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Sarcoplasmic reticulum release channels from frog skeletal muscle display two types of calcium dependence**

Ricardo Bull*, Juan José Marengo

Departamento de Fisiología y Biofísica, Facultad de Medicina, Universidad de Chile, Casilla 70005, Correo 7, Santiago, Chile

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Calcium channels derived from sarcoplasmic reticulum of frog skeletal muscle were fused with planar lipid bilayers. Fractional open times displayed two types of calcium dependence: (i) blockable channels showed a bell-shaped calcium dependence with an activation constant of 4.5 μM, a Hill coefficient for activation of 1.46 and a blocking constant of 226 μM, and (ii) non-blockable channels displayed a sigmoidal calcium dependence with an activation constant of 1.1 μM and a Hill coefficient of 1.42; no blocking effect was seen with calcium up to 0.5 mM. These two types of calcium dependence may underlie the coexistence of two different pathways for calcium release in frog skeletal muscle.

Sarcoplasmic reticulum; Ca^{2+} channel; Ca^{2+} release; Ca^{2+} regulation; Skeletal muscle

1. INTRODUCTION

Calcium release from sarcoplasmic reticulum (SR) is responsible for the transient increment in cytosolic free calcium concentration that determines the contraction of skeletal muscle. The physiological mechanisms that cause calcium release are currently being investigated. Several procedures such as calcium addition [1–3], ionic substitution [4], sulfhydryl oxidation [5,6] and inositol (1,4,5)-trisphosphate addition [7,8] release calcium from SR of the skeletal muscle in vitro and have been proposed to have a physiological role. Calcium release is inhibited by many agents, including Ca^{2+} and Mg^{2+} in the millimolar range [2,3].

Calcium channels present in isolated vesicles from SR of rabbit [9–11], frog [12,13], pig [14] and human [15] skeletal muscle have been studied after fusion with planar lipid bilayers. These channels are activated or blocked by the same agents that modify calcium release from SR vesicles. They are activated by millimolar ATP and micromolar Ca^{2+}, and are blocked by millimolar Mg^{2+} and micromolar Ruthenium red added to the cytosolic side of the channel [9–15]. Nanomolar ryanodine increases fractional open time (P_o), with no change in channel conductance [16,17], whereas micromolar ryanodine locks the channel in a low conductance state with P_o close to unity [16–18].

A bell-shaped calcium dependence has been described for calcium efflux and channel activity in mammalian skeletal muscle [2,3,10]. We found that the SR from frog display calcium release channels with distinct calcium dependences, a fraction of the channels were blocked by millimolar calcium and others displayed a sigmoidal activation curve.

2. MATERIALS AND METHODS

Triads were isolated from skeletal muscle of the Chilean frog *Caudiverbera caudiverbera* as described elsewhere [19]. Briefly, finely minced muscles were homogenized in 0.15 M KCl, 5 mM $MgSO_4$, 20 mM MOPS/Tris, pH 6.8, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.4 mM benzamidine and 1 mM phenyl-methyl-sulfonyl-fluoride. Vesicles sedimenting between 1,500–17,000 × g were collected by differential centrifugation, and were resuspended in the same buffer used for homogenization. After discarding contaminating contractile proteins from this suspension by sedimentation at 1,500 × g, triads collected by sedimentation at 17,000 × g were resuspended in a small volume of 0.3 M sucrose, 20 mM MOPS/Tris, pH 6.8, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.4 mM benzamidine and 1 mM phenyl-methyl-sulfonyl-fluoride. Small aliquots were quickly frozen in liquid N_2, and stored at −80°C.

Planar phospholipid bilayers were painted with a mixture of palmitoyloleoyl phosphatidylethanolamine (POPE), phosphatidylserine (PS) and phosphatidylcholine (PC) in the proportion POPE/PS/PC = 5:3:2. Lipids obtained from Avanti Polar Lipids, Inc., (Birmingham, AL) were dissolved in decane to a final concentration of 50 mg/ml. Vesicles were added to the *cis* compartment which contained 100 mM CsCl, 5 mM $CaCl_2$, 25 mM HEPES/Tris, pH 7.4, where the voltage was applied. The other compartment, called *trans*, contained 25 mM HEPES/Tris, pH 7.4. After observing the cationic current corresponding to the calcium channel from SR, the *cis* compartment was perfused with 5–10 times the compartment volume of a solution containing 225 mM HEPES/Tris, pH 7.4. *Trans* solution, which corresponds to the intrareticular space, was replaced with HEPES/Ca or HEPES/Ba, pH 7.4 to a final Ca or Ba concentration of 37 mM. 0.5 mM total Ca, and sufficient *N*-(2-hydroxyethyl)ethylenediamine-triacetic acid (HEDTA) (for pCa 4.0 to 6.5) or ethyleneglycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) (for pCa 7.0) were added to the *cis* compartment to give the desired free Ca concen-

*Corresponding author. Fax: (56) (2) 7776916.

