CELL CALCIUM

Cell Calcium (1994) 15, 66–78

Inositol 1,4,5-trisphosphate receptors in *Xenopus laevis* oocytes: Localization and modulation by Ca^{2+}

N. CALLAMARAS and I. PARKER

Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, California, USA

Abstract — Inositol 1,4,5-trisphosphate receptors ($InsP_3R$) in *Xenopus laevis* oocytes were localized and their regulation by Ca^{2+} was investigated. Antibodies raised against the C-terminal region of the mouse cerebellar $InsP_3R$ (cAb) cross-reacted with a 255 kD protein in Western blots of *Xenopus* microsomal membranes. Immunolocalization of this protein in cryosections of oocytes revealed diffuse staining of the cytoplasm, intense staining of the sub-plasma membrane region of the animal hemisphere, and punctate staining in association with the germinal vesicle. In the presence of 40 μM free Ca^{2+}, isolated oocyte membranes exhibited a high affinity binding site for Ins 1,4,5-P_3 (K_D = 5nM) and a binding capacity of 450 fmol/mg protein. The specific binding capacity of oocyte membranes for [^{3}H]-Ins 1,4,5-P_3 increased as the level of free Ca^{2+} present in binding assays was raised from < 0.1 nM to 4.0 μM, with an apparent EC_{50} of 60 nM. Increasing the concentration of free Ba^{2+} failed to facilitate [^{3}H]-Ins1,4,5-P_3 binding. Other inositol phosphates competed for Ins1,4,5-P_3 binding sites with approximate IC_{50} values of: Ins1,3,4,5-P_4 = 79 nM, Ins2,4,5-P_3 = 455 nM and L-Ins1,4,5-P_3 = 20 μM. In addition, 150 μg/ml (approximately 12 μM) heparin displaced 50% of bound [^{3}H]-Ins1,4,5-P_3, whereas caffeine (10 mM) had little effect. Functional reconstitution of solubilized $InsP_3Rs$ into lipid bilayers revealed that Ca^{2+} was a necessary co-agonist for activation of the $InsP_3R$. When $InsP_3$ (5 μM) and Ca^{2+} (5 μM) were applied together, conductance steps were observed. $InsP_3$ or Ca^{2+} alone had little effect. These results suggest that the subcellular organization of $InsP_3Rs$ and the facilitation of $InsP_3$ binding and channel opening by Ca^{2+} contribute to the Ins1,4,5-P_3-mediated Ca^{2+} spikes, waves, and oscillations observed in *Xenopus* oocytes.

Inositol 1,4,5-trisphosphate ($InsP_3$) is well established as an intracellular second messenger which binds to $InsP_3$ receptor/channel complexes ($InsP_3Rs$) to release Ca^{2+} from intracellular stores [1, 2]. An $InsP_3R$ highly enriched in cerebellar membranes ($InsP_3R_I$) has been purified to homogeneity [3], cloned [4], localized subcellularly [5, 6], and functionally reconstituted [7]. The $InsP_3R_I$ is a te-

trameric membrane protein composed of identical subunits, each with a relative molecular weight of 260 kD [8]. In Purkinje cells, $InsP_3R_I$s have been localized to a sub-compartment of the smooth endoplasmic reticulum [9], presumably where they function to release sequestered intracellular Ca^{2+} [10]. Binding assays and functional reconstitution of $InsP_3R_I$s have demonstrated that binding of $InsP_3$ and opening of the Ca^{2+} channel are sensitive to free [Ca^{2+}] [11], pH, and ATP levels [11, 12], and can be modified by phosphorylation [13].

Recent molecular characterization of $InsP_3R$ cDNAs from peripheral tissues have detected three additional subtypes of $InsP_3R$ ($InsP_3R_{II-III-IV}$), suggesting that a family of structurally heterogeneous $InsP_3$Rs exists [14]. These additional forms of $InsP_3R$ appear to be localized to different subcellular compartments [15], and also exhibit tissue specific differences in post-translational processing [16], affinity for various inositol phosphates (IPs) [15], and sensitivity to endogenous and pharmacological agents [17]. At present, the physiological relevance of possible subcellular heterogeneity and the complex modulation of $InsP_3$Rs is unclear. However, it has been speculated that the organization of $InsP_3$-sensitive Ca^{2+} stores and modulation of the release mechanism, particularly by Ca^{2+}, are responsible for the spatial and temporal patterns of $InsP_3$-mediated Ca^{2+} release (e.g. Ca^{2+} spikes, waves, and oscillations) observed in many cells [2, 18, 19].

Xenopus laevis oocytes have been used extensively to study $InsP_3$-mediated Ca^{2+} liberation in single intact cells because of their large size (> 1 mm diameter) and simple geometry [20, 21], but until recently little was known about the distribution and biochemical properties of $InsP_3$Rs in the oocyte [22]. In this study, we have localized $InsP_3$Rs in *Xenopus* oocytes, and characterized the effects of Ca^{2+} on the binding properties and channel activity of this receptor, with the aim of correlating histological and biochemical data with the macroscopic properties of $InsP_3$-mediated Ca^{2+} signalling observed in the intact oocyte [23–25]. Specifically, we used an affinity-purified polyclonal antibody raised against a C-terminal epitope of the rat cerebellar $InsP_3R$ (cAb) [4, 26] to determine the subcellular localization of *Xenopus* oocyte $InsP_3$Rs. In addition, assays were conducted to determine the effects of divalent ions, various IPs, and the physiologically active compounds heparin and caffeine on the binding of [^{3}H]-$InsP_3$ to oocyte membranes. Finally, we examined the effect of Ca^{2+} on the single channel activity of solubilized $InsP_3$Rs reconstituted into lipid bilayers.

