Ca^{2+} release by inositol-trisphosphorothioate in isolated triads of rabbit skeletal muscle

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ABSTRACT The effectiveness of the nonmetabolizable second messenger analogue DL-myo-inositol 1,4,5-trisphosphorothioate (IPS_3) described by Cooke, A. M., R. Gigg, and B. V. L. Potter, (1987*b*. *Jour. Chem. Soc. Chem. Commun.* 1525–1526.) was examined in triads purified from rabbit skeletal muscle. A Ca^{2+} electrode uptake-release assay was used to determine the size and sensitivity of the IPS_3-releasable pool of Ca^{2+} in isolated triads. Uptake was initiated by 1 mM MgATP, pCa 5.8, pH 7.5 Release was initiated when the free Ca^{2+} had lowered to pCa ~7. We found that 5–25 μM myo-inositol 1,4,5,-trisphosphate (IP_3), and separately IPS_3, consistently released 5–20% of the Ca^{2+} pool actively loaded into triads. Single channel recording was used to determine if ryanodine receptor Ca^{2+} release channels were affected by IPS_3 at the same myoplasmic Ca^{2+} and IPS_3 concentrations. Open probability of ryanodine receptor Ca^{2+} release channels was monitored in triads fused to bilayers over long periods (200 s) in the absence and following addition of 30 μM IPS_3 to the same channel. At myoplasmic pCa ~7, IPS_3 had no effect in the absence of MgATP (P_o = 0.0094 ± 0.001 in control and P_o = 0.01 ± 0.006 after IPS_3) and slightly increased activity in the presence of 1 mM MgATP (P_o = 0.024 ± 0.03 in control and P_o = 0.05 ± 0.03 after IPS_3). Equally small effects were observed at higher myoplasmic Ca^{2+}. The onset of channel activation by IPS_3 or IP_3 was slow, on the time scale 20–60 s. We suggest that in isolated triads of rabbit skeletal muscle, IP_3-induced release of stored Ca^{2+} is probably not mediated by the opening of Ca^{2+} release channels.

INTRODUCTION

In striated muscle, excitation-contraction coupling is initiated by the depolarization of the plasma membrane which brings about the release of calcium from the sarcoplasmic reticulum (SR). The factors that regulate release from SR in vitro have been extensively documented (Martonosi, 1984), but the actual mechanism that triggers the release in vivo remains conjectural. A key component is the ryanodine receptor, a ~565,000 Da protein specific for the junction between the SR and the transverse tubules (junctional SR). In the SR membrane, the ryanodine receptor forms a channel which is responsible for Ca^{2+} and nucleotide-induced release, hence named the Ca^{2+} release channel (Smith et al., 1985, 1986*a*,*b*; Imagawa et al., 1987; Lai et al., 1988; Smith et al., 1988; Ma et al., 1988*b*). Predictions based on the primary structure of the ryanodine receptor (Takeshima et al., 1989) suggested that ~90% of the protein protrudes from the surface of the SR, presumably to form the "foot" structure (Block et al., 1988; Wagenknecht et al., 1989) that bridges the SR and the *t*-tubules. This large molecular mass may serve as a voltage sensor (if in close proximity to the *t*-system membrane) or as a binding site for a second messenger, whereas the remaining ~10% intramembrane segment may actually form the Ca^{2+} selective pore.

A model of excitation-contraction coupling involves the action of the ubiquitous second messenger, myo-inositol 1,4,5-trisphosphate (IP_3). Similar to the receptor-mediated formation of IP_3 in smooth muscle (Somlyo et al., 1988) it was suggested that IP_3 could be released from the *t*-tubule membrane of striated muscle in response to a depolarization (Vergara et al., 1985). According to this hypothesis, IP_3 would then diffuse across the gap that separates the *t*-system from the SR and induce release by opening a Ca^{2+} channel in the SR (Vergara et al., 1985; Volpe et al., 1985). There are numerous studies that have dealt with the effect of IP_3 in striated muscle and have reported negative results (Movsesian et al., 1985; Scherer and Ferguson, 1985; Lea et al., 1986; Mikos and Snow, 1987; Palade, 1987; Pampe et al., 1988; Hanon et al., 1988) or positive results (Volpe et al., 1985; Rojas et al., 1986; Hidalgo et al., 1986; Donaldson et al., 1987; Nosek et al., 1987). A serious limitation to the IP_3 hypothesis is the time course of contractions evoked by IP_3 (Walker et al., 1987) which were found to be too slow to underlie excitation-contraction coupling in fast twitch muscle. On the other hand, direct support has come from the mea-

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surement of IP_3-mediated opening of Ca^{2+} release channels in the SR membrane of frog skeletal muscle (Suarez-Isla et al., 1988).

In this report we tested the ability of a nonmetabolizable analogue of IP_3, namely DL-myo-inositol 1,4,5-trisphosphorothioate (IPS_3, Cooke et al., 1987*b*; Nahorski and Potter, 1989), to release actively-accumulated Ca^{2+} and to open release channels in triads of rabbit skeletal muscle. Previous studies showed that IPS_3 is resistant to 5-phosphatase activity (Hamblin et al., 1987; Willcocks et al., 1988; Taylor et al., 1989) while being only three-fold less potent than IP_3 in its ability to release Ca^{2+} from intracellular stores in many nonmuscle cells (Strupish et al., 1988; Taylor et al., 1988, 1989). By using IPS_3 we thought to separate the effects of IP_3 from those of other phosphoinositides in the PI cascade (Nahorski and Potter, 1989) and to discard IP_3 hydrolysis by endogenous 5-phosphatases as one source of negative results (Somlyo et al., 1988). The significant observation is that IPS_3 consistently released up to 20% of the actively-loaded Ca^{2+} pool, but under the same conditions activation of Ca^{2+} release channels was none or minimal. This result raises the possibility that the Ca^{2+}-mobilizing activity of IP_3 or IPS_3 in rabbit triads may not be mediated by ryanodine receptor Ca^{2+} release channels but by other channels or mechanisms yet unidentified.

