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Calcium dependence of ryanodine-sensitive calcium channels from brain cortex endoplasmic reticulum

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Abstract Endoplasmic reticulum vesicles isolated from rat brain cortex and fused with lipid bilayers displayed ryanodine-sensitive calcium channels, with three cytoplasmic calcium dependences. A: Channels ($n = 5$) stimulated by Ca^{2+} ($K_{0.5}$ = 1.2 μM and n_{Hill} = 1.9) and not inhibited up to 0.5 mM Ca^{2+}. B: Channels (n = 14) cooperatively activated ($K_{0.5}$ = 6.9 μM and n_{Hill} = 1.8), and inhibited by Ca^{2+} ($K_{0.5}$ = 152 μM and n_{Hill} = 1.8). C: Low P_o (< 0.1) channels (n = 22), non-cooperatively activated and inhibited with the same $K_{0.5}$ = 26.3 μM Ca^{2+}. These three types of responses to cytoplasmic [Ca^{2+}] may underlie separate calcium release pathways in neurons of rat brain cortex.

Key words: Ca^{2+} release; Ca^{2+} regulation; Brain cortex; Neuron; Intracellular calcium; Ryanodine receptor isoform

1. Introduction

The resting free calcium concentration in neurons is ≤100 nM [1]. A large number of cellular processes, including electrogenesis, synaptic transmission, synaptic plasticity, gene expression, embryonic growth and differentiation, are mediated by transient increments in neuronal cytoplasmic [Ca^{2+}] [1]. Calcium release from the endoplasmic reticulum (ER) plays an important role in these cellular responses [2], and the physiological mechanisms that elicit calcium release are currently the subject of active study. Neurons have two separate intracellular calcium release pathways [2], the IP_3 receptors (IP_3-R), which function as inositol-1,4,5-trisphosphate (IP_3) gated channels, and the ryanodine receptors, which are activated by physiological mechanisms not well understood at the present time. Three ryanodine receptor isoforms (ryanodine receptor-1, ryanodine receptor-2 and ryanodine receptor-3) are expressed in rat brain [2–4], and more than one ryanodine receptor isoform may be expressed in a single neuron [4].

Ryanodine binding studies in microsomes indicate that brain ryanodine receptors are modulated by some of the same agents that modify calcium release in other tissues [5–9]. Ryanodine binding is activated by millimolar ATP analogs [7–9], caffeine [5,7,8], and micromolar [Ca^{2+}] [6–9], and is inhibited by millimolar Mg^{2+} [6–9], millimolar [Ca^{2+}] [7–9], and micromolar ruthenium red [9].

Few studies have described the channel properties of brain ryanodine receptors. High conductance (≈100 pS) ryanodine-sensitive calcium channels present in rat [10], bovine [11], and rabbit [5] brain have been studied after fusion with planar lipid bilayers; these channels are activated by millimolar ATP [5,10,11], caffeine [5] and micromolar IP_3 [10,11]. Micromolar calcium activates a ryanodine receptor purified from bovine brain that corresponds to the cardiac isoform [11].

The present work presents a systematic study of the effects of changing cytoplasmic [Ca^{2+}] on the ryanodine-sensitive calcium channels present in a well-defined endoplasmic reticulum fraction isolated from rat brain cortex. These channels were studied at the single channel level after fusion of the isolated microsomes with planar lipid bilayers.