Materials and methods

Materials

Anti-$InsP_3R$ Ab was a generous gift of T.C. Sudhof (The University of Southwestern Texas Medical School, USA). Goat anti-rabbit biotinylated secondary antibody was obtained from Upstate Biotechnology Inc., and alkaline-phosphatase substrate reaction kits were obtained from Vector Laboratories. [^{3}H]-$InsP_3$ was obtained from Amersham, D- and L-$InsP_3$ from LC Services Corp., Ins1,3,4,5-P_4 from Calbiochem, and Ins2,4,5-P_3 was provided by R.F. Irvine (AFRC Institute of Animal Physiology and Genetics Research, Cambridge Research Station, Cambridge, UK). Lipids were obtained from Avanti Polar Lipids. All other reagents were obtained from Sigma.

Preparation of membrane fractions

Following sacrifice of a *Xenopus laevis* by decerebration and pithing, the ovaries were removed and more than 800 Dumont stage V and VI oocytes were manually isolated, defolliculated, and stored as described [27]. Membrane fractions were prepared by a modification of the procedures of Pietri et al. [28]. Briefly, oocytes were homogenized 10% w/v in ice-cold homogenization medium (20 mM HEPES-KOH, 1 mM EGTA, 1mM DTT, 0.5 mM PMSF, 10% sucrose, at pH 7.5) with a Teflon pestle at high speed and spun at 500 *g* for 5 min to remove the yolk, pigment granules and large debris. The supernatant was then centrifuged for 20 min at 30 000 *g*. Pelleted membranes were resuspended and washed twice in homogenization medium, once in homogenization medium supplemented with 1.0 M KCl, and the final membrane pellet was resuspended in medium containing 10% sucrose, 100 mM KCl, 25 mM HEPES-KOH, 5 mM EGTA,

at pH 7.5. Membrane preparations were then frozen at –80°C for later use. Rat cerebella were homogenized, centrifuged, washed, resuspended, and frozen as above, except that the initial 5 min, 500 *g* centrifugation step was omitted.

Immunohistology

Defolliculated oocytes were fixed in 2% paraformaldehyde-Ringers for 3–6 h, cryoprotected in 20% sucrose-Ringers overnight, and quick frozen by submersion in –80°C isopentane. Cryosections 10 μm thick were cut at –18°C and mounted on gelatin coated slides. Consecutive sections mounted on the same slide, which were not exposed to cAb, were used as controls. To reduce background staining, sections were blocked with avidin, biotin, 1% BSA, 2% normal goat serum, levamisole, and 0.2 M α-methyl-mannoside. Sections were rehydrated in PBS, incubated with a 1:300 dilution of cAb overnight, washed, and incubated with a 1:500 dilution of goat anti-rabbit biotinylated secondary antibody for 1 h. Vector-Stain alkaline-phosphate reaction/substrate kits were then used to localize the antigen.

[^{3}H]-InsP$_3$ binding studies

[^{3}H]-InsP$_3$ binding to membranes was measured as described by Pietri et al. [28] with minor modifications. Specifically, 50 μl (50–200 μg protein) of membrane preparation was added to 450 μl of binding medium (100 mM KCl, 25 mM HEPES-KOH, 5mM EGTA, at pH 7.5, and 1–2 x 10^4 cpm of [^{3}H]-Ins1,4,5-P$_3$) to initiate the assay. Non-specific binding was determined in the presence of 4.0 μM unlabelled Ins1,4,5P$_3$. The assay was incubated for 10 min at 4°C and halted by centrifugation at 15 000 *g* for 15 min. Following aspiration, pellets were solubilized, 2 ml of scintillation fluid was added, and radioactivity was counted spectrometrically.

The concentrations of free divalent ions present in the binding medium were set by adding different amounts of chloride salts, calculated using a computer algorithm which employed the equations and dissociation constants for binding to EGTA described by Tsien and Pozzan [29], with appropriate adjustments made for temperature and pH. In displacement studies, the specified concentrations of non-labelled ligand were added to the binding medium, and pH was adjusted if necessary.

Heparin and calmodulin affinity chromatography

Solubilization of oocyte membranes, followed by heparin then calmodulin affinity chromatography, was conducted in order to obtain enriched preparations of InsP$_3$Rs for electrophysiological investigations. Oocyte membranes were solubilized in buffer A (100 mM KCl, 25 mM HEPES-KOH, 1 mM EGTA, 1.8% CHAPS, at pH 7.5) on ice for 2 h, and spun at 30 000 *g* for 1 h. The supernatant was then applied to a 0.5 ml heparin-agarose column equilibrated in buffer A. The column was washed with buffer A, supplemented with KCl to a final concentration of 200 mM. Proteins were eluted with buffer A, supplemented with KCl to a final concentration of 500 mM. Fractions were collected and protein determinations were made. The three peak protein fractions were pooled, diluted 1:5, and supplemented with $CaCl_2$ to a final concentration of 2 mM $CaCl_2$. This was then applied to a 0.3 ml calmodulin-Sepharose column equilibrated in buffer B (100 mM KCl, 25 mM HEPES-KOH, 2 mM $CaCl_2$, 0.1% CHAPS, at pH 7.5). The column was washed with buffer B, and proteins were eluted with buffer C (100 mM KCl, 25 mM HEPES-KOH, 5mM EGTA, 0.1% CHAPS, at pH 7.5). Fractions were collected and analyzed by SDS-PAGE. Peak fractions were then pooled and further concentrated using Amicon-100 microconcentrators (MW cutoff = 100 000) in order to provide a further 10-fold increase in protein concentration and to remove low molecular weight contaminants (e.g. proteins and/or dissociated heparin or calmodulin).