MATERIALS AND METHODS

Purification of triads

Triads were isolated from rabbit skeletal muscle as described by Mitchell et al. (1983), using the pyrophosphate variant. White muscle from the back and hind legs was minced and ground in a food processor. Each of 4–5 portions of 40 g were homogenized for 60 s in 300 ml of 0.3 M sucrose, 0.5 mM EGTA, 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, 1.0 mM $MgCl_2$, pH 7.1, using a blender (Waring Products Div., New Hartford, CT) at maximum speed. The following protease inhibitors were added during homogenization: Pepstatin A (1 μM), Iodoacetamide (1 mM), PMSF (0.1 mM), Leupeptin (1 μM), and Benzamidine (1 mM). The homogenate was spun for 15 min at 9,000 *g* in a Sorvall GSA rotor (DuPont Instruments, Hoffman States, IL). The supernatant was filtered and centrifuged at 14,000 *g* for 30 min in the same rotor. Pellets were resuspended to a final volume of 60 ml in the same medium used for homogenization and layered on top of a step sucrose gradient composed of 7 ml of 27% wt/vol sucrose, 7 ml of 32% sucrose, 14 ml of 38% sucrose, in 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, 1 mM $MgCl_2$, pH 7.1. Gradients were centrifuged for 16 h at 20,000 *g* in a rotor (model SW; Beckman Instruments, Inc., Fullerton, CA) at 4°C. The triad fraction was collected from the 32–38% sucrose interface. After gentle dilution with 3 vol of homogenization medium without sucrose, a pellet was obtained by centrifugation at 30,000 *g* for 40 min using a Beckman Instruments, Inc. 35 rotor. Triads were resuspended in 0.3 M sucrose, 5 mM Hepes-KOH, pH 7.0, aliquoted, frozen in liquid nitrogen, and stored at −80°C until use. Routinely, the [^{3}H]PN200-110 binding capacity was 12 pmol/mg, the [^{3}H]Ryanodine binding capacity was 9 pmol/mg, and the maximum ATP-dependent Ca^{2+} uptake capacity was 15 nmol/mg triad protein.

Ca^{2+} release measurements

Ca^{2+} uptake and release were assayed in a volume of 1 ml at 30°C using a Ca^{2+}-selective electrode (Orion Research, Inc., Cambridge, MA). Uptake medium was 150 mM KCl, 2 mM $MgCl_2$, 50 mM Mes-Tris pH 7.5. Electrodes were calibrated using Ca-EGTA buffers prepared in uptake medium. A computer program was used to determine the free Ca^{2+} concentration using stability constants of Fabiato and Fabiato (1978). Voltage output from the electrode was digitized at 10 points/s and stored on an IBM/PC using Kiethley software (Cleveland, OH). Triads (4 mg/ml) were preincubated for 60 min at 0°C in uptake medium and diluted 20-fold in the electrode vessel (1 ml) which contained uptake medium plus 5 μM free Ca^{2+}. Temperature was kept at 30°C with a recirculating temperature-controlled water bath. Triad suspension in the vessel was vigorously stirred. Calcium uptake was initiated by the addition of 1 mM ATP-KOH pH 7.5. Upon completion of the uptake and leveling of the voltage signal (a typical 16-mV shift), IPS_3 or IP_3 were added from a 1-mM stock solution in uptake medium without Ca^{2+}. ATP, IPS_3, or IP_3 alone had no effect on electrode readings. A total of eight separate triad preparations were tested.

Planar bilayer recordings

Planar bilayers were composed of brain phosphatidylethanolamine and brain phosphatidylserine at a 1:1 weight ratio dissolved in decane. These were formed on a 0.3 mm diam hole in a Lexan cup. Triads preparations (0.1–0.2 mg) were added to the *cis* solution composed of 0.25 M CsCl, 10 μM $CaCl_2$, and 10 mM Hepes-Tris pH 7.5. The trans solution was the same except the CsCl which was 0.05 M (Cs^+ instead of Ca^{2+} was the major carrier of current). Solutions were connected via an Ag/AgCl electrode and an agar/KCl bridge to a List L/M EPC 7 amplifier (List Electronic, Darmstadt, FRG). Trans solution was held at ground. Recordings were filtered through a low-pass Bessel filter (Frequency Devices, Haverhill, MA) at a front panel setting of 1.5 kHz and digitized at 4 kHz. Acquisition, storage, and analysis were done on a PC/AT computer using Keithley software (Cleveland, OH). The total recording time was ~120 min from three preparations of triads.

Chemicals and abbreviations

Phospholipids were purchased from Avanti Polar Lipids (Birmingham, AL). NaATP was purchased from Sigma Chemical Co. (St. Louis, MO). Ionophore A23187 and ryanodine were purchased from Calbiochem (La Jolla, CA). Sucrose, salts, and buffers were reagent grade (Fisher Scientific Co., Pittsburgh, PA; Behring Diagnostics, San Diego, CA). CsCl was analytical grade (Johnson Matthey Co., Hereforshire, England). DL-IP_3 and DL-IPS_3 were synthesized as described (Cooke et al., 1987*a*,*b*) and were purified by ion exchange chromatography on DEAE Sephadex A-25. The concentration of D-(1,4,5)IP_3 or D-(1,4,5)IPS_3, and not that of the racemate are reported in all experiments. Abbreviations were as follows, ATP(adenosine 5′ trisphosphate); EGTA (ethylenediamine tetraacetic acid); Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid); IP_3 (DL-myo-inositol 1,4,5-trisphosphate); IPS_3 (DL-myo-inositol 1,4,5-trisphosphorothioate); Mes (2*N*-Morpholino ethanesulfonic acid); PMSF (phenylmethylsulfonyl fluoride); Tris (Tris-hydroxymethyl aminomethane).

RESULTS

IP_3-induced release of Ca^{2+} in triads of skeletal muscle

Triads derived from the *t*-tubule SR junction of skeletal muscle can be purified without apparent loss of structural integrity and with retention of Ca^{2+} uptake capacity (Ikemoto et al., 1984; Caswell et al., 1979; Mitchell et al., 1983). We previously used this preparation as a convenient source of functional ryanodine receptors (Imagawa et al., 1987; Ma et al., 1988*b*; Smith et al., 1988; Valdivia et al., 1989, 1990). Fig. 1 describes the release of Ca^{2+} induced by IP_3 and IPS_3 in a suspension of triads. Ca^{2+} was actively loaded into the SR via the Ca^{2+} pump as described (Miyamoto and Racker, 1982). Low free Ca^{2+} (≤ 3 μm) was used during the uptake phase so that the free Ca^{2+} concentration at the moment of release would be within the resting range of myoplasmic Ca^{2+}, ~0.1–0.2 μM. Uptake, shown as a downward deflection in the top trace of Fig. 1, was always initiated by the addition of 1 μmol ATP-KOH pH 7.5 (1 mM final concentration) after equilibration of 200 μg triad protein in 1 ml of 150 mM KCl, 2 mM $MgCl_2$, 5 μM total $CaCl_2$, 50 mM Mes-Tris, pH 7.5. The average decrease in free Ca^{2+} was from 1.8 ± 0.19 μM Ca^{2+} (±SD, $n = 12$) to 0.2 ± 0.01 μM Ca^{2+} (±SD, $n = 12$) in ~25 s with an initial rate of ~18 nmol Ca^{2+}/mg triad protein/min. The absolute voltage of the Ca^{2+}-sensitive electrode was −117 ± 3 mV ($n = 11$) and −131 ± 3 mV ($n = 11$) before uptake and at the end of the uptake period, respectively. The total Ca^{2+} accumulated during the uptake, measured 2 min after the addition of ATP, was 10.5 ± 2.7 nmol Ca^{2+}/mg triad protein ($n = 8$). After uptake, free Ca^{2+} remained constant below ~0.2 μM for up to 200 s and then slowly returned to micromolar levels with a $t_{1/2}$ ~ 250 s (not shown). This was presumably due to a slow leakage of vesicular Ca^{2+} after ATP consumption. The following controls, i) through vi) (not shown, see Valdivia et al., 1989*a*), ensured that the observed Ca^{2+} waveforms in Fig. 1 were due to sequestration of Ca^{2+} via the SR Ca^{2+} pump and to release of Ca^{2+} from the interior of sealed vesicles. i) Uptake could be initiated by 1 mM ATP but not by the same concentration of the non-hydrolyzable analogue AMP-PMP; ii) the duration of the uptake phase was increased ~five-fold by addition of 5 mM creatine phosphate and 50 units creatine kinase as an ATP-regenerating system; iii) free Ca^{2+} could be increased to pre-ATP levels by addition of 1 μM of the Ca^{2+}-ionophore A23187; iv) uptake was not affected by 10 μM oligomycin, the mitochondrial ATP-ase inhibitor; and v) uptake was decreased by the Ca^{2+} release channel-specific alkaloid ryanodine, at concentrations of 1–100 μM; vi) no release was observed when IP_3 or IPS_3 were added in the absence of ATP or in triads incubated with Ca^{2+} and ATP but in the presence of Ca^{2+} ionophore A23187.