2. Materials and methods

2.1. Isolation of membrane fractions

Six week old male Sprague-Dawley rats, weighing on average 250 g, were killed by decapitation; brains were removed and placed in ice-cold buffer A (20 mM MOPS/Tris, pH 7.0, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.4 mM benzamidine, 1 mM phenyl-methyl-sulfonyl-fluoride, 10 μg/ml trypsin inhibitor). All subsequent procedures were carried out at 4°C in a cold room. Finely minced pieces of dissected cortex were homogenized in a glass Potter homogenizer, using 10 ml of buffer A per g wet tissue. After addition of 3 M sucrose to a final sucrose concentration of 0.3 M, the homogenate was sedimented at 1000×g for 10 min. The resulting supernatant was sedimented at 20000×g for 20 min; the pellet was discarded and microsomes were collected by sedimentation of the supernatant at 100000×g for 1 h. The microsomal pellet (P_3) was resuspended in ice-cold buffer B (buffer A plus 0.3 M sucrose), solid KCl was added to a final concentration of 0.5 M KCl, and the microsomes were incubated at 0°C for 15 min. This suspension was loaded on top of a discontinuous sucrose gradient made of equal volume layers of 19% and 27.5% sucrose solutions (w/v, adjusted by refractometry), containing 20 mM MOPS/Tris, pH 7.0, 0.15 M KCl and the above mixture of protease inhibitors. The fraction (P_{31}) banding at the 19–27.5% sucrose interface was collected by aspiration and, after dilution in buffer B, it was sedimented at 100000×g for 1 h and resuspended in a small volume of buffer B. The same procedure was followed with the pellet fraction (P_{32}). Membrane fractions, in aliquots of <0.1 ml, were quickly frozen in liquid N_2 and stored at −80°C for up to one month. Protein was determined according to Bradford [12]. Standard procedures were followed for SDS-PAGE gels and Western blot analysis of membrane fractions [13], using 2G6 anti-ryanodine-receptor antibody [14] kindly provided by Drs. J. Airey and J. Sutko.

2.2. Binding experiments

Determination of binding site density for [^{3}H]IP_3 was performed as described [15]. Binding of [^{3}H]ryanodine was assayed as described previously [16], except that in some experiments 10 μM [Ca^{2+}] was present in the incubation solution (adjusted with 2 mM HEDTA and 1.56 mM $CaCl_2$). Binding of [^{3}H]saxitoxin ([^{3}H]STX) was assayed using a modification of a previous protocol [17]; briefly, membranes at 0.5 mg of protein per ml were incubated for 1 h at 22°C in 0.3 ml of a solution containing 120 mM choline chloride, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, and 10 nM [^{3}H]STX. To unmask latent sites, 0.4 mg/ml of saponin was added during the incubation. Non-specific binding was determined in the presence of 100 nM tetrodotoxin. [^{3}H]IP_3 and [^{3}H]Rya were obtained from Dupont Co. (Boston, MA) and [^{3}H]STX from Amersham Int. (Buckinghamshire, UK). Protease inhibitors and other analytical reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

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2.3. Channel recording and analysis

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 37.5 mg/ml. Fusion of vesicles to negatively charged Mueller-Rudin membranes was performed as described in previous work [18], with slight modifications. Vesicles were added to the *cis* compartment, which contained 200 mM CsCl, 100 μM $CaCl_2$, 25 mM HEPES/Tris, pH 7.4. The other compartment (*trans*) contained 25 mM HEPES/Tris, pH 7.4. After fusion, the *cis* compartment was perfused with 5 times the compartment volume of a solution containing 225 mM HEPES/Tris, pH 7.4. To obtain the desired cytoplasmic free calcium concentration, 0.5 mM Ca-HEPES plus enough *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA) were added to the *cis* compartment. Free $[Ca^{2+}]$ values were always checked with a calcium electrode. The *trans* solution, which corresponds to the intrareticular space, was replaced with 40 mM Ca- or Ba-HEPES, 10 mM Tris-HEPES, pH 7.4. All experiments were carried out at room temperature (22–24°C). Voltage was applied to the *cis* compartment, and the *trans* compartment was held at virtual ground through an operational amplifier in a current-to-voltage configuration. Current signals were recorded on tape. All experiments were done with membranes held at 0 mV.

Data analysis was done as described in detail in previous work [18]. Values given for P_o were calculated from single channels records lasting at least 180 s.