Bilayer formation and channel incorporation

Artificial lipid bilayers were formed on the tips of standard patch clamp pipettes by the 'double dip' method [30]. Briefly, stock solutions of phosphatidylethanolamine (10 mg/ml) and phosphatidylserine (10 mg/ml) were combined at a ratio of 3:1, dried under nitrogen, and resuspended in an equal volume of n-pentane, as a solvent. Bilayers were formed in the presence of asymmetrical solutions (pipette —

50 mM BaOH, 250 mM HEPES, pH 7.4: bath — 250 mM HEPES-Tris, pH 7.4) by twice passing a patch clamp pipette through a monolayer of lipid solution layered onto 3 ml of bath solution in a standard petri dish. Channel incorporation into bilayers was achieved by the addition of 1 μg of protein directly to the bath with constant stirring. Fusion events were detected as a rapid transient drop of bilayer resistance in the presence of an applied voltage. $InsP_3$, $CaCl_2$, or heparin were added directly to the bathing medium, and were removed by exchanging the bathing medium with at least 20 bath volumes. Current recordings were made with an Axopatch IC amplifier and digitally recorded. Recorded data were filtered at 1 kHz, digitally sampled, and analyzed using Strathclyde Electrophysiological Software (© John Dempster, Dept of Physiology and Pharmacology, University of Strathclyde, Glasgow, UK) running on an IBM compatible personal computer.

Other procedures

5% SDS-PAGE gel electrophoresis, transfer of proteins to nitrocellulose, and Western blot hybridizations using cAb were carried out by the methods of Sambrook et al. [31]. Protein determinations were conducted with the Coomassie blue Bio-Rad protein determination kit.

Results

Western blot analysis of Xenopus laevis microsomal membranes

Western blot analysis revealed that microsomal membranes from *Xenopus laevis* oocytes possessed a high molecular weight protein (255 kD) which cross-reacted with cAb and migrated at a slightly lower relative molecular weight (M_r) than the rat cerebellar $InsP_3R$ (Fig. 1). Although equal amounts of cerebellum and oocyte membrane protein were loaded (10 μg protein), greater specific staining was observed in the cerebellar lane. It appeared that cAb had a higher reactivity with the cerebellar $InsP_3R$, since qualitatively similar amounts of 260/255 kD protein were observed in parallel silver stained SDS gels (data not shown).

Immunohistological localization of $InsP_3Rs$ in Xenopus oocyte sections

Immunolocalization with cAb in sections through the animal hemisphere of the oocyte revealed a general diffuse staining of the cytoplasm, intense staining of the plasma membrane region which extended approximately 5 μm into the cytoplasm, as well as punctate staining in the sub-plasma membrane region (Fig. 2A). No significant staining of control sections was observed (Fig. 2B). In contrast to the intense staining in the animal hemisphere, sections through the vegetal hemisphere showed little or no staining of the cytoplasm or plasma membrane regions (Fig. 2C). Staining of punctate bodies was also observed in the cytoplasmic envelope surrounding the germinal vesicle (Fig. 2D).

Effects of free Ca^{2+} on the affinity of [^{3}H]-$InsP_3$ binding to oocyte membranes

Preliminary experiments established that little [^{3}H]-

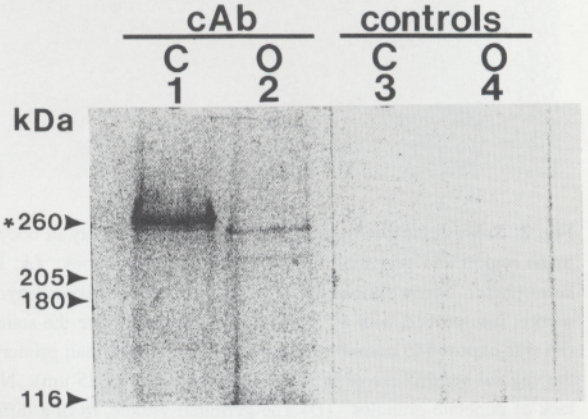

Fig. 1 Western blot analysis of *Xenopus* oocyte and cerebellar microsomal membrane proteins using cAb. Affinity purified polyclonal antibodies raised against the mouse $InsP_3R$ (cAb) recognize a 255 kD protein in *Xenopus* oocyte microsomal membrane preparations. High molecular weight proteins of 260 kD from cerebellar (lane 1, C) and 255 kD from oocyte (lane 2, O) microsomal membrane, reacted with cAb. Parallel blots which were not exposed to primary antibody, but to normal pre-immune serum, show no detectable staining of cerebellar (lane 3, C) or oocyte (lane 4, O) proteins. All lanes were loaded with 30 μg of total protein and primary antibodies were localized using horseradish peroxidase conjugated secondary antibodies with DAB as a substrate.

