INOSITOL (1,4,5)-TRISPHOSPHATE ACTIVATES A CALCIUM CHANNEL IN ISOLATED SARCOPLASMIC RETICULUM MEMBRANES

BENJAMÍN A. SUÁREZ-ISLA,*‖ VERÓNICA IRRIBARRA,§ ANDRÉS OBERHAUSER,§ LAURA LARRALDE,¶ RICARDO BULL,‡ CECILIA HIDALGO,*§ AND ENRIQUE JAIMOVICH§

**Centro de Estudios Científicos de Santiago, Casilla 16443, Santiago 9, Chile; ‡Department of Physiology and Biophysics, Faculty of Medicine, University of Chile, Casilla 70055, Santiago 7; §Department of Biology, Faculty of Sciences, University of Chile; and ¶School of Biology, Faculty of Sciences, Universidad Central de Venezuela, A.P. 21201 Caracas, Venezuela*

ABSTRACT Sarcoplasmic reticulum membrane vesicles isolated from frog skeletal muscle display high conductance calcium channels when fused into phospholipid bilayers. The channels are selective for calcium and barium over Tris. The fractional open time was voltage-independent (−40 to +25 mV), but was steeply dependent on the free *cis* $[Ca^{2+}]$ (P_0 = 0.02 at 10 μM *cis* Ca^{2+} and 0.77 at 150 μM Ca^{2+}; estimated Hill coefficient: 1.6). Addition of ATP (1 mM; *cis*) further increased P_0 from 0.77 to 0.94. Calcium activation was reversed by addition of EGTA to the *cis* compartment. Magnesium (2 mM) increased the frequency of rapid closures and 8 mM magnesium decreased the current amplitude from 3.4 to 1.2 pA at 0 mV, suggesting a reversible fast blockade. Addition of increasing concentrations of inositol (1, 4, 5)-trisphosphate (*cis*), increased P_0 from 0.10 +/− 0.01 (mean +/− SEM) in the control to 0.85 +/− 0.02 at 50 μM in an approximately sigmoidal fashion, with an apparent half-maximal activation at 15 μM inositol (1, 4, 5)-trisphosphate in the presence of 40 μM *cis* Ca^{2+}. Lower concentrations of this agonist were required to produce a significant increase in P_0 when 10 μM or less *cis* Ca^{2+} were used. The channel was blocked by the addition to the *cis* compartment of either 0.5 mM lanthanum, 0.5 μM ruthenium red, or 200 nM ryanodine, all known inhibitors of Ca^{2+} release from sarcoplasmic reticulum vesicles. These results demonstrate the presence of calcium channels in the sarcoplasmic reticulum from frog skeletal muscle with a pharmacological profile consistent with a role in excitation-contraction coupling and with the hypothesis that inositol (1, 4, 5)-trisphosphate is a physiological agonist in this process.

INTRODUCTION

Several models of excitation-contraction coupling (EC-coupling) have been put forward to explain how the depolarization of the transverse tubule membrane triggers calcium release from the sarcoplasmic reticulum (SR) (1–6). Inositol (1, 4, 5)-trisphosphate ($InsP_3$) has been proposed as an internal agonist that would elicit calcium release from the SR by opening calcium channels (4–8). Calcium channels of large (9) and small conductance (10) have been detected in SR membrane fractions from rabbit skeletal muscle, and have been proposed to participate in calcium release. However, a direct effect of $InsP_3$ on channels present in SR, a central feature of the hypothesis that $InsP_3$ is the agonist activating calcium release, has not been observed. We report here that micromolar concentrations of $InsP_3$ activate a high conductance calcium channel by increasing channel fractional open time (P_0) without effect on the single-channel conductance.

MATERIALS AND METHODS

Biochemical

Highly purified SR membranes were prepared from the leg muscles of the Chilean frog *Caudiverbera caudiverbera* by homogenization of the tissue followed by sucrose density gradient centrifugation essentially as described (11), except that the SR fraction banding at the 43–50% (wt/vol) interface of a sucrose gradient was used. Planar phospholipid bilayers were formed from mixtures of palmitoyloleoyl phosphatidylethanolamine (POPE) and phosphatidylcholine (PC) or POPE and phosphatidylserine (PS) obtained from Avanti Polar Lipids, Inc., Birmingham, AL. Lipids were dissolved in decane at 25 mg/ml. All chemicals used were of reagent grade.

Single-channel Recording and Analysis

Sarcoplasmic reticulum membrane vesicles (50 to 100 μg protein) were added to the *cis* side (corresponding to the cytoplasmic side) with stirring.