3. Results and discussion

The procedure followed in this work to isolate purified microsomes from rat brain cortex was highly reproducible, and consistently yielded vesicular fraction of similar characteristics (n = 15). The purified vesicular fraction P_{31} (see section 2.1), displayed a ryanodine receptor density of 2.86 ± 0.43 pmol/mg of protein at 10 μM $[Ca^{2+}]$ (Table 1). This ryanodine receptor density is several-fold higher than other values reported in brain microsomes [5–10]. Since ryanodine receptors seem to be absent in glial cells [2], the high ryanodine receptor density of fraction P_{31} suggests that these vesicles most likely originate from neuronal cells. A lower density of IP_3 receptors was obtained (Table 1), with values that are in the range reported for rat brain microsomes [7]. The low density of binding sites for STX indicates that fraction P_{31} is not significantly contaminated with plasma membrane fractions.

Fusion of fraction P_{31} with planar lipid bilayers was a reproducible and frequent event. High conductance (100 ± 7 pS; mean ± S.E.M.) calcium channels were obtained (n = 41). The channels shown in this work were modulated by ryanodine; addition of 1–4 μM ryanodine locked the channels in the lower (≈40%) conductance level with a fractional open time near unity (not shown), which is the characteristic sub-conductance open state produced by this alkaloid [19].