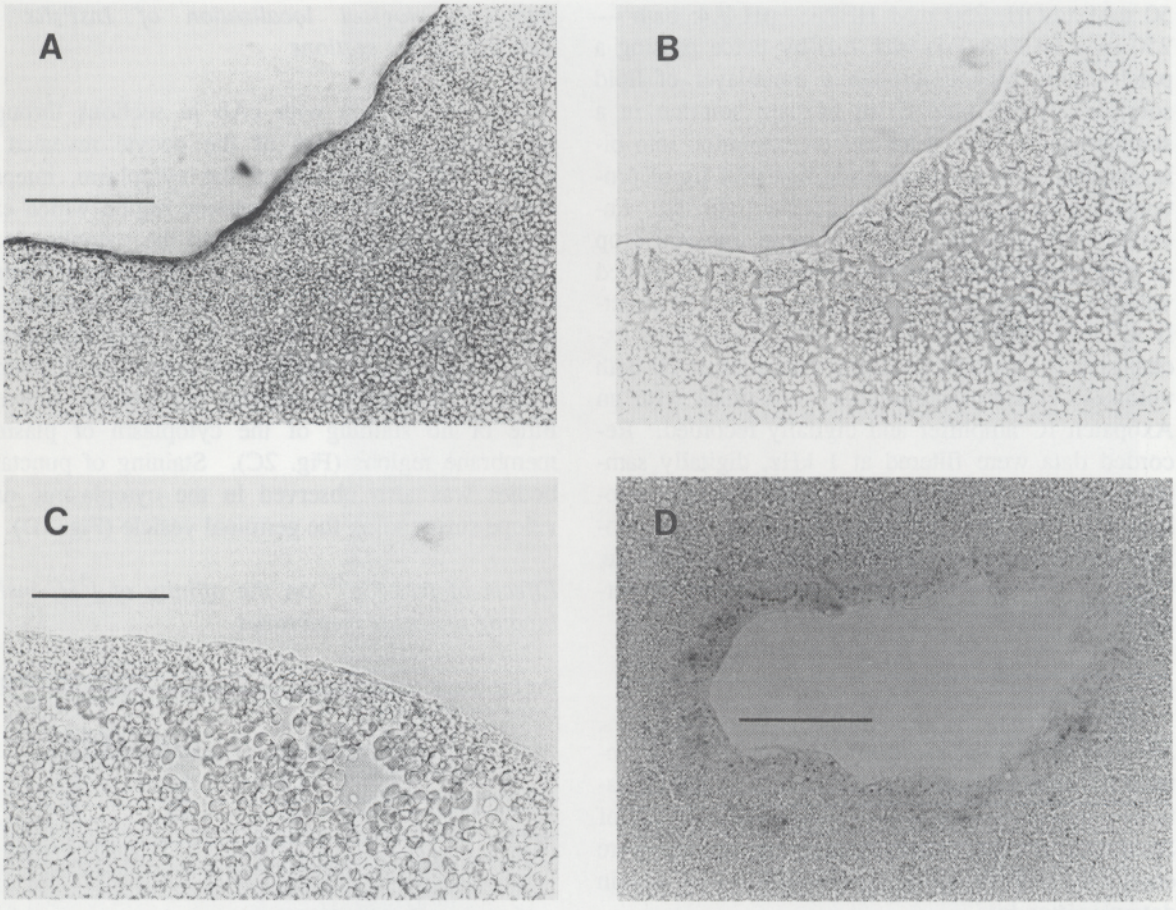

Fig. 2 Subcellular distribution of cAb immunoreactivity in oocyte sections. $InsP_3R$ immunoreactivity is localized to the plasma membrane region and germinal vesicle in oocyte cryosections. (A, B) Consecutive sections of the plasma membrane region of the animal hemisphere. Some shrinkage of the oocyte resulted from dehydration during cryoprotection (bar in A represents 50 μm). In (A) the section was probed with a 1:300 dilution of cAb. Note the staining of plasma and sub-plasma membrane regions. The control section (B) was exposed to normal pre-immune serum rather than primary antibody. Note the lack of staining. (C) Plasma membrane region of the vegetal hemisphere of section (A) (bar represents 25 μm). Note the lower levels of staining in the plasma membrane region and the lack of punctate granules. (D) The germinal vesicle, from a different section, reveals a diffuse staining, in addition to punctate staining near the germinal envelope (bar represents 25 μm). All plates are bright field photomicrographs of 15 μm thick *Xenopus* oocyte cryosections. Primary antibodies were visualized using alkaline-phosphatase conjugated secondary antibodies with Vector Red as a substrate.

$InsP_3$ binding to oocyte membranes could be detected under Ca^{2+} free assay conditions. Therefore, radioligand displacement assays with 40 μM Ca^{2+} present in the assay medium, as well as under Ca^{2+} free conditions, were conducted to determine the sensitivity of $InsP_3$ binding sites in oocyte membranes. Non-specific binding was determined by the addition of 4 μM unlabelled $InsP_3$, and was always less than 10% of the total [^{3}H]-$InsP_3$ bound. In the absence of free Ca^{2+}, little [^{3}H]-$InsP_3$ could be detected (Fig. 3). However, when 40 μM free Ca^{2+} was present in the assay medium, displaceable [^{3}H]-$InsP_3$ was detected. Scatchard plot analysis of these data demonstrates that in the presence of 40 μM free