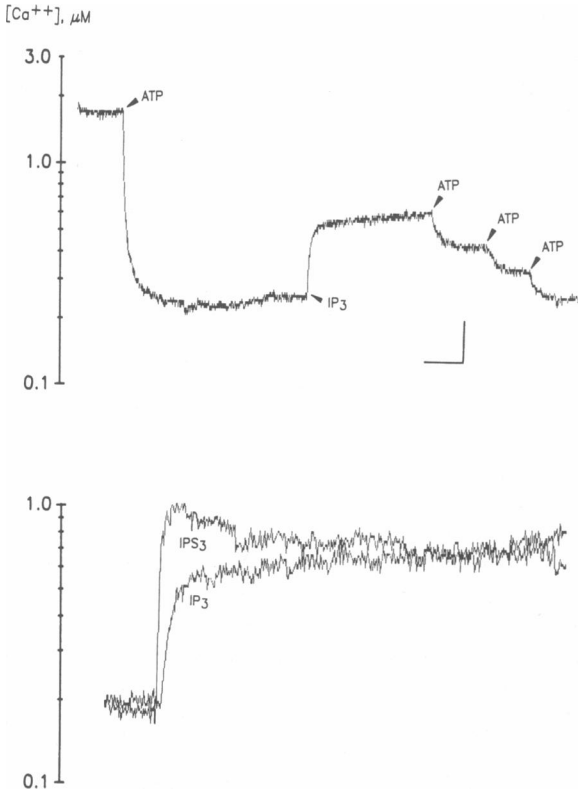

FIGURE 1 IP_3 and IPS_3-induced Ca^{2+} release in triad vesicles measured with a Ca^{2+} electrode. Uptake corresponds to a downward deflection (decrease in solution free Ca^{2+}) and release to an upward deflection (increase in solution free Ca^{2+}). Left scales give calibrated free Ca^{2+}. (*Top*) Ca^{2+} uptake was initiated by 1 μmol at ATP-KOH (*arrow*) added to a volume of 1 ml containing 150 mM KCl, 2 mM $MgCl_2$, 5 μM Ca^{2+}, 50 mM Mes-Tris, pH 7.5 and 200 μg triad protein. Release was initiated by 25 nmol of IP_3 (*arrow*). Subsequent additions of 1 μmol of ATP-KOH are indicated by arrows. Voltage and time marks are 2 mV and 90 s. (*Bottom*) Ca^{2+} release induced by 25 μM IP_3 or 25 μM IPS_3 from two separate experiments are compared on expanded scales. Voltage and time marks are 1 mV and 30 s.

The top and bottom panels of Fig. 1 show that 25 μM D-IP_3 or D-IPS_3 added as 50 μM DL-racemate were equally effective in releasing a fraction of the ATP-dependent Ca^{2+} sequestered into triads. The IP_3 and IPS_3 used in all experiments contained equal amounts of D-(1,4,5) and L-(1,4,5) isomers of which only the D-(1,4,5) isomer was previously shown to be biologically active (Taylor et al., 1988, 1989; Strupish et al., 1988). Accordingly, we reported the concentration of D-(1,4,5)IP_3 or D-(1,4,5)IPS_3, and not that of the racemate. As shown in Fig. 1 (*top*), successive additions of 1 μmol

ATP-KOH, after addition of IP_3, could stimulate a net reuptake of Ca^{2+}. Although the total Ca^{2+} released by IP_3 or IPS_3 was approximately the same, the overlapped traces in Fig. 1 (*bottom*) indicated that IPS_3 elicited release at a slightly higher rate. Total release at different doses of IPS_3 is shown in Fig. 2. Approximately 20% of the sequestered Ca^{2+} was released at a concentration of 25 μM whereas 3–5% was released at 5 μM. The concentrations of IPS_3 that mobilized SR Ca^{2+} were 5 to 10-fold higher than previously used in three other cell lines. In Swiss 3T3 cells D-(1,4,5)IP_3 and DL-(1,4,5)IPS_3 released ~50% of the intracellular Ca^{2+} pool with EC_{50}s of 0.7 μM and 5 μM, respectively (Taylor et al., 1988). In hepatocytes, 60% of the Ca^{2+} pool was mobilized with EC_{50}s of 0.4 μM and 2.1 μM, respectively, whereas in GH3 cells, the EC_{50}s for the same compounds were 0.22 μM and 3 μM, respectively (Strupish et al., 1988). It has been argued that a strong hydrolysis of IP_3 by endogenous 5-phosphatases could explain the low effectiveness of exogenously added IP_3 in some fragmented or in vivo muscle preparations (Hidalgo et al., 1986; see Somlyo et al., 1988 for a review). We discarded this possibility because the amount of Ca^{2+} released by IPS_3 was the same as that released by IP_3.