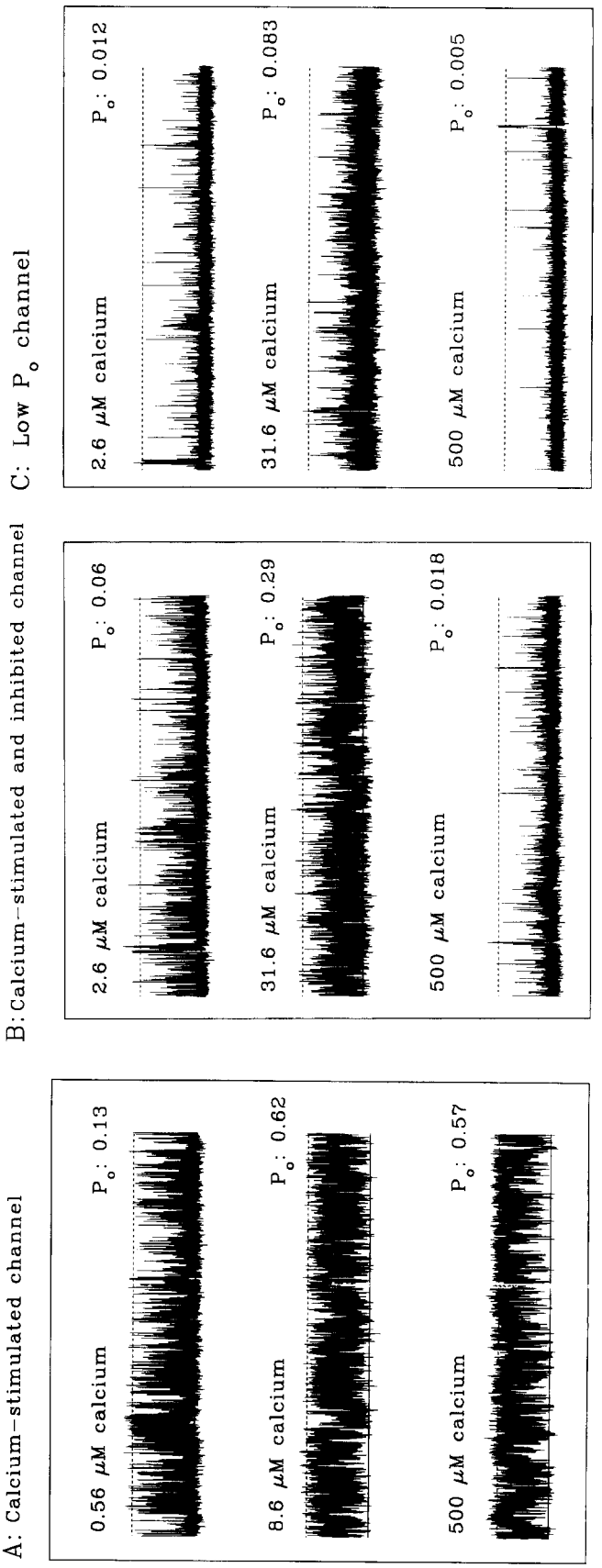

Fig. 1. Examples of single channels records that show the different effects of *cis* calcium on P_o. A: A channel stimulated by low $[Ca^{2+}]$ and not inhibited by high $[Ca^{2+}]$ (up to 500 μM). B: A channel stimulated by low $[Ca^{2+}]$ and inhibited by high $[Ca^{2+}]$. C: A channel with $P_o < 0.1$ at low and high $[Ca^{2+}]$ and thus defined as a low P_o channel. Current amplitude for the open state of all channels measured at 0 mV did not change in the range of 1–100 μM free calcium (3.5 pA), but decreased 15% at 500 μM calcium, in correspondence with an increased calcium counterflux from the *cis* to the *trans* compartment. *Cis* and *trans* solutions are described in the text; the *cis* free $[Ca^{2+}]$ is indicated at the left of each trace. P_o values shown were calculated from records lasting at least 180 s. All traces last 10 s.

These channels were classified into three groups (Fig. 1), according to the effect of *cis* (cytoplasmic) $[Ca^{2+}]$ on their fractional open time (P_o).

The less frequent response to changes in *cis* $[Ca^{2+}]$ (n = 5, out of 41 channels) corresponded to the calcium-stimulated channels (Fig. 1A). Significant channel activity (P_o = 0.13) was observed at 0.56 μM *cis* $[Ca^{2+}]$ (top trace). Increasing *cis* $[Ca^{2+}]$ to 8.6 μM (middle trace) produced further channel activation (P_o = 0.62), which persisted on further increasing *cis* $[Ca^{2+}]$ to 500 μM (lower trace). The calcium-dependence curve of P_o showed that these calcium-stimulated channels (Fig. 2, open triangles) displayed sigmoidal activation by low cytosolic $[Ca^{2+}]$, in the range of 0.3 μM to 10 μM, and were not inhibited by increasing $[Ca^{2+}]$ up to 0.5 mM. The experimental P_o data, fitted to a Hill equation, yielded an activation constant of 1.2 μM $[Ca^{2+}]$, a Hill coefficient of 1.9 and a maximal P_o of 0.73 (Fig. 2, solid line through open triangles). These results suggest that calcium binds cooperatively with a high affinity to two or more sites in order to activate these channels. This calcium dependence has been described for native [20,21] and purified [22] cardiac sarcoplasmic reticulum (SR) channels derived from dog hearts (ryanodine receptor-2) [23], for one calcium channel isoform present in fish skeletal muscle [24], and for 50% of the channels present in SR isolated from frog skeletal muscle [18]. Inhibition of cardiac ryanodine receptors by much higher cytoplasmic $[Ca^{2+}]$ has been recently reported, with a $K_{0.5}$ of 15 mM [25]. Since we routinely used calcium concentrations up to 0.5 mM, we cannot discard the possibility that higher calcium concentrations will inhibit the calcium-stimulated channels of brain cortex as well. It remains to be established whether this channel behavior reflects the presence in our isolated vesicles of the ryanodine receptor-2 isoform, which is present in high density in rat brain cortex [9,11].