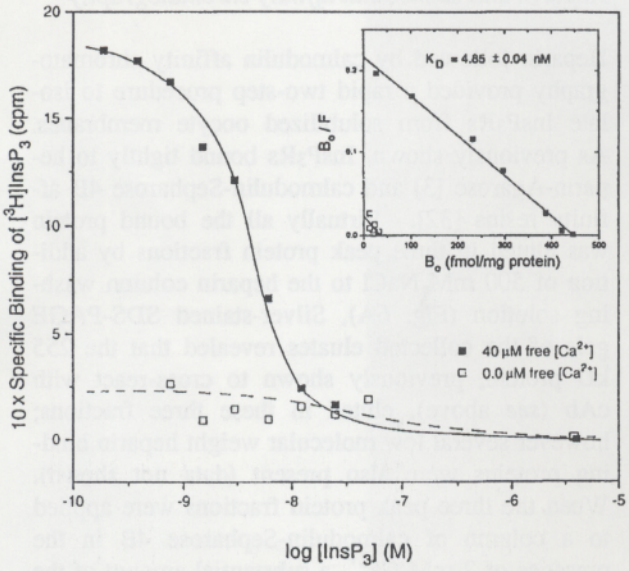

Fig. 3 Effect of Ca^{2+} on [^{3}H]-$InsP_3$ saturation binding. Ca^{2+} facilitates $InsP_3$ binding to oocyte membranes. Displaceable [^{3}H]-$InsP_3$ in the presence of 40 µM free Ca^{2+} (filled squares) or under Ca^{2+} free conditions (open squares) are shown. Scatchard analysis (*see* inset) was conducted to determine the K_D (5 nM) and B_{max} (450 fmol/mg protein) of [^{3}H]-$InsP_3$ binding sites in the presence of 40 µM free Ca^{2+} (filled squares) and the K_D (6 nM) and B_{max} (40 fmol/mg protein) of [^{3}H]-$InsP_3$ binding sites in the absence Ca^{2+} (open squares). Non-specific binding was determined in the presence of 4 µM $InsP_3$. Data points are the means of duplicate determinations from a single experiment, repeated twice more with similar results.

Ca^{2+}, $InsP_3$ bound to an apparently homogenous population of binding sites with a K_D = 5 nM and a B_{max} = 450 fmol/mg of protein. In the absence of free Ca^{2+} the B_{max} decreased more than 10-fold to 40 fmol/mg, without a measurable change in the affinity of the binding site (Fig. 3, inset).

Effect of Ca^{2+} and Ba^{2+} on the [^{3}H]-$InsP_3$ binding capacity of oocyte membranes

The effects of Ca^{2+} and Ba^{2+} on [^{3}H]-$InsP_3$ binding to oocyte membranes were investigated. Non-specific binding at each [Ca^{2+}] or [Ba^{2+}] was always less than 10% of the total [^{3}H]-$InsP_3$ bound. Increasing free [Ca^{2+}] in the assay medium (< 1.0 nM to 4.0 µM) increased the specific binding capacity of oocyte membranes for [^{3}H]-$InsP_3$ from virtually zero to 510 fmol/mg protein. The dose-response relation for this effect provided an apparent EC_{50} of 60 nM Ca^{2+} (Fig. 4). Ba^{2+} did not alter [^{3}H]-$InsP_3$ binding at concentrations between 1.0 nM to 4.0 µM. In marked contrast, specific binding of [^{3}H]-$InsP_3$ to cerebellar membranes decreased dramatically as free [Ca^{2+}] was increased from < 1.0 nM to 4.0 µM (Fig. 4).

Displacement of [^{3}H]-$InsP_3$ binding by various IPs, heparin and caffeine

All displacement assays were carried out with 40 µM free Ca^{2+} present in the assay medium, with non-specific binding determined by the addition of 4 µM $InsP_3$. Unlabelled D-$InsP_3$ displaced bound

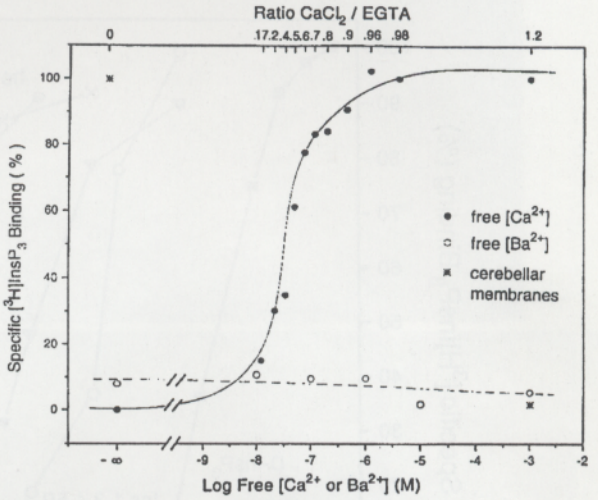

Fig. 4 Effect of increasing [Ca^{2+}] on [^{3}H]-$InsP_3$ binding capacity. Raising free [Ca^{2+}] over a physiological range increased the specificity binding capacity of oocyte membrane for [^{3}H]-$InsP_3$ (filled circles), whereas increasing [Ba^{2+}] had little effect (open circles). In contrast, Ca^{2+} potently inhibited the binding of [^{3}H]-$InsP_3$ to cerebellar membranes (stars). Data from oocyte membranes were expressed as percentages of maximal binding with 40 µm free Ca^{2+} present, and cerebellar data as a percentage of maximal binding in the absence of free Ca^{2+}. Data are the means of duplicate determinations from a single experiment, repeated twice more with similar results.

[^{3}H]-InsP$_3$ in a dose dependent manner with an apparent IC$_{50}$ = 4 nM. Furthermore, displacement of [^{3}H]-InsP$_3$ binding was highly stereospecific. L-InsP$_3$ was approximately 5000-fold less effective (IC$_{50}$ = 20 μM) than D-InsP$_3$ at displacing [^{3}H]-InsP$_3$. Ins1,3,4,5-P$_4$ displayed a relatively high affinity for InsP$_3$ binding sites with an IC$_{50}$ of 79 nM, approximately 20-fold less potent than D-InsP$_3$. Ins2,4,5-P$_3$, a poorly metabolizable InsP$_3$ analog, was less effective at displacing [^{3}H]-InsP$_3$, with an IC$_{50}$ =455 nM (Fig. 5).

