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Calcium Release from the Nucleus by $InsP_3$ Receptor Channels

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Summary

The nucleus is surrounded by a double membrane separating it from the cytoplasm. The perinuclear space is continuous with endoplasmic reticulum, and the nuclear outer membrane shares many features with the reticular membrane. We now show that inositol 1,4,5-trisphosphate ($InsP_3$) receptors associated with the nucleus release Ca^{2+} from isolated Xenopus laevis oocyte nuclei. Electrophysiological measurements of the intracellular $InsP_3$ receptor in its native membrane have not been possible on the fine filamentous endoplasmic reticulum. In this paper, we directly measure $InsP_3$-dependent receptor channels in isolated nuclei. The nuclear $InsP_3$ receptor is activated by $InsP_3$ and modulated by Ca^{2+}. The channel is weakly regulated by ATP, is mildly voltage dependent, and has a greater conductance with monovalent cations than with divalent cations.

Introduction

Scores of tyrosine kinase and G protein–linked receptors release the ubiquitous second messenger Ca^{2+} via the intracellular inositol 1,4,5-trisphosphate receptor ($InsP_3R$). The receptor has been purified and reconstituted in lipid bilayers, documenting its dependence on $InsP_3$ (Ehrlich and Watras, 1988; Mayrleitner et al., 1991). The purified $InsP_3R$ is regulated by cytoplasmic Ca^{2+} (Bezprozvanny et al., 1991) and ATP (Maeda et al., 1991; Bezprozvanny and Ehrlich, 1993). Plant vacuole $InsP_3Rs$ differ from mammalian channels in the bilayer in their voltage sensitivity (Alexandre et al., 1990). Whereas the endoplasmic reticular (ER) $InsP_3R$ has been well characterized in artificial bilayers, the electrophysiological properties have not been studied with the receptor in its native membrane, owing to the inaccessibility of the ER for direct patch-clamp experiments. Questions remain concerning the effects of removing the channel from regulatory factors that may exist in the native membrane and are not present in an artificial lipid bilayer, as has been shown for the ER ryanodine channel (Brillantes et al., 1994).

The nuclear membrane is continuous with the ER (Dale et al., 1994; Bachs et al., 1992; Bustamante, 1994) and contains phosphatidyl inositol metabolic enzymes (Divecha et al., 1993). The $InsP_3R$ has been localized to the perinuclear region based on immunofluorescence studies with antibodies specific to the $InsP_3R$ (Parys et al., 1992; Kume et al., 1993). In addition, $^{45}Ca^{2+}$ is released from isolated nuclei in response to $InsP_3$ (Malviya et al., 1990). In this paper, we directly measure the single-channel gating properties of the $InsP_3R$ in the nuclear membrane.

Results and Discussion

Following isolation of nuclei, we found that the anti-$InsP_3R$ antibody specifically immunostained the surface of the nuclear envelope (Figures 1A and 1B). No fluorescence was observed in control nuclei exposed to preimmune sera. The $InsP_3Rs$ identified on the surface of the oocyte nuclei functionally released Ca^{2+} from isolated nuclei. Figure 1C shows that single oocyte nuclei loaded with the Ca^{2+}-sensitive dye Indo-1 released Ca^{2+} in response to the (1,4,5) isoform of $InsP_3$. In contrast, the (1,3,4) form of $InsP_3$, $InsP_4$, and caffeine failed to induce release (Figure 1D). Heparin, an inhibitor of $InsP_3R$ channels (Ehrlich and Watras, 1988; Mayrleitner et al., 1991), blocked the $InsP_3$-induced release. Half of the available Ca^{2+} was released from the nuclear store by 50 nM $InsP_3$ (Figure 1E). We conclude from the results shown in Figure 1 that isolated Xenopus laevis nuclei contain a high density of functional $InsP_3Rs$.