Effect of IP_3 and IPS_3 on Ca^{2+} release channels at micromolar cytosolic Ca^{2+}

To identify the mechanism of release we tested the effect of IP_3 and IPS_3 on the Ca^{2+} release channel described by Smith et al., (1985, 1988). Recordings were made using a gradient of CsCl as current carrier, as described previously (Vilven et al., 1989; Valdivia et al., 1990). 250 mM CsCl was present on the *cis* solution and 50 mM CsCl, on the *trans* solution. Both solutions were buffered to pH 7.4 with 10 mM Hepes-Tris and to pCa 5 with 10 mM Ca_2EGTA buffers. The use of CsCl permitted the blockade of SR K^+ channels (Coronado and Miller, 1979) and allowed Ca^{2+} to be maintained within a physiological range on either side of the channel. Dihydropyridine-sensitive *L*-type Ca^{2+} channels present in the transverse tubule of triads (Valdivia et al., 1990) would conduct Cs^+ when Ca^{2+} is maintained in the micromolar range (Coronado and Smith, 1987). However, their contribution to the recordings was discarded because the conductance of *L*-channels is ~10 times smaller than that of release channels and because the activity of *L*-channels in planar bilayers is dependent on the constant presence of Bay K8644 dihydropyridine agonist. Observations (*a*) through (*d*) described below, established that Ca^{2+} release channels were functional in CsCl solutions. (*a*) In *cis* 250 mM CsCl and *trans* 50 mM CsCl, release channels had a linear current-voltage relationship with a slope conductance of 450 pS and a reversal at potentials more negative than −30 mV. This result is the same as in a study of purified ryanodine receptors from rabbit heavy SR (Smith et al., 1988); (*b*) 10 μM ryanodine produced an irreversible decrease in conductance to 250 pS and an increase in mean open time from 0.5 ms in control to 50 ms in the presence of ryanodine; (*c*) *Cis* Ca^{2+} increased channel activity from P_o = 0.002 ± 0.002 at pCa = 9 to P_o = 0.4 ± 0.1 at pCa 6; further increase in *cis* Ca^{2+} decreased channel activity to P_o = 0.01 ± 0.01 at pCa 3. This result is in excellent agreement with ^{45}Ca efflux measurements in heavy SR vesicles (Meissner, 1986); (*d*) *Cis* 5 mM ATP increased channel activity ~five-fold, *cis* 1 mM free Mg^{2+} decreased activity 10-fold, and *cis* 1 μM ruthenium red decreased activity 100-fold. These results are the same reported previously using high trans Ca^{2+} as current carrier in heavy SR fused to planar bilayers (Smith et al., 1986*a*,*b*). Fig. 3 *A* and Fig. 4 *A* show activity of single release channels at +20 mV, 10 μM free Ca^{2+} (labeled control pCa 5) and the same channels after addition of 30 μM IP_3 (Fig. 3 *A*, labeled IP_3 pCa 5) or 30 μM IPS_3 (Fig. 4 *A*, labeled IPS_3 pCa 5). At this concentration, which was the highest tested in the uptake and release experiments of Fig. 2, IP_3 and IPS_3 produced a modest increase in bursting activity.

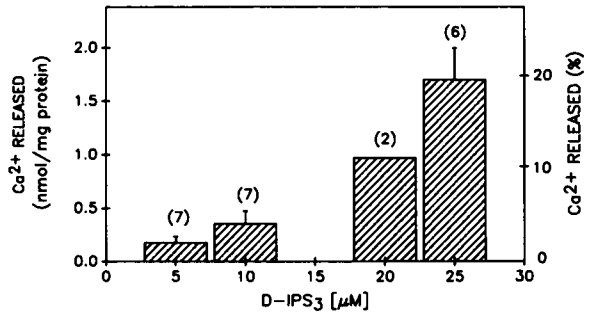

FIGURE 2 Doses of IPS_3 required to induce Ca^{2+} release in actively-loaded triads. Triads were actively loaded at constant protein (200 μg/ml), MgATP, and free Ca^{2+} described in Fig. 1 and text. The free Ca^{2+} concentration in the reaction medium averaged 2.0 ± 0.5 μM before uptake and 0.25 ± 0.05 μM after completion of uptake (n = 22 experiments). Bars represent mean and 1 SD for the number of experiments indicated in parentheses. Ca^{2+} release (%), corresponds to the percentage of the Ca^{2+} uptake which is released by IPS_3. Ordinate gives concentration of the D-(1,4,5)IPS_3 isomer.

To describe the kinetic effect of both phosphoinositides quantitatively, channels were monitored during relatively long periods (80–100 s) during control and after addition of 30 μM IP_3 or IPS_3. The data showed that the increase in bursting activity translated into a small increase in the total time in which channels spent open. The open probability, P_o, was calculated from peak current histograms (see Fig. 2 of Ma and Coronado, 1988*a*) and corresponds

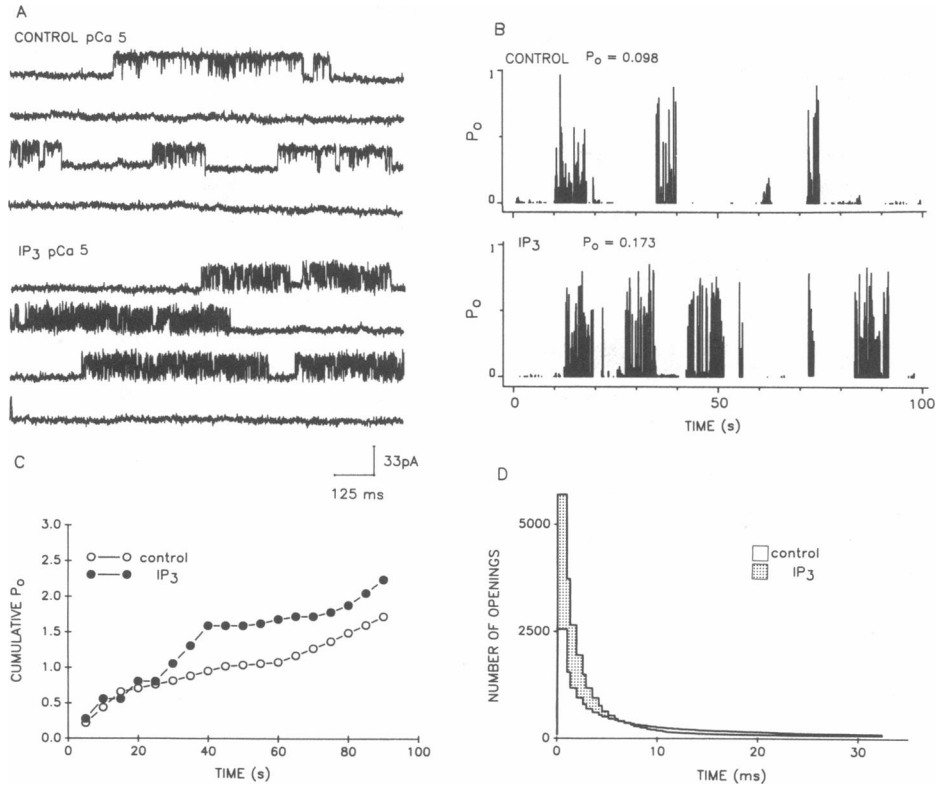

FIGURE 3 Effect of IP_3 on Ca^{2+} release channels activated by 10 μM Ca^{2+}. (*A*) through (*D*) are from the same experiment. (*A*) Representative traces at a holding potential of 20 mV and 10 μM *cis* free Ca^{2+} before (labeled control pCa 5) and after (labeled IP_3 pCa 5) addition of *cis* 30 μM IP_3. (*B*) P_o measured every 100 ms appears as a bar of length 0 to 1. Empty spaces represent 100-ms sweeps without openings. Average P_o from the entire recording is shown for control and test periods. (*C*) The cumulative sum of P_o measured every 5 s appears continuously as a function of recording time; $t = 0$ corresponds to the beginning of the control period or to the time immediately after addition of IP_3. (*D*) % of open events of duration time t, or longer are plotted as a function of time, t. Total events are $n = 2057$ for control and $n = 6252$ following addition of IP_3.