A second response to changes in *cis* $[Ca^{2+}]$ was found more frequently (n = 14) and characterized the calcium-stimulated and calcium-inhibited channels (Fig. 1B). Cytosolic $[Ca^{2+}]$ stimulated these channels in the range of 3 μM (Fig. 1B, upper trace); further increasing *cis* $[Ca^{2+}]$ to 30 μM increased I_o to 0.29 (Fig. 1B, middle trace). Channel inhibition was observed at *cis* $[Ca^{2+}]$ >30 μM (Fig. 2, solid circles), reaching almost complete inhibition at 0.5 mM $[Ca^{2+}]$ (Fig. 1B, lower trace). The best fit to the P_o data was obtained with the following equation (Eq. 1):

$$I_o = P_{o\,max}/(1 + (K_a/[Ca^{2+}])^n + ([Ca^{2+}]/K_i)^n) \quad (1)$$

where $P_{o\,max}$ corresponds to the P_o value of maximal activation by calcium, K_a is the calcium concentration for half-maximal P_o activation and is also defined as $K_{0.5}$, n is the Hill coefficient for calcium binding, and K_i is the $[Ca^{2+}]$ that produced half-maximal inhibition of P_o. The other symbols have their conventional meanings. The best fit was achieved with K_a = 6.9 μM, K_i = 152 μM, n = 1.8, and $P_{o\,max}$ = 0.49 (Fig. 2, solid line through filled circles). These results indicate that this second channel behavior also reflects cooperative calcium binding to activation sites, as observed with the calcium-stimulated channels (Fig. 2). However, lower calcium affinity, as evidenced by the 6-fold higher $K_{0.5}$ values for activation, was observed in this case. The response to *cis* Ca of the calcium stimulated and calcium inhibited channels of brain cortex microsomes is similar to that found previously for the calcium channels of mammalian skeletal SR [21,26], which correspond to the ryanodine receptor-1 isoform [23]. A similar effect of Ca was also found in one of the two channel types present in fish skeletal muscle [24], and in half of the channels present in SR from frog skeletal muscle [18]. Cooperative binding to activation sites has previously been described for purified rab-

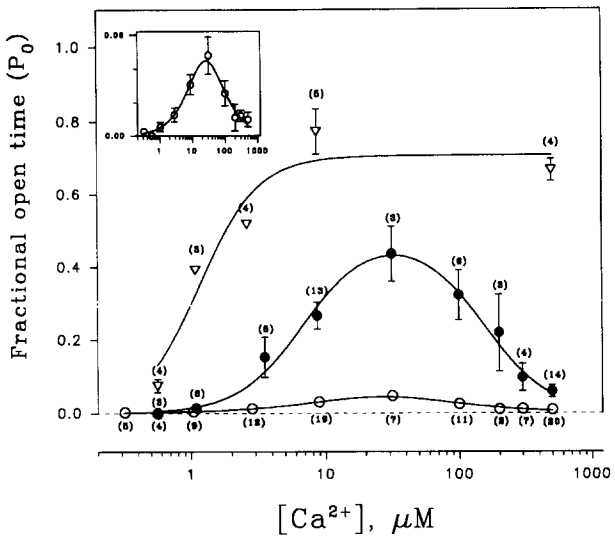

Fig. 2. Calcium dependence of calcium channel open probability. Open circles: low P_o channels; filled circles: calcium-stimulated and calcium-inhibited channels; open triangles: calcium-stimulated channels. Numbers in parentheses represent the number of records averaged at each calcium concentration. *Cis* and *trans* solutions were as detailed in the text. Symbols represent mean values plus S.E.M. The solid lines represent the best non-linear fit of the data to the equations defined in the text. The inset illustrates the behavior of the low P_o channels shown in an amplified scale.

Table 1
Receptor density in different membrane fractions isolated from brain cortex

	[^{3}H]Ryanodine (pmol/mg)	[^{3}H]IP_3 (pmol/mg)	[^{3}H]Saxitoxin (pmol/mg)
P_3	0.15 ± 0.04 (4)	0.14 ± 0.07[b]	4.9 ± 0.60[b]
P_{31}	1.07 ± 0.07 (5)	0.38 ± 0.03 (4)	0.6 ± 0.40[b]
	2.86 ± 0.43 (5)[a]		
P_{32}	0.18 ± 0.04 (2)	0.08 ± 0.02 (2)	16.7 ± 6.38[b]

The density of binding sites for [^{3}H]ryanodine, [^{3}H]IP_3 and [^{3}H]saxitoxin was measured in fraction P_3, which corresponds to the 100000×g microsomal pellet obtained from brain cortex, and in subfractions P_{31} and P_{32}, which were obtained by fractionation of P_3 in discontinuous sucrose density gradients as described in detail in the text. Data represent mean ± S.E.M. of several different preparations, as indicated by the numbers in parentheses.

