation of the inositol 1,4,5-trisphosphate receptor by cytosolic Ca. *Journal of Biological Chemistry.* 267: 22961–22966.

Mourey, R. J., A. Verma, S. Supattapone, and S. H. Snyder. 1990. Purification and characterization of the inositol 1,4,5-trisphosphate receptor protein from rat vas deferens. *Biochemical Journal.* 272:383–389.

Muallem, S., S. J. Pandol, and T. G. Beeker. 1989. Hormone-evoked calcium release from intracellular stores is a quantal process. *Journal of Biological Chemistry.* 264:205–212.

Neher, E. 1986. Concentration profiles of intracellular calcium in the presence of a diffusible chelator. *Experimental Brain Research.* 14:80– 96.

Nunn, D. L., and C. W. Taylor. 1992. Luminal Ca^{2+} increases the sensitivity of Ca^{2+} stores to inositol 1,4,5-trisphosphate. *Molecular Pharmacology.* 41:115–119.

Otsu, K., H. F. Willard, V. K. Khanna, F. Zorzato, N. M. Green, and D. H. MacLennan. 1990. Molecular cloning of cDNA encoding the Ca^{2+} release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *Journal of Biological Chemistry.* 265:13472–13483.

Pallotta, B. S. 1991. Single ion channel's view of classical receptor theory. *FASEB Journal.* 5:2035–2043.

Parker, I., and I. Ivorra. 1990. Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: a possible mechanism for oscillatory release of Ca^{2+}. *Proceedings of the National Academy of Sciences. USA.* 87:260–264.

Richardson, A., and C. W. Taylor. 1993. Effects of Ca^{2+} chelators on purified inositol 1,4,5-trisphosphate ($InsP_3$) receptors and $InsP_3$-stimulated Ca^{2+} mobilization. *Journal of Biological Chemistry.* 268:11528–11533.

Ross, C. A., J. Meldolesi, T. A. Milner, T. Satoh, S. Supattapone, and S. H. Snyder. 1989. Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje neurons. *Nature.* 339:468–470.

Shuttleworth, T. J. 1992. Ca^{2+} release from inositol trisphosphate-sensitive stores is not modulated by intraluminal Ca^{2+}. *Journal of Biological Chemistry.* 267:3573–3576.

Smith, J., R. Coronado, and G. Meissner. 1986. Single-channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. Activation by Ca^{2+} and ATP and modulation by Mg^{2+}. *Journal of General Physiology.* 88:573–588.

Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium release-channel of sarcoplasmic reticulum. *Journal of General Physiology.* 92:1–26.

Somlyo, A. V., H. Shuman, and A. P. Somlyo. 1977. Elemental distribution in striated muscle and effects of hypertonicity: electron probe analysis of cryosections. *Journal of Cell Biology.* 74:828–857.

Stern, M. D. 1992. Buffering of calcium in the vicinity of a channel pore. *Cell Calcium.* 13:183–192.

Stojilkovic, S., M. Kukuljan, M. Tomic, E. Rojas, and K. J. Catt. 1993. Mechanism of agonist-induced $[Ca^{2+}]_i$ oscillations in pituitary gonadotrophs. *Journal of Biological Chemistry.* 268:7713–7720.

Sudhof, T. C., C. L. Newton, B. T. Archer, Y. A. Ushkaryov, and G. A. Mignery. 1991. Structure of a novel $InsP_3$ receptor. *EMBO Journal.* 10:3199–3206.

Supattapone, S., S. K. Danoff, A. Theibert, S. K. Joseph, J. Steiner, and S. H. Snyder. 1988*a*. Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. *Proceedings of the National Academy of Sciences, USA.* 85:8747–8750.

Supattapone, S., P. F. Worley, J. M. Baraban, and S. H. Snyder. 1988*b*. Solubilization, purification, and characterization of an inositol trisphosphate receptor. *Journal of Biological Chemistry.* 263:1530–1534.

Takeshima, H., S. Nishimura, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Matsuo, M. Ueda, M. Hanaoka, T. Hirose, and S. Numa. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature.* 339:439–445.

Tinker, A., A. R. G. Lindsay, and A. Williams. 1992. A model for ionic conduction in the ryanodine receptor channel of sheep cardiac muscle sarcoplasmic reticulum. *Journal of General Physiology.* 100:495–517.

Tinker, A., A. R. G. Lindsay, and A. J. Williams. 1993. Cation conduction in the calcium release channel of the cardiac sarcoplasmic reticulum under physiological and pathophysiological conditions. *Cardiovascular Research.* 27:1820–1825.

Tinker, A., and A. J. Williams. 1992. Divalent cation conduction in the ryanodine receptor of sheep cardiac muscle sarcoplasmic reticulum. *Journal of General Physiology.* 100:479–493.

Tsien, R. W., P. Hess, E. W. McCleskey, and R. L. Rosenberg. 1987. Calcium channels: mechanisms of selectivity, permeation, and block. *Annual Review of Biophysics and Biophysical Chemistry.* 16:265–290.

Tsien, R. W., and R. Y. Tsien. 1990. Calcium channels, stores and oscillations. *Annual Review of Cellular Biology.* 6:715–760.

Volpe, P., and B. H. Alderson-Lang. 1990. Regulation of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. II. Effect of cAMP-dependent protein kinase. *American Journal of Physiology.* 258:C1086–C1091.

Walker, J. W., A. V. Somlyo, Y. E. Goldman, A. P. Somlyo, and D. R. Trentham. 1987. Kinetics of smooth and skeletal muscle activation by laser pulse photolysis of caged inositol 1,4,5-trisphosphate. *Nature.* 327:249–252.

Watras, J., and D. Benevolensky. 1987. Inositol 1,4,5-trisphosphate induced calcium release from canine aortic sarcoplasmic reticulum vesicles. *Biochimica et Biophysica Acta.* 731:354–363.

Watras, J., I. Bezprozvanny, and B. E. Ehrlich. 1991. Inositol 1,4,5-trisphosphate-gated channels in cerebellum: presence of multiple conductance states. *Journal of Neuroscience.* 11:3239–3245.

Williams, A. J. 1992. Ion conduction and discrimination in the sarcoplasmic reticulum ryanodine receptor/calcium-release channel. *Journal of Muscle Research and Cell Motility.* 13:7–26.

Yue, D. T., and E. Marban. 1990. Permeation in the dihydropyridine-sensitive calcium channel. Multi-ion occupancy but no anomalous mole-fraction effect between Ba and Ca. *Journal of General Physiology.* 95:911–939.