Of over 160 patches of nuclear membrane that possessed channel activity, 93% contained characteristic $InsP_3$ channels when $InsP_3$ (≥ 1 μM) was in the pipette. In the absence of $InsP_3$ in the pipette, none of the same channels were observed ($n = 17$). Previously identified Cl^- channels were also seen in patches (Tabares et al., 1991). At low concentrations of $InsP_3$ (<0.1 μM), channel activity ran down within 20 s to 1 min after activation, in contrast to persistent channel activity measured with the purified $InsP_3R$ in the bilayer. At higher $InsP_3$ concentrations, no run down was noted for the nuclear $InsP_3$-dependent current. To test directly whether the channel events we observed were activated by $InsP_3$, the cytoplasmic face of excised patches was exposed to control solution, followed by perfusion of the interior of the recording pipette with a solution containing $InsP_3$. The $InsP_3$ perfusion resulted in activation of characteristic $InsP_3R$ channels ($n = 22$; Figure 2A). It is unlikely that mechanical stimulation during perfusion activated the channels, since perfusion of the patch with control solution alone did not cause channel openings ($n = 4$). Perfusion of the patch with heparin inhibited channel activity that had been initiated previously by $InsP_3$ ($n = 3$; Figure 2B).

The conductance of the $InsP_3$-dependent channel was measured using K^+, Na^+, Ca^{2+}, and Ba^{2+} as cationic charge carriers. Representative channel openings are shown in Figure 3A with K^+ as the charge carrier at the specified membrane potentials. The third conductance level (172 pS) predominated, but two smaller conducting substates (126 and 90 pS; Figure 3B) and a large conductance level (244 pS) were also observed. A linear current–voltage relation was apparent for all substates with equimolar K^+ as the charge carrier (Figure 3C). Replacement of 140 mM KCl with 80 mM $CaCl_2$ in the bath (10 μM $InsP_3$ in pipette) increased channel activity (Np_o) an average of 13-fold in

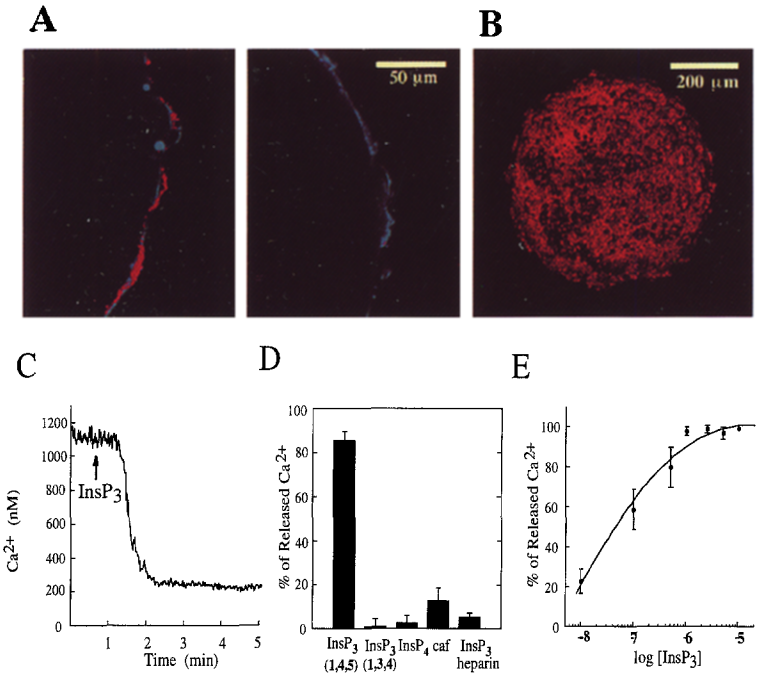

Figure 1. Oocyte Nuclei Contain Functional $InsP_3Rs$

(A) Fixed oocyte nuclei were incubated with affinity-purified polyclonal antibody to the rat $InsP_3R$ (red punctate staining) and to rhodamine B, a membrane dye (blue), to show the distribution of the $InsP_3R$ on the nuclear surface (left; n = 14). Control nuclei were exposed only to the fluorescein-conjugated secondary antibody (right) or preimmune sera followed by secondary antibodies, illustrating little nonspecific staining (n = 12).

(B) Three-dimensional reconstruction of anti-$InsP_3R$ antibody staining of the surface of the nucleus.

(C) A single isolated nucleus released Ca^{2+} in response to $InsP_3$ (5 μM; added to the bath at the arrow).