Caffeine and heparin, compounds known to inhibit InsP$_3$-mediated Ca^{2+} release in the oocyte [32, 33], inhibited [^{3}H]-InsP$_3$ binding to oocyte membranes. Caffeine was a relatively weak inhibitor, with an apparent IC$_{50}$ value > 10 mM (Fig. 5). As in other tissues, heparin was a potent inhibitor of InsP$_3$ binding. Low molecular weight heparin (M_r = 5000) inhibited [^{3}H]-InsP$_3$ binding by 50% at 150 μg/ml (approximately 12 μM) (Fig. 5).

Heparin and calmodulin affinity chromatography

Heparin followed by calmodulin affinity chromatography provided a rapid two-step procedure to isolate InsP$_3$Rs from solubilized oocyte membranes. As previously shown, InsP$_3$Rs bound tightly to heparin-Agarose [3] and calmodulin-Sepharose 4B affinity resins [32]. Virtually all the bound protein was eluted in three peak protein fractions by addition of 500 mM NaCl to the heparin column washing solution (Fig. 6A), Silver-stained SDS-PAGE gels of the collected eluates revealed that the 255 kD protein, previously shown to cross-react with cAb (*see* above), eluted in these three fractions; however several low molecular weight heparin binding proteins were also present (data not shown). When the three peak protein fractions were applied to a column of calmodulin-Sepharose 4B in the presence of 2 mM Ca^{2+}, a substantial amount of the applied protein was not retained on the column

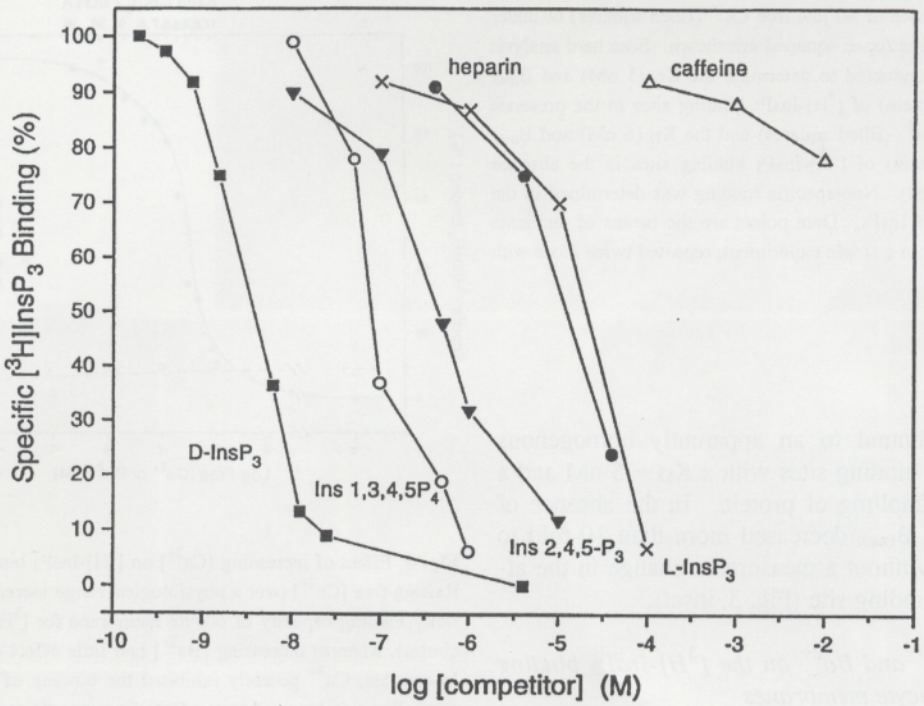

Fig. 5 Specificity of [^{3}H]-InsP$_3$ binding to oocyte membranes. Assay conditions were identical to those described in Figure 3. Displacement of [^{3}H]-InsP$_3$ binding by increasing concentrations of D-InsP$_3$ (filled squares), Ins1,3,4,5-P$_4$ (open circles), Ins2,4,5-P$_3$ (filled triangles), L-InsP$_3$ (crosses), heparin (filled circles), and caffeine (open triangles). Data points are means of duplicate or triplicate determinations which varied by less than 10% of the mean, and are expressed as a percentage of maximal binding in the absence of competitor.

(Fig. 6B). Addition of 10 mM EGTA to the column washing buffer rapidly eluted all bound $InsP_3R$ in two fractions as determined by the presence of the 255 kD protein in silver-stained SDS-PAGE gels (Fig. 6B, inset).