Please address all correspondence to Benjamin A. Suárez-Isla.

Vesicle fusion to neutral (POPE/PC; 4:1) or negatively charged (POPE/PS; 1:1) "painted" bilayers was promoted by initiating the experiment in a gradient of 100 mM choline chloride, 5 mM $CaCl_{2+}$ (*cis*) vs. 25 mM Hepes Tris (*trans*, corresponding to the intravesicular space). Recording conditions were subsequently established after extensive washing of the *cis* compartment with 225 mM Hepes Tris containing variable concentrations of calcium, and replacement of the solution in the *trans* compartment with 37 mM Ba Hepes or Ca Hepes. All Hepes buffers were titrated to pH 7.4 with Tris base or the corresponding hydroxides. The experiments were carried out at 22–24°C. Current was measured with an operational amplifier (type LF157-AH; National Semiconductor Corp., Santa Clara, CA) in a current-to-voltage converter configuration that included a 200-Mohm feedback resistor (12). Data were recorded on tape at 1 kHz (−3dB) and digitized at 5 kHz with a laboratory microcomputer and software implemented by Dr. Osvaldo Alvarez or with a version of the Axolab/Axess acquisition and analysis system (Axon Instruments, Inc., Burlingame, CA). P_0 varies considerably from channel to channel and in some recordings open-close transitions of the Ca^{2+} channel show nonstationary behavior ("gearshifts") (13). Thus, drug effects were compared in the same channel during recordings of 240 s or longer in experiments where the standard deviation of P_0 in the steady state was not >15%. With these criteria it was found that P_0 was voltage-independent, a characteristic that was preserved in all conditions studied.

Ins(1, 4, 5)P_3 was supplied by Calbiochem-Behring Corp., La Jolla, CA. 1 mM stock solutions in water were prepared shortly before use and contained <10 μM-free Ca^{2+} contamination, as measured with a calcium electrode. The minimal dilution used to attain the maximal concentration of *cis* $InsP_3$ tested (50 μM) was 20-fold; this would have provided a total extra [Ca^{2+}] of 0.5 μM, insufficient to elicit the observed agonist effects of $InsP_3$ reported here.

RESULTS AND DISCUSSION

Single-channel events corresponding to the activation of a cationic conductance with a current amplitude of 3.2 pA at 0 mV (Fig. 1 *A*) were observed at 15 μM *cis* Ca^{2+} and 37 mM *trans* Ba^{2+} as current carrier. The current-voltage relationship (Fig. 1 *B*) was linear between −25 and +25 mV in neutral and charged bilayers but showed a significant sublinearity at potentials more positive than +25 mV. The single-channel conductance was 100 +/− 4 pS with 37 mM *trans* Ba^{2+} (mean +/− SEM; $n = 6$) and 103 pS with 37 mM *trans* Ca^{2+}; in both cases 225 mM Hepes Tris was present in the *cis* compartment. The direction of the current could not be reversed. Extrapolation of the linear part of the current-voltage curve gave a pseudo-reversal potential of +30.0 +/− 1.2 mV ($n = 6$), indicative of a minimum permeability ratio of Ba^{2+} over Tris of 9.2 (14).

The fractional open time, P_0, showed a steep dependence on the free *cis* Ca^{2+} concentration. Consecutive additions of Ca^{2+} to the *cis* side (Fig. 1 *C*) increased P_0 of a channel incorporated into a POPE/PC bilayer, from 0.02 at 10 μM Ca^{2+} to 0.77 at 150 μM Ca^{2+}, with an estimated Hill coefficient of 1.6. Application of 1 mM *cis* ATP further increased P_0 to 0.94. The effect of calcium was reversed by adding similar micromolar concentrations of EGTA (not shown).

Addition of *cis* Mg^{2+} (2 mM) increased the frequency of rapid closures (not shown) while P_0 decreased slightly. Higher Mg^{2+} levels (up to 8 mM) decreased the current amplitude from 3.4 pA to 1.2 pA at 0 mV (cf, Fig. 3 *C*), suggesting a fast blockade by *cis* Mg^{2+} of the current