(D) Percentage of Ca^{2+} released from nuclei in response to 5 μM (1,4,5) $InsP_3$ (n = 21), (1,3,4) $InsP_3$ (n = 8), and (1,3,4,5) $InsP_4$ (n = 11). Heparin (10 μg/ml; n = 16) blocked the $InsP_3$-dependent release. In contrast, caffeine (5 mM) released little Ca^{2+} (n = 14). The value for 100% release of Ca^{2+} was defined as the difference between the maximal $[Ca^{2+}]$ following ATP application (5 mM) and the resting level of Ca^{2+} in the bath (clamped at 200 nM free Ca^{2+}).

(E) Dose–response relation between the concentration of $InsP_3$ and the percentage of Ca^{2+} released by the nuclei (n ≥ 4 nuclei for each point).

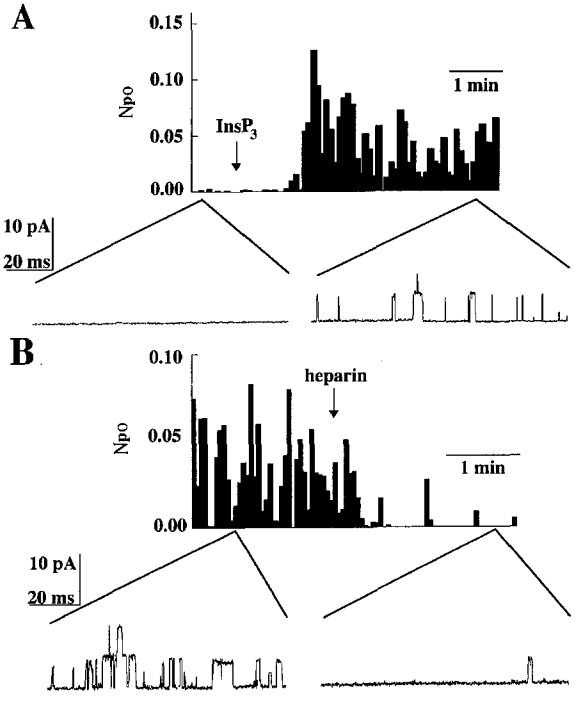

Figure 2. $InsP_3$-Dependent, Heparin-Sensitive Channel Activity in Excised Patches of Nuclear Membrane

(A) The recording pipette contained stock solution without $InsP_3$; positive pressure was applied (arrow) to the internal perfusion pipette containing 10 μM $InsP_3$. This example is representative of 22 perfusion experiments; 3 patches had no $InsP_3$-dependent channel activity following recording pipette perfusion. V = +30 mV.

excised patches (main substate = 85 pS in 80 mM Ca^{2+}; Figure 3D). Ba^{2+} (75 mM) conductance of the predominant substate was 130 pS. Relative single-channel conductances at the predominant substates were $K^+ \approx Na^+ > Ba^{2+} > Ca^{2+}$. Equimolar Cl^- replacement by SO_4^{2-} had no obvious effect on channel behavior. Ion substitution experiments showed that the channel was more permeant to K^+ than Ca^{2+}. The $InsP_3$-dependent channel activity in the nuclear membrane was voltage dependent in the physiologic range of potentials (~0 mV), with highest activity displayed at more positive potentials (Figure 4A). The mean closed time of single-channel recordings was 5-fold greater at −40 mV compared with +40 mV. In contrast, the mean open time did not change significantly with membrane potential (n = 10 patches).

Previous studies of the $InsP_3R$ in permeabilized cells (Iino and Endo, 1992), microsomes (Finch et al., 1991), and lipid bilayers (Mayrleitner et al., 1991; Bezprozvanny et al., 1991) showed that the $InsP_3$-dependent channel exhibits a bell-shaped sensitivity to cytoplasmic $[Ca^{2+}]$. However, these experiments all measured the effect of Ca^{2+} on Ca^{2+} flux, a situation in which it is difficult to determine the actual $[Ca^{2+}]$ at the site of the receptor (Stern, 1992). We determined the Ca^{2+} sensitivity of the $InsP_3R$ with K^+ as the charge carrier and found maximal channel

(B) Heparin (10 μg/ml) perfusion blocked channel activity. $InsP_3$ was present in the patch-clamp pipette during seal formation to induce channel activity and was also in the heparin-containing solution.