to the fraction of total digitized current falling within 4 SD above and below the mean amplitude of the open channel. In the experiment of Fig. 3 *A*, P_o was 0.098 in control and 0.173 after addition of 30 μM IP_3, whereas in Fig. 4 *A*, P_o was 0.099 in control and 0.167 after addition of 30 μM IPS_3. Clearly the level of activation produced by either compound was the same in each case, ~60% above control. Panels *B* in Figs. 3 and 4 describe the appearance of events during the 80–100 s that lasted each recording. The bars of length 0 to 1 indicate open probability on a sweep by sweep basis (100 ms/sweep). There was an obvious clustering of events in time either in controls or after addition of IP_3 or IPS_3. Clusters of events or bursts were operationally defined as a collection of sweeps containing openings flanked by at least one sweep without openings. Either in Fig. 3 *B* or Fig. 4 *B* there was a clear increase in the number of bursts per unit time after addition of the phosphoinositide. However, a plot of cumulative P_o shown in Fig. 3 *C* and Fig. 4 *C* revealed that this effect was not instantaneous but developed 30–40 s after exposure of channels of IP_3 or IPS_3. Thus, the kinetic effect of IP_3, and similarly IPS_3, was not only small but also sluggish. Despite the increase in P_o, we found that the mean open time, fitted from the time histograms (Figs. 3 *D* and 4 *D*), actually remained the same or it decreased but it did not increase. In controls and in the presence of IP_3 or IPS_3, the exponential fitting of the distributions of open events required two time constants. A fast time constant, τ_1, was necessary to fit the brief and mostly unresolved events $\leq$2 ms, and a slower time constant, τ_2, was needed to fit events $\geq$5 ms. In Fig. 3 *D*, the time constants that best described the histogram were $\tau_1 = 1.9$ ms and $\tau_2 = 13$ ms in control; and $\tau_1 = 1.4$ ms and $\tau_2 = 4.6$ ms during the 80-s period after addition of 30 μM IP_3. In Fig. 4 *D*, $\tau_1 = 1.1$ ms and $\tau_2 = 7.8$ ms in control; and $\tau_1 = 1.1$ ms and $\tau_2 = 7.0$ ms during the 80-s period after addition of 30 μM IPS_3. A summary of the kinetic data averaged from three separate experiments under the same conditions that tested IPS_3 are shown in Table 1. The most significiant effects were a shortening of the mean open time, a shortening of the mean closed time, an increase in the total number of events, and an increase

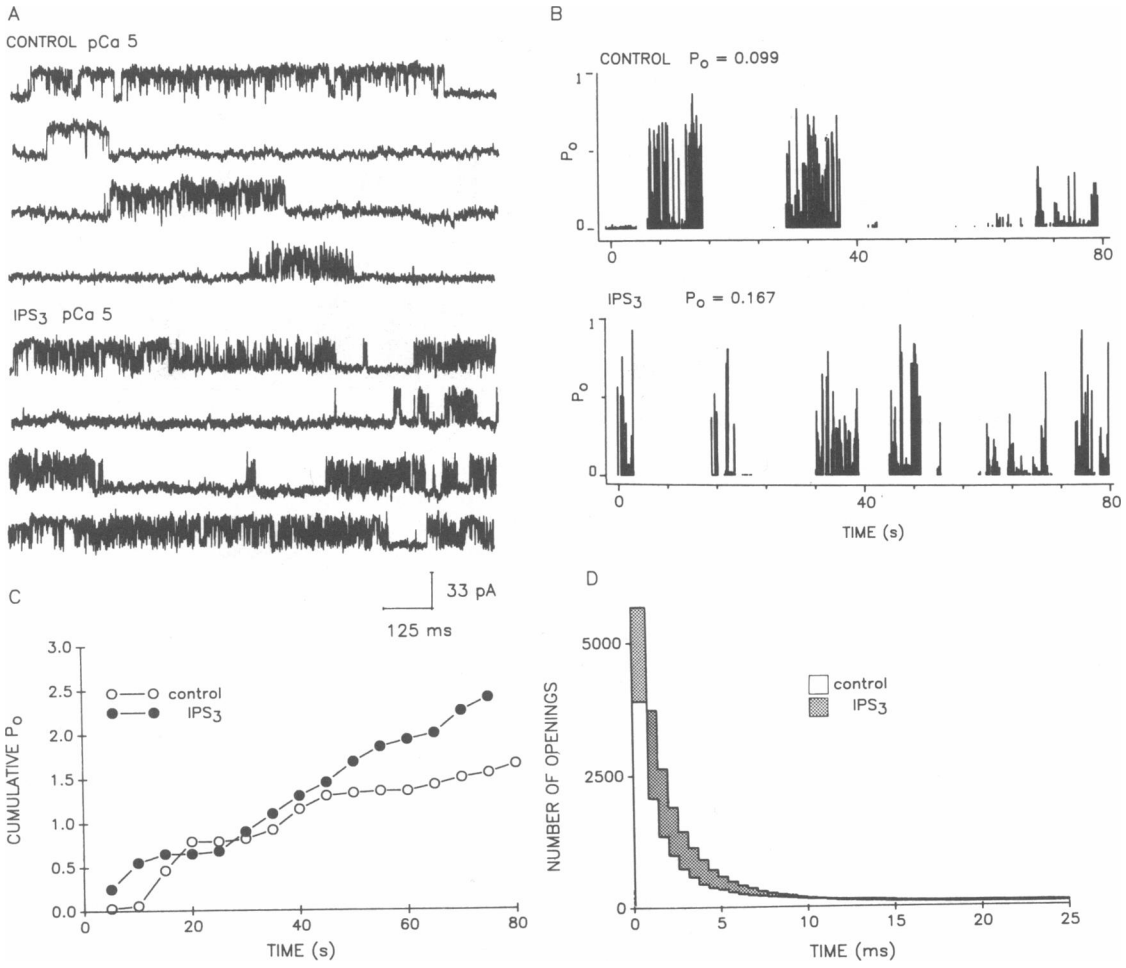

FIGURE 4 Effect of IPS_3 on Ca^{2+} release channels activated by 10 μM Ca^{2+}. (*A*) through (*D*) are from the same experiment. (*A*) Representative traces at a holding potential of 20 mV and 10 μM *cis* free Ca^{2+} before (labeled control pCa 5) and after (labeled IPS_3 pCa 5) addition of *cis* 30 μM IPS_3. (*B*) through (*D*) are the same as in Fig. 3. (*D*) Total events are $n = 2648$ for control and $n = 5580$ following addition of IPS_3.

in the mean duration of bursts. Thus, the effects of IPS_3 are complex and probably occur by modification of several kinetic steps. However, the average P_o computed from three separate recordings changed only slightly from 0.12 ± 0.03 in control to 0.19 ± 0.03 after addition of 30 μM IPS_3. P_o of control and IPS_3 were not equal to a level of significance $t \leq 0.1$.