[a]Data measured at pCa 5.0.

[b]Data given as mean ± range of duplicate determinations done in a single preparation.

bit skeletal channels [27] and native frog skeletal muscle [18]. Cooperative inhibition is also present in rabbit skeletal muscle channels [25].

The most frequent response to changes in *cis* $[Ca^{2+}]$ ($n = 22$) defined the low P_o channels (Fig. 1C). These channels were activated by increasing *cis* $[Ca^{2+}]$ from 2.6 μM to 31.6 μM (Fig. 1C, upper and middles traces), and were inhibited by higher $[Ca^{2+}]$ (Fig. 1C, lower trace). At all $[Ca^{2+}]$ studied, the low P_o channels displayed $P_o < 0.1$ (Fig. 2, open circles). The best non-linear fit to these data was obtained with the following equation (Eq. 2):

$$P_o = P_{o\,max}/(1 + K/[Ca^{2+}] + [Ca^{2+}]/K) \quad (2)$$

This equation defines non-cooperative activation and inhibition of P_o by *cis* $[Ca^{2+}]$, with only one constant, K, for half-maximal activation and inhibition of P_o; the other symbols have the same meanings as in Eq. 1. The best non-linear fit to the data yielded $K = 26.3$ μM and $P_{o\,max} = 0.13$ (Fig. 2, solid line through filled circles; for amplification see inset).

The fact that the data were well fitted to Eq. 2 (Fig. 2, inset) implies that the low P_o channels were simultaneously activated and inhibited by increasing $[Ca^{2+}]$, ruling out the possibility that they represent a stable low P_o mode of the calcium stimulated and inhibited channels. This third pattern of calcium modulation has not been described in any tissue. One ryanodine receptor isoform that is expressed in chicken skeletal muscle shows modal behavior, with low and high P_o modes [28]. However, the low P_o channels never showed $P_o > 0.1$ in continuous records lasting up to 20 min, making it unlikely that the low P_o values found are due to modal channel behavior.

The simultaneous stimulation and inhibition by Ca of the low P_o channels would explain why low P_o values were obtained at all *cis* $[Ca^{2+}]$, since channel activation and inhibition would compete with each other in the steady-state conditions used in this work to record channel activity. It is possible that under physiological conditions channel activation and inhibition by *cis* $[Ca^{2+}]$ had different kinetic responses. If, for instance, channel activation was faster than inhibition, these low P_o channels would open only briefly following increases in cytoplasmic $[Ca^{2+}]$, producing a brief calcium release, which may underlie transient physiological cellular responses.

The possibility that variable proteolysis of a single channel isoform produced the three types of channel behavior is unlikely. High concentrations of a mixture of protease inhibitors were used throughout membrane isolation, and Western blot analysis indicated that only one ryanodine receptor immunoreactive band was present when tested with an antibody specific for one ryanodine receptor isoform (not shown). We cannot rule out that other chemical changes occurred during membrane isolation, such as changes in the phosphorylation state of the channel that regulate ryanodine receptor channels in other cells [23]. However, it is not known whether phosphorylation affects the response of the channel to changes in *cis* $[Ca^{2+}]$.

Since in the brain cortex three isoforms of the ryanodine receptor are expressed [2–4], it is possible that the three different calcium sensitivities observed in this work represent the channel activity of these three isoforms. Although further studies are needed to test this possibility, we tentatively propose that the three types of calcium sensitivity of ryanodine receptor calcium channels observed in brain cortex microsomes represent different pathways for calcium release in neurons, that are differentially modulated by intracellular $[Ca^{2+}]$.

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