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tration; pCa values were measured with a calcium electrode. The experiments were carried out at room temperature (22–24°C). The *trans* compartment was held at virtual ground through an operational amplifier in a current-to-voltage configuration. Current signals were recorded on tape.

For analysis, data were filtered at 400 Hz using a four-pole low-pass Bessel type filter and digitized at 2 kHz with a 12 bit A/D converter (Labmaster DMA interface, Scientific Solutions, Inc., Solon, OH) using Axotape software (Axon Instruments, Inc., Burlingame, CA). Fractional open times were computed from records of 120 s or longer using pClamp software (Axon Instruments, Inc., Burlingame, CA). For event detection, a threshold level halfway between the open and closed current levels was used.

3. RESULTS

High conductance calcium channels in SR from frog [13] were classified according to their calcium dependence of fractional open time (P_o) into blockable channels and non-blockable channels. Current traces obtained at different cytosolic free calcium concentrations

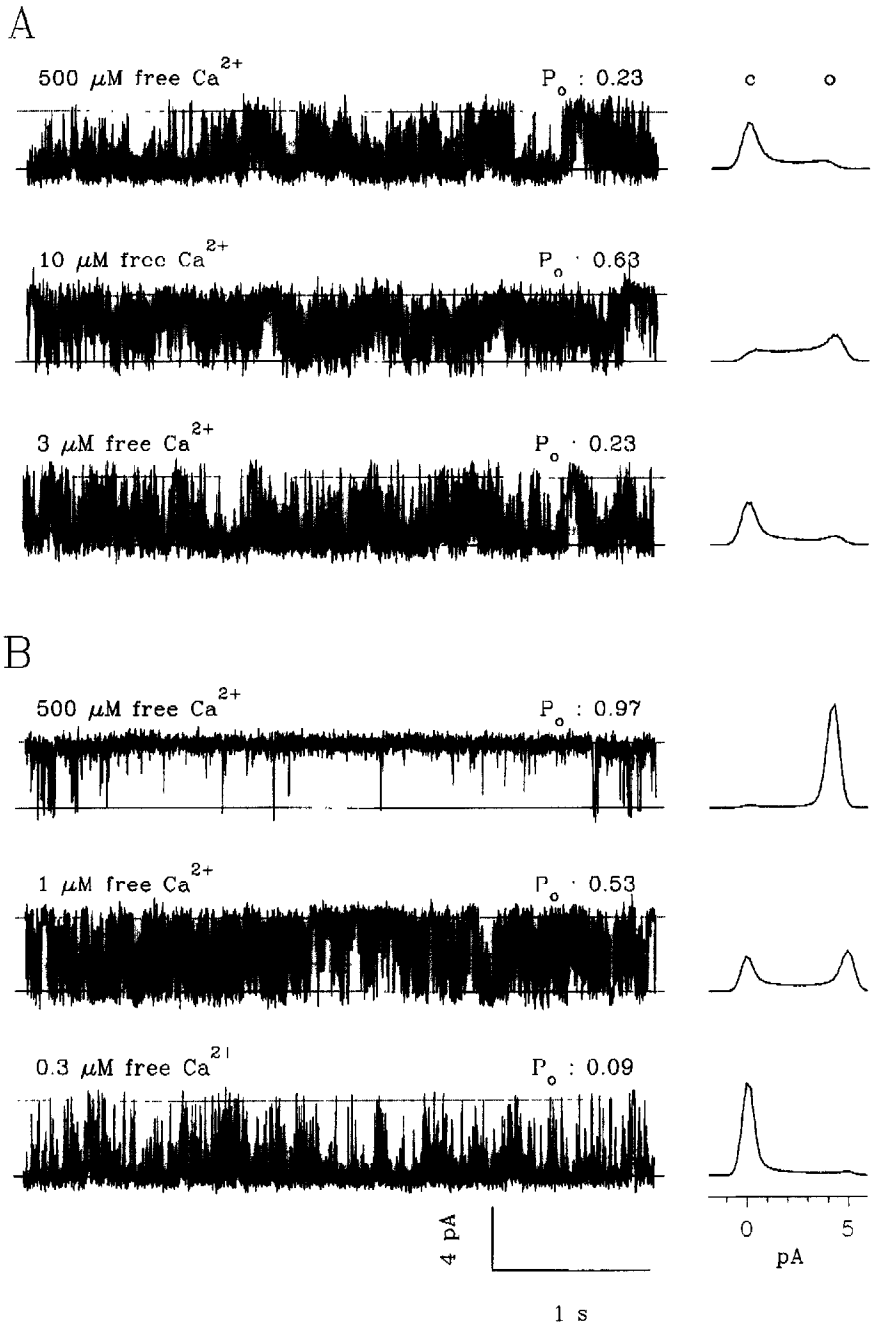

Fig. 1. Current records of two single channels with different calcium dependence. (A) Example of a blockable channel. (B) Example of a non-blockable channel. *Cis* solution: 225 mM HEPES/Tris, pH 7.4, 0.5 mM total Ca^{2+}. HEDTA was added to give free Ca^{2+} concentrations indicated above the traces. *Trans* solution: 37 mM Ba^{2+}/HEPES, 10 mM Tris/HEPES, pH 7.4. P_o and current histograms obtained from at least 120 s of continuous records are shown. (c: closed; o: open). Membrane was held at 0 mV; channels open upwards.

from a blockable and from a non-blockable single channel are depicted in Fig. 1 (parts A and B, respectively). Current amplitude, measured at 0 mV, for the open state of both channels did not change in the range of 0.3–10 μM free calcium, but decreased 15% with 500 μM calcium, in correspondence with an increased calcium counterflux from the *cis* to the *trans* compartment. The blockable channel showed an important reduction in P_o at 500 μM calcium (Fig. 1A, upper trace), whereas the non-blockable channel displayed P_o near unity (Fig. 1B, upper trace). The non-blockable channel was activated by lower calcium concentrations than the blockable channel (see Fig. 2). Substantial channel activity could be seen at calcium concentrations as low as 0.3 μM free calcium with the non-blockable channel (Fig. 1B, lower trace). At 1 μM calcium, a concentration where scarce activity was seen with blockable channels (see Fig. 2, filled symbols), P_o for the non-blockable channel reached 0.53 (Fig. 1B, middle trace). 10 μM calcium was needed to activate the blockable channel to the same extent (Fig. 1A, middle trace).