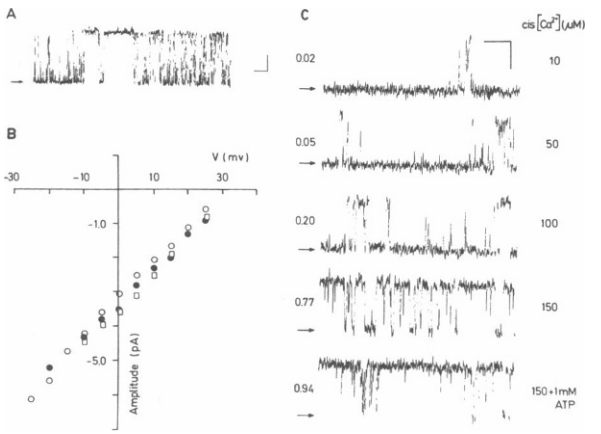

FIGURE 1 (*A*) Single-channel current fluctuations at 0 mV in the presence of 15 μM *cis* Ca^{2+}. Vertical calibration: 1 pA. Horizontal calibration: 15 s. Channel incorporated in charged bilayers (POPE/PS 1:1). Channel openings corresponding to cation flux in the *trans-cis* direction are plotted upward. Voltages were applied in the *cis* compartment, while the *trans* chamber was kept at virtual ground. The horizontal arrow indicates the closed state. In this segment the overall P_0 was 0.51. (*B*) Current-voltage relationship. Channel incorporated in charged bilayers (POPE/PS 1:1). (*Open circles*) 37 mM *trans* Ba^{2+}. (*Closed circles*) 37 mM *trans* Ca^{2+}. (*Open squares*) channel incorporated into neutral bilayers (POPE/PC 4:1), 37 mM *trans* Ba^{2+}. (*C*) Effect of *cis* [Ca^{2+}] on the fractional open time at 0 mV. Vertical calibration: 2 pA. Horizontal calibration: 120 ms. The lowest trace depicts the effect of adding 1 mM *cis* ATP. The P_0 values are indicated to the left-hand side of each trace and were evaluated from an uninterrupted trace of at least 240 s. Arrows on the left indicate closed state.

carried by barium. These effects were reversed by extensive washing of the *cis* side.

$InsP_3$ has been recently proposed as an agonist of EC coupling in skeletal muscle (4, 5). We tested whether $InsP_3$ could be an activator of the calcium channel present in frog SR membranes and found that micromolar concentrations of this compound, added to the *cis* compartment, activated this channel by increasing P_0 without alteration of the single-channel conductance (Fig. 2).

In charged bilayers formed by POPE/PS (1:1), addition of 10 μM $InsP_3$ to the *cis* side in the presence of 40 μM *cis* Ca^{2+} (Fig. 2, *open circles*) produced a small but significant increment of P_0 from 0.10 +/− 0.01 (mean +/− SEM) in the control to 0.18 +/− 0.02 ($p < 0.01$, two-tailed Student t-test). P_0 was evaluated from continuous records of at least 280 s. Further additions of $InsP_3$ increased P_0 up to 0.85 +/− 0.02 at 50 μM in an approximately sigmoidal fashion (Fig. 3 *A*), indicating an apparent half-maximal activation at 15 μM $InsP_3$, in the presence of 40 μM Ca^{2+}.

Histograms of distribution of open and closed times were adequately described by the sum of two exponential functions if all events lasting <1 ms were excluded from the analysis (not shown). In the example of Fig. 2, the addition of 20 μM $InsP_3$ to the *cis* chamber decreased mean closed times from 6.1 and 40.6 ms (fast and slow time constants), to 4.1 and 12.3 ms, respectively. In contrast, mean open times increased from 1.6 and 5.7 ms to 4.0 and 20.6 ms. As