Small effect of IPS_3 at submicromolar cytosolic Ca^{2+} plus MgATP

The lack of a larger effect of IPS_3 on the P_o could have resulted from the relatively high *cis* Ca^{2+} used to activate channels during control because binding of IP_3 to its receptor, at least in brain, is actually inhibited by micromolar Ca^{2+} (Worley et al., 1987). We therefore repeated the same recordings above at pCa 7. In Fig. 5, control activity was first recorded at pCa 5 and this was followed by the lowering of *cis* free Ca^{2+} to pCa 7 using a calibrated solution of EGTA. Control activity at this lower Ca^{2+} resulted in a decrease in channel open probability of ~six-fold due to the Ca^{2+} dependence of the release channel (Smith et al., 1986*a*; Vilven et al., 1989). Following the test period, the *cis* free Ca^{2+} concentration was increased to confirm that an active channel was present in the bilayer even though IPS_3 was unable to open it. In Fig. 6, control in pCa 5 was followed by addition of ATP and Mg^{2+} which resulted in a decrease in P_o and a decrease in open channel current due to Mg^{2+} blockade (Smith et al., 1986*a*). Mg^{2+} and ATP were added to simulate the ligand conditions used in uptake and release experiments in Fig. 1. However, either in the absence of Mg^{2+} and ATP (Fig. 5) or in the presence of Mg^{2+} and ATP (Fig. 6), 30 μM IPS_3 had virtually no effect on the P_o of release channels. This is described

TABLE 1 Kinetic effect of IPS_3 on calcium release channels of rabbit skeletal muscle triads

		Control	30 μM IPS_3
Recording time	*ms*	292,500	308,750
Number of open events		9,553	18,131
Mean open time	τ_1, *ms*	1.6 ± 0.4	1.4 ± 0.2
	τ_2, *ms*	12.5 ± 3.8	8.2 ± 3.5
Mean closed time	τ_1, *ms*	2.6 ± 0.78	1.6 ± 0.2
	τ_2, *ms*	36.7 ± 11.5	29.5 ± 6.9
Fraction open time	P_0	0.12 ± 0.03	0.19 ± 0.03
Number of bursts		272	343
Mean burst duration	t_1, *ms*	38.4 ± 19.6	45.9 ± 19.7
	t_2, *ms*	776 ± 217	728 ± 150
Fraction burst time		0.27 ± 0.12	0.35 ± 0.12

Mean ± SD are from three separate recordings at a holding potential of +20 mV and *cis* 10 μM free Ca^{2+}. Records were filtered at 1.5 KHz and digitized at 4 KHz. Openings were identified by two threshold detectors placed between baseline and open current levels. Burst were identified as one or more openings flanked by closings of duration >50 ms. Fraction of open time or burst time corresponds to the probability of occurrence of an open event or burst event within the recording period. Recording time in each experiment was exp. 1 control 97.5 s, exp. 1 test 97.5 s; exp. 2 control 130 s, exp. 2 test 130 s; exp. 3 control 66 s, exp. 3 test 82.5 s.

quantitatively by the bar histograms next to each recording, and by the P_o calculated for each condition. In three recordings such as in Fig. 5, P_o averaged 0.0094 ± 0.001 in control pCa 7 and 0.01 ± 0.006 after addition of IPS_3. In three recordings such as in Fig. 6, P_o averaged 0.024 ± 0.03 in control pCa 7 and 0.05 ± 0.03 after addition of IPS_3. A test for two means of independent samples was used to establish if the P_o of control and that following IPS_3 were unequal for a specified level of significance, t. In the absence of MgATP, P_os of control and following IPS_3 were not unequal for all levels of significance $t \leq 0.1$. In the presence of MgATP, P_os of control and following IPS_3 were unequal for a level of significance $t \geq 0.1$ but could not be demonstrated to be unequal for a level of significance $t \leq 0.05$.

DISCUSSION

Because Ca^{2+} release channels are the major Ca^{2+} conductance pathway of the junctional SR of skeletal muscle, the issue of whether they mediate IP_3-induced Ca^{2+}release is important to resolve. Based on a comparison of $^{45}Ca^{2+}$ flux density and single channel open probability, we estimated previously a minimum number of three