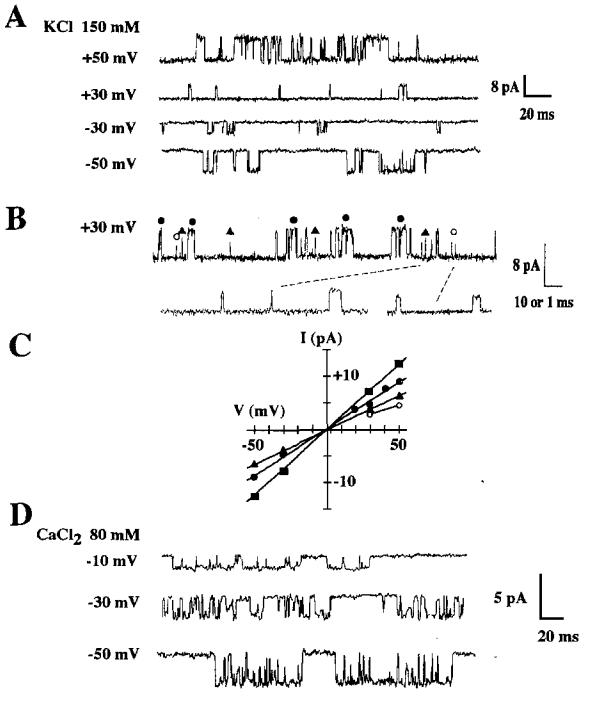

Figure 3. Current–Voltage Relation of $InsP_3$-Dependent Channels

(A) Nuclear-attached, single-channel recordings at membrane potentials from −50 to +50 mV.

(B) Examples of three of the four conductance states of the $InsP_3R$ are shown. The two smallest conductance states (closed triangles and open circles) are shown in expanded time in the bottom traces.

(C) Current–voltage relation for all four conductance substates shows the large conductance substate (244 pS; closed squares), the most frequently observed conductance state (172 pS; closed circles), and the two smaller states (126 pS [closed triangles] and 90 pS [open circles]).

(D) Single-channel recordings with 80 mM Ca^{2+} as the charge carrier show bursts of high activity at membrane potentials from −10 to −50 mV (n = 8). Single-channel conductances were calculated from amplitude histograms for each substate and permeability experiment. Ion replacement was accomplished by complete perfusion of the bath with the replacement solution while measuring inward current in excised patch-clamp mode.

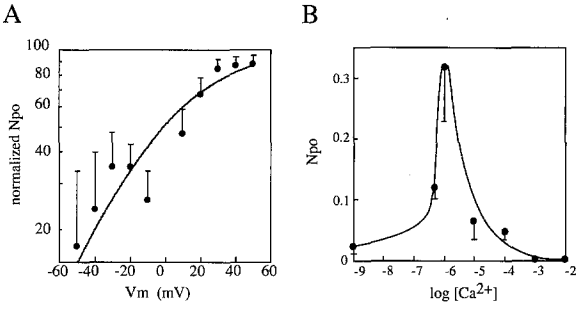

Figure 4. Regulation of $InsP_3R$ Channel Activity

(A) $InsP_3R$ activity was sensitive to membrane potential (pipette solution contained 1 μM calculated free Ca^{2+}). The highest channel activity in each patch was normalized to 100 (n = 5), and mean values were fit to the Boltzmann equation: $1/[1 + \exp(-ze/kT)]$, z = 0.8. At limiting slope an e-fold increase in channel activity (Np_o) required a 12 mV change in membrane potential.

(B) Ca^{2+} dependence of the $InsP_3R$. Holding potential = +30 mV; K^+ (140 mM) was the charge carrier (n ≥ 7 patches for each point).

activity at ~1 μM free Ca^{2+} (Figure 4B). Lowering the cytoplasmic [Ca^{2+}] from 1.0 to 0.01 μM reduced the single-channel mean open time from approximately 6 ms to 1 ms. Mean closed times increased at low (<100 nM) and high (>50 μM) Ca^{2+} concentrations, but the variability was too great to determine a statistically significant difference. The bell-shaped Ca^{2+} dependence of the receptor has been questioned recently with reports of direct competition between $InsP_3$ and the Ca^{2+} chelators at the receptor binding site (Richardson and Taylor, 1993; Combettes et al., 1994). In these experiments we used low concentrations of chelators (75 μM or less) that have been reported not to interfere with $InsP_3$ binding (10 μM $InsP_3$; Richardson and Taylor, 1993).