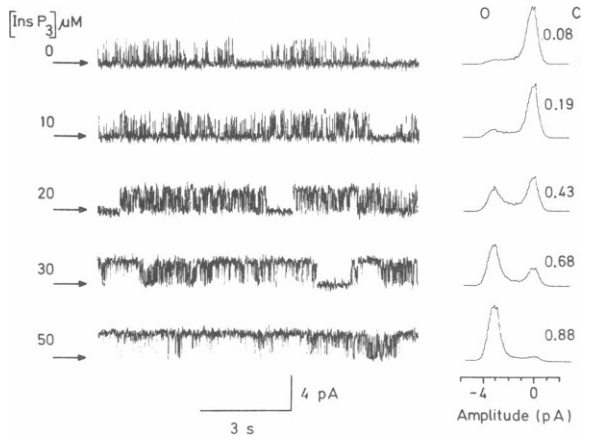

FIGURE 2. Effect of Ins(1, 4, 5)P3 on SR channel fractional open time at 0 mV (*left*). SR vesicles were incorporated into a charged POPE/PS bilayer and recordings were obtained with 37 mM *trans* Ba^{2+} and 40 μM *cis* Ca^{2+}. The twelve second traces shown are representative of the average P_0 values in each condition. Horizontal calibration: 3 s. Vertical calibration: 4 pA. Arrows on the left indicate closed state. (*Right*) corresponding amplitude histograms depicting changes in fractional open time and a constant single-channel current amplitude of 3.4 pA. (The probability of fusion of SR vesicles with neutral planar bilayers is much lower than in charged bilayers, a fact that makes difficult a systematic study of single-channel properties). Time distribution histograms of open and closed times (not shown), could be approximately described by double-exponential functions, corresponding to fast and slow kinetic components.

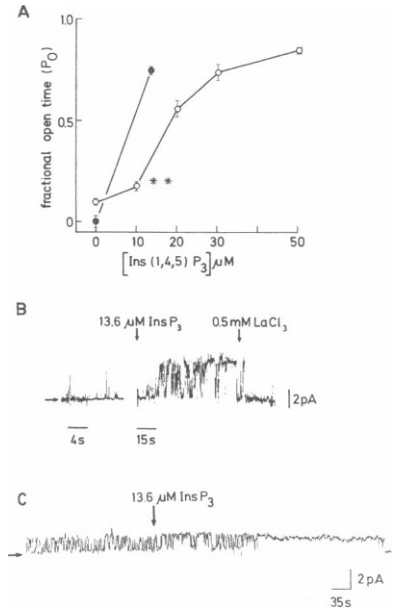

FIGURE 3 (*A*) Fractional open time v.s. $InsP_3$ concentration in the presence of 2 *cis* Ca^{2+} concentrations. Channel incorporated in charged bilayers (POPE/PS 1:1). (*Open circles*) (40 μM *cis* Ca^{2+}). Symbols correspond to average P_0 values of the experiment shown in Fig. 2 given as mean SEM (*Closed circles*) (10 μM *cis* Ca^{2+}) before and after addition of 5 μM *cis* $InsP_3$. (*B*) Effect of 13.6 μM *cis* $InsP_3$ on single-channel activity at 0 mV in neutral POPE/PC bilayers in the presence of 10 μM *cis* Ca^{2+}. P_0 increased from 0.01 to 0.75. The channel was irreversibly blocked upon addition of 0.5 mM $LaCl_3$ (shown at arrow). (*C*) Effect of 13.6 μM *cis* $InsP_3$ in the presence of 8 mM Mg^{2+}, 1 mM ATP and 150 μM Ca^{2+} added before to the *cis* compartment. The decrement in current amplitude is due to the high *cis* $[Mg^{2+}]$. Horizontal arrow indicates closed state. Vertical calibration is 2 pA for *B* and *C*. Similar observations were made in all experiments corresponding to incorporations of different SR preparations into charged or neutral bilayers ($n = 22$).

indicated by the amplitude histograms in Fig. 2, the kinetic effects of $InsP_3$ were reflected as an increase in P_0 but they took place without change of the single-channel current amplitude.

Experiments carried out in charged bilayers at 10 μM *cis* Ca^{2+} or less, indicate that lower $InsP_3$ concentrations are needed to open the channel. In the example shown in Fig. 3 *A* (*closed circles*), addition of only 5 μM *cis* $InsP_3$ increased P_0 from 0.03 to 0.51 in the presence of 10 μM *cis* Ca^{2+}. These results suggest that a critical calcium concentration is required to allow for maximal agonist potency, but further experiments are needed to obtain a detailed description of the effects of $InsP_3$ on channel gating as a function of the calcium concentration.

The stimulating effect of $InsP_3$ was also observed after channel incorporation into neutral POPE/PC bilayers. In this case, addition of 13.5 μM $InsP_3$ to the *cis* side in the presence of 10 μM-free *cis* calcium produced also a significantly greater increase in P_0 than that observed in 40 μM *cis* Ca^{2+} in charged bilayers from 0.01 to 0.75 (not shown).

As the internal resting Mg^{2+} concentration has been determined to be between 1 and 2 mM (15), we studied the influence of millimolar Mg^{2+} on the stimulating effects of $InsP_3$. We found that even in the presence of a rather high Mg^{2+} (8 mM) in the *cis* compartment, addition of $InsP_3$ could still activate the channel (Fig. 3 *C*), suggesting that both agents may exert their pharmacological effects at distinct sites. In this particular example, P_0 increased from 0.55 to a value close to 1.0.