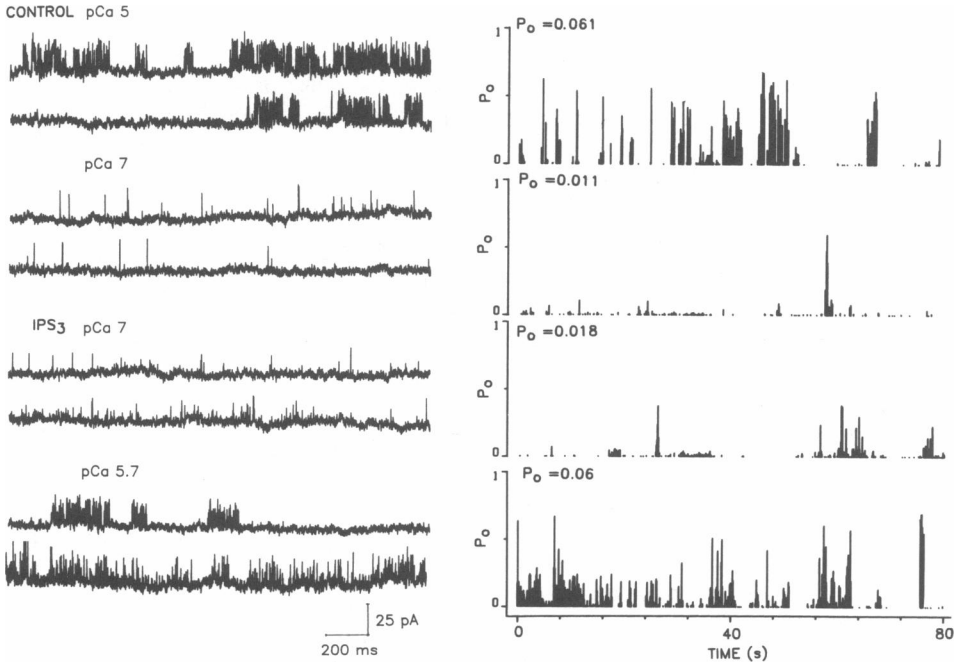

FIGURE 5 Effect of IPS_3 on Ca^{2+} release channels held closed by submicromolar Ca^{2+}. (*Left*) Representative traces following sequential additions to the *cis* chamber, shown from top to bottom. All traces from the same experiment. (*Right*) P_o measured every 100 ms is shown for 80 s following each addition. Average P_o during each 80-s period is indicated. Control activity was in *cis* 10 μM free Ca^{2+}, pH 7.4. Free Ca^{2+} was next lowered to pCa 7 with *cis* 30 μM K_2EGTA pH 7.4. *Cis* 30 μM IPS_3 was next added. Free Ca^{2+} was next raised to pCa 5.7 with *cis* 41.6 μM $CaCl_2$. Holding potential was 20 mV in all records.

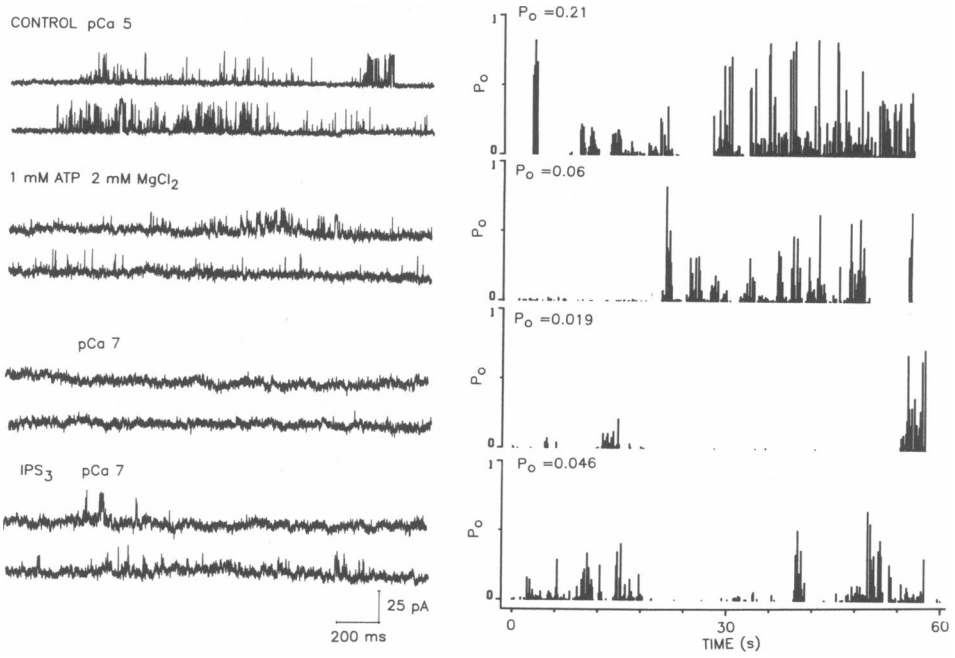

FIGURE 6 Effect of IPS_3 on Ca^{2+} release channels held closed by submicromolar Ca^{2+} plus MgATP. (*Left*) Representative traces following sequential additions to the *cis* chamber, shown from top to bottom. All traces from the same experiment. (*Right*) P_o measured every 100 ms is shown for 60 s after each addition. Average P_o during each 60-s period is indicated. Control was *cis* 10 μM free Ca^{2+}, pH 7.4, Cis 1 mM total ATP and 2 mM total $MgCl_2$ were next added. Free Ca^{2+} was next lowered to pCa 7 with *cis* 300 μM K_2EGTA pH 7.4. *Cis* 30 μM IPS_3 was next added. Holding potential was 20 mV in all records.

channels per each junctional SR vesicle in ~90% of the vesicle population and a density of ~20 channels/μ^2 (Smith et al., 1986*b*). This large density and the large unitary conductance of release channels raised the possibility that even the small changes in P_o observed upon addition of IPS_3 (from 0.019 to 0.046 in Fig. 6), could be sufficient to explain the release of the ~20% of Ca^{2+} actively stored into triads (Fig. 1). For this explanation to be consistent with Fig. 1, it would be necessary to assume (*a*) that the steady-state level of intravesicular Ca^{2+}, seen at the end of the uptake period, is set by an equal Ca^{2+} pump and leak rates; (*b*) that upon addition of IPS_3, a lower steady-state level of intravesicular Ca^{2+} is set by an increase in the leak rate, i.e., a more active Ca^{2+} release channel; and (*c*) that in the presence of IPS_3, the pump rate can overcome the leak rate if sufficient ATP is present. The latter would explain the recovery of intravesicular Ca^{2+} when ATP is increased in a dose-dependent manner after release by IPS_3 (Fig. 1, *top*).

Comparison of influx and efflux rates of Ca^{2+} in triads

Although assumptions (*a*) through (*c*) are generally valid, a straightforward calculation given below shows that the efflux rate through the total population of release channels in our preparation of triads is ~10^3 times larger than the influx rate of Ca^{2+} via the Ca^{2+} pump measured experimentally. Thus, a finely tuned balance between influx and efflux rates that would satisfy (*a*) through (*c*) is unlikely to be set by Ca^{2+} release channels given their high density in triads and prodigious turnover rate. The total Ca^{2+} efflux through Ca^{2+} release channels in the population of triads can be estimated with a minimum number of assumptions as,

$$J^e = inP_o/zF, \tag{1}$$

where J^e has units of moles of Ca^{2+}/s/mg triad protein; i is the single channel Ca^{2+} current at the operating SR voltage and intravesicular Ca^{2+} concentration; F is Faraday's constant; z is the valence; n is the density of channels per mg triad protein; and P_o is the open probability per channel. The single channel current is a function of voltage, V, and is calculated in terms of the channel equilibrium potential as,

$$i = \gamma_{Ca}(V - V_{eq}), \tag{2}$$

where γ_{Ca} is the Ca^{2+} conductance at a given concentration of intravesicular Ca^{2+}, and V_{eq} is the reversal potential of the release channel under the ionic gradients set

across the SR. γ_{Ca} is estimated from the Ca^{2+} binding isotherm described for the ryanodine receptor (Smith et al., 1988) given by,