The non-blockable behavior was observed in 37 channels, and the blockable calcium-dependence in 35 channels. The detailed calcium dependence of fractional open times for these two populations of channels is depicted in Fig. 2.

Non-blockable channels displayed sigmoidal activation by cytosolic free calcium without blocking effect at calcium concentrations up to 500 μM (Fig. 2, open circles). Full activation (P_o near unity) was achieved with calcium concentrations equal or higher than 10 μM. Data were fitted with the following Hill equation (Eq. **(1)**):

$$P_o = P_{o\,max} * [Ca^{2+}]^n / ((K_a)^n + [Ca^{2+}]^n) \quad \text{Eq. (1)}$$

where $P_{o\,max}$ corresponds to P_o value at maximal activation by calcium, K_a is the calcium concentration for half-maximal activation of the channel, and n is the Hill coefficient for calcium binding to activation sites. The least squares fit to our data was obtained with an activation constant of 1.1 μM, a Hill coefficient of 1.42 and maximal P_o of 0.94 (Fig. 2, solid line through open symbols). These results suggest that calcium binds cooperatively to at least two sites in order to activate the channel.

Blockable channels showed bell-shaped calcium dependence (Fig. 2, filled circles), with activation and inhibition of channel activity by cytoplasmic free calcium. In contrast to non-blockable channels, maximum P_o of only 0.57 was reached at 30 μM free calcium (Fig. 2, filled circles). Clear blockade was seen at 500 μM calcium concentration (Po = 0.23; Fig. 2, filled symbols). Data were fitted with the following equation (Eq. **(2)**):

$$P_o = P_{o\,max} * [Ca^{2+}]^n / ((K_a)^n + [Ca^{2+}]^n + [Ca^{2+}]/K_b) \quad \text{Eq. (2)}$$

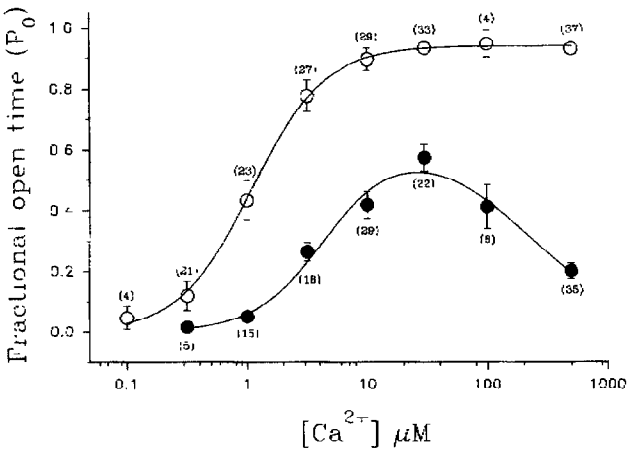

Fig. 2. Calcium dependence of calcium channels at 0 mV. Open circles: non-blockable channels; filled circles: blockable channels. The number of channels at each calcium concentration is given in parenthesis. Symbols represent the mean value and error bars the S.E.M. values. Solid lines depict the fits performed with Eq. **(1)** and Eq. **(2)** for non-blockable and blockable channels respectively (see the text).