The agonistic effect of $InsP_3$ was also seen in a channel selective for calcium and barium present in heavy SR membranes isolated from rabbit muscle. Highly purified heavy SR vesicles were obtained as described (16) and channels were incorporated into POPE/PS bilayers. This channel had a single-channel conductance of 110 pS in 37 mM *trans* Ba^{2+}, was activated by micromolar *cis* Ca^{2+} and by millimolar ATP, was blocked by Mg^{2+} and lanthanum, and its kinetic properties resembled closely the high conductance calcium channel described by Smith et al. (9).

Lanthanum and ruthenium red have been used extensively as blockers of Ca^{2+} efflux from SR vesicles (17). We found that the high conductance channel described here was irreversibly blocked by 0.2 mM lanthanum added to the *cis* chamber (Fig. 3 *B*, last addition). Likewise, ruthenium red (0.5 μM) blocked the channel by increasing the frequency of brief closures (not shown). Ryanodine, another inhibitor of Ca^{2+} release from the SR (18), had a complex effect on channel gating. At a concentration of 10 nM, ryanodine increased P_0. However, at 50 nM the

frequency of very brief closures was significantly augmented and at 200 nM ryanodine the channel was locked in a state of low conductance (~⅓ of the original value) that showed very infrequent closures. The complete description of blocker effects and mechanisms will be the subject of another report (Bull, R., Irribarra, V., Marengo, J. J. and Suarez-Isla, B. A. manuscript in preparation).

The findings reported here demonstrate the presence of calcium channels in SR vesicles that are activated by micromolar $InsP_3$ in the presence of calcium, magnesium, and ATP. This channel can be added to the recently proposed category of agonist-operated calcium conductances (19, 20), with $InsP_3$ being a direct agonist.

The existence of $InsP_3$ activated channels in SR is part of the hypothesis of chemical transmission in EC coupling in skeletal muscle (4, 21, 22). $InsP_3$ elicits contractures in amphibian (4), mammalian (7), and crustacean (8) muscle fibers, as well as calcium release from isolated SR membranes obtained from rabbit muscle (5), and it has been shown that the intracellular concentration of $InsP_3$ increases in muscles stimulated to contract (4). A triadic delay long enough to allow for diffusion of an intracellular messenger has been demonstrated (23) and the enzymes responsible for the production of the membrane-bound precursors of $InsP_3$, namely the phosphatidylinositol and phosphatidylinositol 4-phosphate (PIP) kinases, have been shown to be present in highly purified transverse tubule membranes isolated from frog muscle (24). In contrast, SR membranes do not contain PIP kinase and therefore, the $InsP_3$ available must necessarily be generated in the transverse tubule membrane (21, 22). The activity of these enzymes in isolated membranes (24) seems adequate to generate enough phosphatidylinositol (4, 5)-bisphosphate (PIP_2, the direct precursor of $InsP_3$) so that upon hydrolysis of PIP_2, $InsP_3$ could reach micromolar concentrations in the triadic space (21, 22). Thus, several criteria that support the role of $InsP_3$ as an internal agonist of EC coupling seem to be fulfilled.

However, the involvement of $InsP_3$ in EC coupling in skeletal muscle has been challenged by some authors. Recently, Lea et al. (25) did not observe tension development or aequorin light signal upon addition of $InsP_3$ to skinned frog muscle fibers able to respond to caffeine or calcium. Walker et al. (26) have obtained tension development with "caged" $InsP_3$ applied to frog skeletal muscle fibers but they have reported kinetics of tension development too slow to assign a physiological role to $InsP_3$ activation. It is possible that the complex modulation profile of the high conductance channel described here, that displays sensitivity to calcium, ATP, magnesium, as well as $InsP_3$, may explain why the effect of $InsP_3$ was not observed in all cases (25). In addition, more than one calcium release pathway could coexist and participate in EC coupling of skeletal muscle. In fact, a different small conductance calcium channel sensitive to caffeine and dantrolene but insensitive to $InsP_3$ has been found in native mammalian SR membranes (10) and we have also observed a calcium selective channel of lower conductance present in SR membranes from frog, that is activated by caffeine but not by Ca^{2+}, ATP, or $InsP_3$ (27 and unpublished observations). These channels may have different roles in the calcium release process.

Taken together, our results are consistent with the proposed role of inositol (1, 4, 5)-trisphosphate as a physiological agonist of EC-coupling in skeletal muscle.

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