$$\gamma_{Ca} = \gamma_{max}/[1 + Kd/(Ca_i)], \quad (3)$$

where γ_{max} is the saturating Ca^{2+} conductance, Kd is the Ca^{2+} binding affinity, and $[Ca_i]$ is the intravesicular Ca^{2+} concentration. We assumed $[Ca_i] = 5$ mM for the intravesicular concentration during constant pump activity (see Hasselbach and Oetliker, 1983). The γ_{max} and Kd, determined previously from single channel experiments, were 172 pS and 3 mM, respectively (Smith et al., 1988). The Ca^{2+} conductance of a single release channel (Eq. 3) is $\gamma_{Ca} = 108$ pS. To estimate the single channel current (Eq. 2) we assumed no voltage drop across the triad membrane, $V = 0$ mV (see Hasselbach and Oetliker, 1983) and $V_{eq} = 5$ mV determined previously in physiological solutions, i.e., *cis* 150 mM KCl, 10 μM Ca^{2+} and *trans* 150 mM KCl, 5 mM Ca^{2+} (Smith et al., 1988). The Ca^{2+} current per single open channel (Eq. 2) is $i = 0.54$ pA. The number of channels expressed in milligrams of triad protein is directly obtained from [^{3}H]Ryanodine binding studies in the same preparation. The estimated ryanodine receptor density was 9 pmol/mg triad protein (Valdivia et al., 1990). Assuming one active channel per ryanodine binding site (Imagawa et al., 1987), $n = 5.4 \times 10^{12}$ channels/mg triad protein. Thus, for a typical activation of release channels by IPS_3 such as in Fig. 6, where P_o (test minus control) = 0.027, we estimated the Ca^{2+} efflux rate through release channels in the population of triads (Eq. 1) to be $J^e = 0.41$ μmol Ca^{2+}/s/mg triad protein. This efflux is three orders of magnitude larger than the initial rate of Ca^{2+} influx during ATP-dependent activation of the SR Ca^{2+} pump in our preparation or J^i = 0.3 nmol Ca^{2+}/s/mg triad protein (calculated from Fig. 1 and indicated in text).

This gross mismatch between Ca^{2+} influx via the Ca^{2+} pump and efflux via release channels has important consequences. Based on this calculation, for example, the recovery of uptake after the stepwise reactivation of the Ca^{2+} pump with ATP, in the presence of IPS_3 (Fig. 1, *top*), cannot be explained under the asumption that IPS_3 opens Ca^{2+} release channels. This is simply because the Ca^{2+} pump rate would have to increase by a factor of 10^3 to overcome efflux rate due to the constant activation of release channels by IPS_3. It is therefore likely that a separate channel, presumably of low conductance and/or low density, would be responsible for the IPS_3-induced release seen in the Ca^{2+} electrode experiments. That IP_3-induced release could be mediated by a receptor other than the ryanodine receptor is not surprising. The purified IP_3 receptor is a 260-kDa protein (Supattapone et al., 1988), dissimilar to the 567-kDa ryanodine receptor (Takeshima et al., 1989; Furuichi et al., 1989). Furthermore, in smooth muscle microsomes, the $^{45}Ca^{2+}$ efflux rate through Ca^{2+} release channels was ~10 times larger than the IP_3-induced Ca^{2+} release (Watras et al., 1989). Thus, the kinetics of both release mechanisms are quite distinct, and at least in smooth muscle, they appear to be mediated by entirely different channels (Ehrlich and Watras, 1988).

Differences between frog and rabbit release channels

A direct effect of IP_3 on Ca^{2+} release channels was described by Suarez-Isla et al. (1988) in frog SR, albeit at high Ca^{2+} and IP_3 concentrations. In their study, 50 μM IP_3 (purchased from Calbiochem) could open release channels from $P_o \sim 0.10$ to $P_o \sim 0.85$ with a half-maximal dose of 15 μM IP_3 at a cytosolic free Ca^{2+} of 40 μM. This result differs substantially from those summarized in Table 1. Essentially, we found weak effects of IP_3 at micromolar Ca^{2+} and notoriously small effects at physiological resting levels of Ca^{2+} and MgATP. However, there are differences in recording solutions that should be mentioned. Suarez-Isla et al., (1988) used 37 mM *trans* (lumenal) Ca^{2+} or Ba^{2+} as current carrier whereas we kept *trans* Ca^{2+} at 10 μM. Low *trans* Ca^{2+} prevented Ca^{2+}-dependent inactivation which drastically decreases P_o even when the *cis* Ca^{2+} was kept in the micromolar range (Ma et al., 1988*b*). For example, in 10 μM *cis* Ca^{2+} the P_o was ~0.2 (Table 1) whereas in their controls at the same *cis* Ca^{2+}, activity was virtually null ($P_o \sim 0.03$, Fig. 3 *A* of Suarez-Isla et al., 1988). Therefore, it could be argued that the IP_3 sensitivity of the release channel is increased when the channel is inactivated by high trans Ca^{2+}. We considered this possibility unlikely because in previous experiments IP_3 had no effect in rabbit release channels in high trans Ca^{2+}, either in the absence of Cs^+ (Smith et al., 1986*a*) or in the presence of the CsCl solutions used in the present work (not shown). Cs^+ itself did not inhibit IPS_3 induced release in the Ca^{2+} electrode assay (not shown). Thus, neither the absence of high trans Ca^{2+} nor the presence of Cs^+ may entirely account for the difference in IP_3 sensitivity between the frog and the rabbit release channels. One hypothesis is that the IP_3 sensitivity is conferred to the SR by a putative "IP_3 receptor" which in mammalian skeletal muscle would be fairly dilute. For this to apply specifically to Ca^{2+} release channels, the putative "IP_3 receptor" and the ryanodine receptor could only be loosely associated because in sucrose gradients, the CHAPS-solubilized ryanodine receptor migrates as an oligomer with a monomer subunit MW of ~400 KDa (Valdivia et al., 1990). In our hands, the preparation of purified ryanodine receptors does not have contaminants abundant enough to suggest the pres-

ence of putative "IP_3 receptor" subunits. However, a 71-kDa protein was selectively cross-linked to ryanodine receptors in rabbit triads suggesting that loosely associated proteins can interact with Ca^{2+} release channels (Chadwick et al., 1988). Thus, an "IP_3 receptor" abundant in amphibian muscle, but not in mammalian muscle, may account for the comparatively higher IP_3 sensitivity reported for the frog release channel (Suarez-Isla et al., 1988) relative to that found here for the rabbit release channel.

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Note added in proof: No effect of IP_3 was observed by Penner, R., E. Neher, H. Takeshima, S. Nishimura, and S. Numa (1989. Functional expression of the calcium release channel from skeletal muscle ryanodine receptor cDNA. *FEBS [Fed. Euro. Biochem. Soc.] Lett.* 259:217–221) in calcium release channels expressed in Chinese hamster ovary cells transformed with rabbit ryanodine receptor cDNA. This further suggests that ryanodine receptors and IP_3 receptors of skeletal muscle are separate proteins.

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