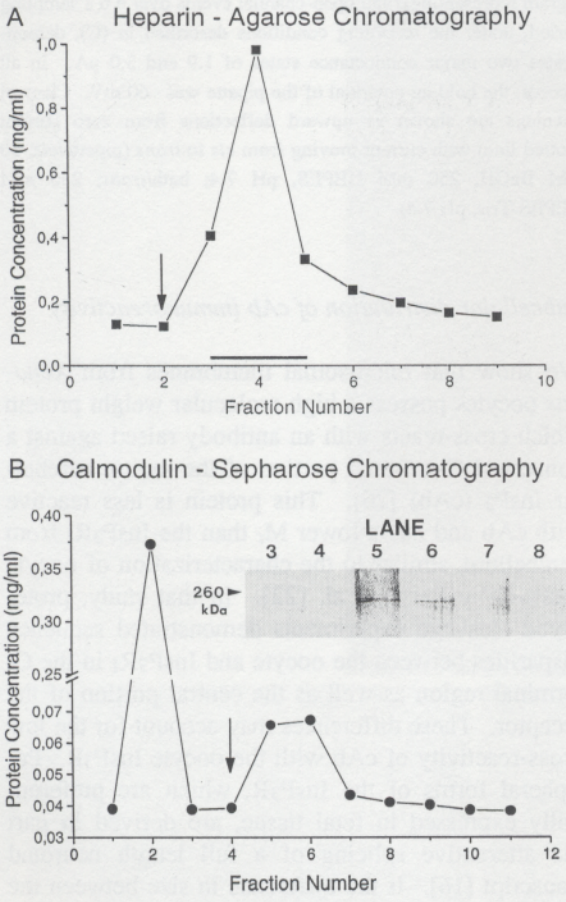

Fig. 6 Heparin followed by calmodulin affinity chromatography of CHAPS solubilized membranes. **(A)** Solubilized proteins from oocyte membranes were absorbed to a heparin-Agarose column and eluted with high salt (arrow), and the three peak protein fraction were collected and pooled (bar). Pooled heparin column eluate was applied to a calmodulin-Sepharose 4B column **(B)**. Fraction two represents pooled column flow-through. Proteins were eluted by the addition of 5 mM EGTA to the column washing buffer (arrow). Subsequent SDS-PAGE analysis of collected fractions revealed that fractions 5 and 6 were highly enriched in the 255 kD protein previously shown to cross-react with cAB (inset).

Reconstitution of $InsP_3$-activated channels

Addition of affinity purified $InsP_3R$ (1 μg protein) to the *trans* side of lipid bilayers resulted in multiple fusion events with the membrane. After several such fusion events had occurred, the success rate for observing functionally incorporated channels was approximately 50% when both $InsP_3$ and Ca^{2+} were present in the bath. In the absence of $InsP_3$, 5 μM free Ca^{2+} in the bath did not elicit current steps, demonstrating the absence of Ca^{2+}-activated channels in the bilayer (Fig. 7A). Following washout of Ca^{2+}, $InsP_3$ (5 μM) was added to the bath solution. Addition of $InsP_3$ alone was also without effect (Fig. 7B). Subsequent addition of Ca^{2+} to the bath induced current steps of about 1.9 and 5.0 pA (corresponding to chord conductances of 31 pS and 83 pS, respectively) at a holding potential of –60 mV (Fig. 7C), as determined by amplitude histograms of the channel open state (Fig. 7E). Abrupt transitions from zero current to 5.0 pA and back again to zero, as well as intermediate steps from 5.0 to 1.9 pA, suggest that these two current levels represent multiple conductance states of a single channel. Further addition of 200 μg/ml of heparin to the bath inhibited all channel activity (Fig. 7D), consistent with the pharmacology of the $InsP_3R$.

Discussion

Recent studies have underscored the roles of Ca^{2+} modulation and the organization of the $InsP_3$-sensitive stores in generating the complex patterns of the $InsP_3$-mediated Ca^{2+} release observed in *Xenopus* oocytes [18, 25, 33]. In this study we have examined the subcellular localization of oocyte $InsP_3Rs$ at the light microscopic level, and tested the effects of Ca^{2+} on the binding of $InsP_3$ to oocyte membranes as well as the gating properties of the functionally reconstituted channel. Following the completion of our immunological and biochemical studies a similar report by Parys et al. appeared [22]. In that study, binding data were obtained using purified $InsP_3Rs$ in the absence of free Ca^{2+}, and the effects of Ca^{2+} on the functional properties of the receptor were investigated by assaying Ca^{2+} release from microsomes. In the present study receptor