When studied in the bilayer, $InsP_3R$ channel activity was strongly modulated by cytoplasmic levels of ATP (Maeda et al., 1991; Bezprozvanny and Ehrlich, 1993). We saw little effect of perfusion with ATP (5 mM) on $InsP_3$-dependent currents with 10 μM $InsP_3$ and 1 μM Ca^{2+} in the pipette (n = 7). The average initial Np_o of these channels was 0.21, which may have been sufficiently high to preclude further activation by ATP. In three patches that had low initial channel activity (Np_o < 0.1), perfusion of the pipette with 5 mM ATP increased activity an average of 4-fold.

We conclude that the nucleus contains a functional Ca^{2+} store responsive to $InsP_3$. Second messenger–stimulated channel activity had not been recorded previously on the nuclear envelope. We suggest that this intact nuclear preparation may provide a unique tool for studying receptors and channels typically classified as ER. We have defined the properties of the nuclear $InsP_3$-dependent current and find some differences when compared with the purified receptor in artificial lipid bilayers. We cannot duplicate all the conditions used for bilayer recordings; thus, precise comparisons cannot be made. However, in terms of $InsP_3$ dependence, block by heparin (Ehrlich and Watras, 1988; Mayrleitner et al., 1991), mean open time, presence of four substates with the third substate predominant (Watras et al., 1991), and regulation by cytoplasmic Ca^{2+} (Mayrleitner et al., 1991; Bezprozvanny et al., 1991), the bilayer and native nuclear $InsP_3R$ channel properties are surprisingly similar. The higher conductance of the native nuclear $InsP_3R$ (85 pS in 80 mM Ca^{2+}) versus that reported previously for the receptor in the bilayer (26–53 pS in ~50 mM Ca^{2+}; Maeda et al., 1991; Bezprozvanny and Ehrlich, 1994; Mayrleitner et al., 1991) suggests that the $InsP_3R$ may be closer to the ryanodine receptor (90–140 pS; Coronado et al., 1994) in its conductance properties than previously speculated. Relative K^+ and divalent permeability reported for the bilayer ($Ba^{2+} > Ca^{2+} > K^+$; Bezprozvanny and Ehrlich, 1994) differs from that of the native $InsP_3$ channel ($K^+ > Ca^{2+}$). The difference may be due to the amino acid sequence differences found between Xenopus and mammalian $InsP_3Rs$ (Yamamoto-Hino et al., 1993). But, cerebellar bilayer reconstitutions also contain micro-

somal K^+ channels that cannot be easily distinguished from $InsP_3$ channels in K^+-containing solutions, necessitating assumptions in determining monovalent permeability (Bezprozvanny and Ehrlich, 1994).

Nuclear Ca^{2+} regulation must play a role in the Ca^{2+} dependence of nuclear transcriptional regulators, the Ca^{2+} control of DNA synthesis (for review, see Dale et al., 1994), Ca^{2+}-sensitive endonuclease activity (Jones et al., 1989), and fusion of nuclear vesicles bound to chromatin (Sullivan et al., 1993). Nuclei also contain Ca^{2+}-dependent contractile proteins, and nuclear Ca^{2+} has been implicated in DNA repair (Dale et al., 1994). The location of an $InsP_3$-sensitive Ca^{2+} store in the nucleus and the recognition of phosphoinositide pathway enzymes associated with the nucleus (Divecha et al., 1993) may provide important clues for understanding the Ca^{2+} dependence of the cell cycle (Lu and Means, 1993; Whitaker and Patel, 1990).

Experimental Procedures

Xenopus laevis oocytes were removed from the animals and defolliculated manually (Lechleiter and Clapham, 1992) or with collagenase. Cells from which nuclei were to be used for Ca^{2+} imaging were injected with 50 nl of 1 mM Indo-1 (final concentration $\approx$ 50 μM). After 30 min, cells were manually split open at the equator, and nuclei were teased free of cytoplasm into a mock intracellular solution containing 140 mM KCl, 10 mM HEPES, and 3 mM $MgCl_2$ (pH 7.3); free Ca^{2+} was clamped at 200 nM using 4.43 mM CaK_2EGTA, 5.57 mM H_2K_2EGTA (Neher, 1988). Nuclei were washed twice in the same buffer and placed in the recording chamber for confocal imaging or patch-clamp experiments. All experiments were performed in this pipette and bath solution at room temperature (22°C ± 2°C) unless otherwise noted.