where K_b is the calcium concentration for half-maximal block of the channel and the other symbols have the same meaning as in Eq. **(1)**. No cooperativity was assumed for blocking sites. The best fit was achieved with an activation constant of 4.5 μM and a Hill coefficient for activation of 1.46, a theoretical maximal P_o of 0.63, and a blocking constant of 226 μM (Fig. 2, solid line through filled circles).

Very often two channels fused to the bilayer. Interestingly, usually one channel displayed the non-blockable behavior and the other the blockable calcium dependence (data not shown), indicating that the difference resides in the channel itself and not in environmental conditions.

4. DISCUSSION

In this work, we have found that calcium channels of skeletal muscle from frog displayed two different calcium sensitivity patterns: (i) a bell-shaped calcium dependence with activation at micromolar concentrations and inhibition at millimolar concentrations; and (ii) a sigmoidal calcium dependence, with higher calcium sensitivity and no channel block up to millimolar calcium concentrations. The first pattern of calcium sensitivity is similar to the calcium dependence found previously for calcium channels from skeletal SR [10,12–14]. Cooperative binding to activation sites was also found by Smith et al. [20] for purified rabbit skeletal channels. However, Meissner [3], measuring calcium efflux in mammalian SR vesicles, found cooperative calcium binding to activation sites only in the presence of Mg^{2+} and adenine nucleotides. Half-maximal inhibition of channel activity was attained with the same calcium concentration that blocked calcium efflux from mammalian SR vesicles [3].

The second type of calcium dependence has only been described for native [21,22] and purified [23] cardiac SR channels derived from dog hearts. Non-blockable channels seem to lack the inhibitory site. Alternatively, its blocking constant may be higher than the calcium concentrations tested. These channels show similar cooperative calcium binding to activation sites as the blockable channels but display higher affinity.

Since SR from frog expresses two isoforms of the ryanodine receptor [24,25] it is possible that the two different calcium sensitivities may represent the channel activity of these two isoforms. We found both patterns of calcium dependence with equal frequency, in accordance with the fact that both isoforms occur in approximately equal amounts [26]. These isoforms have been purified, and after incorporation into planar bilayers they express channel activity that is activated by ATP and blocked by ruthenium red [26]. However, both isoforms display different calcium sensitivity toward ryanodine binding; lower calcium concentrations are needed to activate ryanodine binding to the lightest isoform [26]. The non-blockable channels described in this work could correspond to the lightest isoform, since they are activated by cytosolic calcium concentrations lower than those needed to activate the blockable channels, that could correspond to the heaviest isoform. The heaviest isoform is recognized by antibodies raised against the rat skeletal ryanodine receptor, and the lightest by a canine cardiac antibody [25].

Our experiments were carried out using a highly purified triad preparation, which contains 10% transverse tubules [19]. Native tubular membranes incorporated into planar lipid bilayers show calcium channels with much lower conductance and marked voltage dependence [27–29], ruling out the likelihood that the channels studied here correspond to these tubular calcium channels. Moreover, addition of an activating dihydropyridine, such as Bay K 8644 is needed to obtain stable recordings of measurable activity of tubular channels [27,29]. Furthermore, we found modulation by ryanodine for channels which displayed the blockable or the non-blockable behavior (not shown), indicating that the channels studied in this work are indeed ryanodine-sensitive SR calcium channels. The use of high concentration of several protease inhibitors throughout the preparation, as well as in the storing solution, make unlikely the possibility that the two types of channel behavior arises from the proteolysis of a single channel type. Moreover, SDS gels of this preparation [19] show the high molecular weight doublet characteristic of the ryanodine receptor from amphibian skeletal muscle [24–26]. Western blot analysis indicated that ryanodine receptor immunoreactivity was only present in these two isoforms with no evidence for lower molecular weight bands, discarding the presence of proteolytic products (Hidalgo, C., personal communication). In spite of the fact that the isolation procedure of the triads is mild and short, we cannot completely rule out that other chemical changes can take place during isolation.

Further studies are needed to elucidate whether the two types of Ca^{2+} dependence do relate to the different isoforms of the ryanodine receptor found in amphibian skeletal muscle. We tentatively propose that the two calcium sensitivities of calcium channels observed may underlie the coexistence of two different pathways for calcium release activated by different physiological mechanisms.

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