Imaging

Nuclei to be used for antibody immunostaining were fixed by incubation in 80 mM PIPES, 50 mM KCl, 5 mM EGTA, 3 mM $MgCl_2$, 3.7% paraformaldehyde for 4 hr. Nuclei were rinsed in Tris-buffered saline (TBS), then exposed to polyclonal affinity-purified antibodies to rat $InsP_3R$ (Sharp et al., 1993) diluted in TBS with 2% bovine serum albumin for 24 hr. Nuclei were washed 6 times in TBS over 24 hr and then labeled with secondary anti-goat fluorescein isothiocyanate antibodies in TBS with 2% bovine serum albumin for 18–24 hr at 4°C, followed by 5–6 TBS washes over 16–18 hr. Control nuclei were exposed to preimmune sera and subsequent secondary antibody or to the secondary antibody alone. Following imaging of antibody immunostaining, the nuclei were exposed to the lipid dye rhodamine B (250 ng/ml) for 20 min, and imaging was repeated. Images were collected on a Bio-Rad MRC 600 scanner adapted to an inverted microscope. The microscope objective was an Olympus 10× UV Planapo. ANALYZE (Mayo Foundation) and Adobe Photoshop software were used for overlay of the two sets of images and for three-dimensional reconstruction.

Ca^{2+} images were collected every 2 s at wavelengths of 495 and 405 nm, digitized to 8 bits (255 grey scale), and stored on optical disk. Fluorescence values were converted to ratios (R) by averaging the pixel values within the boundary of the nucleus and dividing the values for each image. Calculated Ca^{2+} concentrations were obtained from R_{min} and R_{max} values collected from nuclei exposed to 1 μM ionomycin in 10 mM EGTA ($[Ca^{2+}] < 100$ nM) or 1 mM Ca^{2+}, respectively. In addition, control nuclei injected with Ca^{2+} and EGTA (50 μM Ca^{2+} or 100 μM EGTA intracellular concentrations) were used to obtain R_{max} and R_{min} values for each set of experiments. Ca^{2+} concentrations were calculated according to $[Ca^{2+}] = K_d(S_{f2}/S_{b2})[(R - R_{min})/(R_{max} - R)]$, where S_{f2}/S_{b2} is the fluorescence ratio for free and Ca^{2+}-bound forms of Indo-1 at 495 nm and assuming a K_d of 250 nM for Indo-1 (Grynkiewicz et al., 1985). All nuclei were loaded with Ca^{2+} by exposing them to 5 mM ATP prior to release experiments, as described previously (Malviya et al., 1990; Nicotera et al., 1989). All error bars reflect ± SEM.

Electrophysiology

Electrophysiological experiments were carried out using conventional patch-clamp procedures including nuclear-attached (Mazzanti et al., 1990) and excised-patch recording (Hamill et al., 1981; cytoplasmic side of membrane inside the pipette). We define the membrane potential with respect to the cytoplasmic side of the membrane; V_{nuc} membrane = $V_{pipette}$ in both configurations. The pipette contained the stock solution described above, except that EGTA concentrations used were less than 75 μM to avoid block of $InsP_3$ binding (Richardson and Taylor, 1993; Combettes et al., 1994). The total contaminating Ca^{2+} in the stock buffer solution prior to the addition of the chelator was determined using atomic absorption spectroscopy (Thermo-Garrel Ash) at a wavelength of 422 nm. Single-channel recordings were sampled at 10–20 kHz and stored on VCR tape. Data were filtered at 1–2.5 kHz for analysis and display. The product of the number of channels in the patch (N) and the probability of channel opening (p_o) was calculated as the integral of channel current over time (Logothetis et al., 1987). Np_o values were averaged and shown in 5 and 3 s bins (Figures 2A and 2B, respectively). For measurements summarized in Figure 4, Np_o was obtained from >30 s long segments of recordings at each Ca^{2+} concentration and membrane potential shown.

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