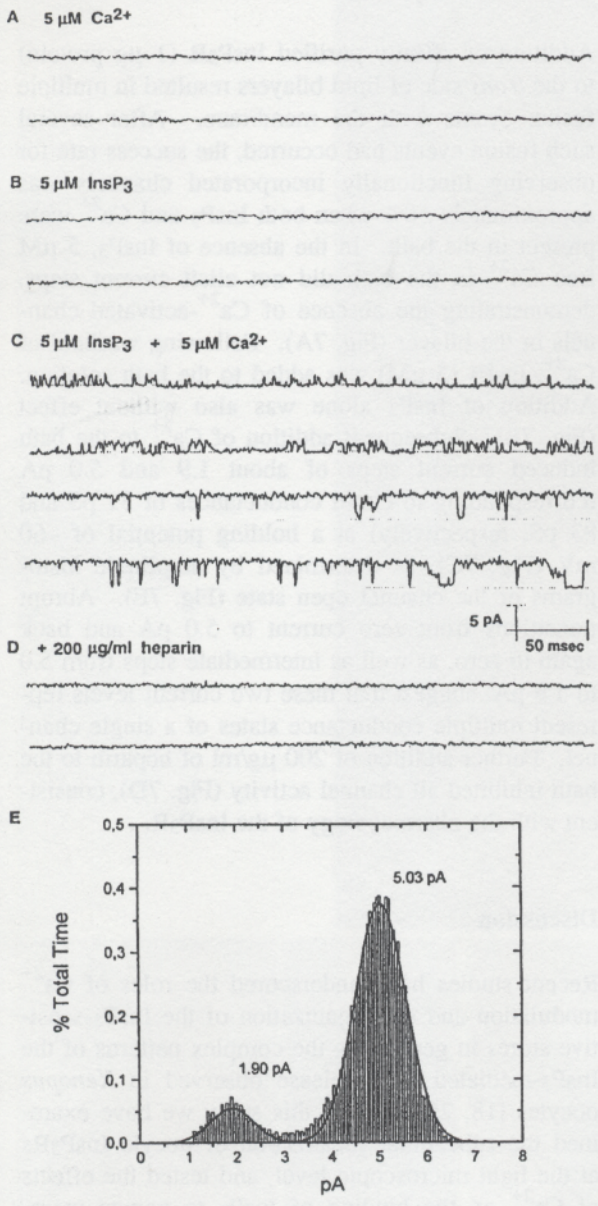

Fig. 7 Functional reconstitution of $InsP_3$-activated Ca^{2+}-dependent channels into lipid bilayers. (A) Addition of Ca^{2+} (5 μM free Ca^{2+}) or (B) $InsP_3$ (5 μM) alone to the bath did not induce channel activity, as seen in consecutive 425 ms records, respectively. (C) Subsequent addition of $InsP_3$ (5 μM) and Ca^{2+} (5 μM) to the bath chamber activated a membrane current with two major conductance states. Representative channel openings are shown in four consecutive records. (D) The further addition of heparin (200 μg/ml) inhibited all channel activity. (E) An amplitude histogram representing 1450 open-channel events over a 6 s sampling period, under the recording conditions described in (C), demonstrates two major conductance states of 1.9 and 5.0 pA. In all records, the holding potential of the pipette was –60 mV. Channel openings are shown as upward deflections from zero current (dotted line) with current moving from *cis* to *trans* (pipette/*cis*; 50 mM BaOH, 250 mM HEPES, pH 7.4; bath/*trans*; 250 mM HEPES-Tris, pH 7.4).

binding assays employed microsomal membranes and single channel records were obtained following reconstitution of solubilized $InsP_3Rs$. Comparison of these results obtained using different methodologies may provide insight into the molecular mechanisms of $InsP_3$-mediated Ca^{2+} release in the intact cell, particularly with respect to modulation by Ca^{2+}.

Subcellular distribution of cAb immunoreactivity

We show that microsomal membranes from *Xenopus* oocytes possess a high molecular weight protein which cross-reacts with an antibody raised against a conserved C-terminal portion of the mouse cerebellar $InsP_3$ (cAb) [26]. This protein is less reactive with cAb and has a lower M_r than the $InsP_3R_I$ from cerebellum, similar to the characterization of oocyte $InsP_3Rs$ by Parys et al. [22]. In that study, proteolytic cleavage experiments demonstrated sequence disparities between the oocyte and $InsP_3R_I$ in the C-terminal region as well as the central portion of the receptor. These differences may account for the low cross-reactivity of cAb with the oocyte $InsP_3R$. Peripheral forms of the $InsP_3R$, which are preferentially expressed in fetal tissue, are derived in part via alternative splicing of a full length neuronal transcript [16]. If the difference in size between the oocyte and neuronal $InsP_3Rs$ is the result of such an alternative splicing event, then the oocyte $InsP_3R$ could correspond to one of the previously described fetal/peripheral forms, or perhaps represent a novel isoform of the $InsP_3R_I$. Southern blot analysis, or *in situ* hybridization using subtype-specific probes, are needed to clarify which $InsP_3R$ subunit RNA, or RNAs, are present in the oocyte.

The subcellular distribution of cAb immunoreactivity ($InsP_3Rs$) in the oocyte (Fig. 2) was similar to that of the cortical endoplasmic reticulum (CER)

[34], and consistent with the distribution of $InsP_3Rs$ observed in the oocyte and other cells [9, 22, 34]. Electron micrographic studies have shown that the CER is a dense network of smooth endoplasmic reticulum concentrated in the animal hemisphere, in close proximity (5–10 μm) to the plasma membrane [34]. We observed a more intense staining of the sub-plasma membrane region of the animal hemisphere as compared to the vegetal hemisphere, suggesting an increased density of $InsP_3Rs$ in this region. This is consistent with the greater sensitivity of the animal hemisphere to injected $InsP_3$ [35], and further supports the hypothesis that the CER represents the $InsP_3$ sensitive Ca^{2+} pool [36, 37].

In addition to homogenous staining, punctate staining was observed in association with the sub-plasma membrane region and peri-nuclear envelope. This may represent a clustering of $InsP_3Rs$ at specific sites on continuous intracellular membrane systems or on discrete organelles (e.g. calciosomes) [38]. In the case of the sub-plasma membrane region, these clusters could account for the functionally discrete Ca^{2+} hot spots observed in optical studies of $InsP_3$-mediated Ca^{2+} release in the oocyte [18]. Localized $InsP_3R$ immunoreactivity around the germinal vesicle suggests that it may also act as an $InsP_3$ sensitive Ca^{2+} store, as shown for hepatocyte nuclei [39]. In addition, recent observations suggest that the nuclear membrane may possess the enzymatic machinery necessary to generate $InsP_3$ from nuclear phosphatidyl inositol 1,2-bisphosphate [40].

Displacement of $InsP_3$ binding by IPs and physiologically active compounds

The high affinity and stereo-specificity of the binding site present in oocyte microsomal membranes for D-$InsP_3$ over L-$InsP_3$, as well as its selectivity profile for other IPs, make it unlikely that this binding represents an $InsP_3$-phosphatase or other non-$InsP_3R$ protein [11], and strongly suggest that it is the physiological $InsP_3R$. The affinity of Ins1,3,4,5-P_4 for the $InsP_3$ binding site was approximately 20-fold less than that of $InsP_3$, similar to its physiological potency in the intact oocyte [41]. This result supports the idea that Ins1,3,4,5-P_4 facilitates $InsP_3$-induced Ca^{2+} release by a direct activation of the $InsP_3R$ and may release intracellular Ca^{2+} from $InsP_3$-sensitive pools [41, 42]. Ins2,4,5-P_3, a poorly metabolized isomer of $InsP_3$, has been shown to be only 5-times less potent that $InsP_3$ in releasing intracellular Ca^{2+} in the oocyte [41]. However, our binding studies show that, although Ins2,4,5-P_3 displaces $InsP_3$ from its binding site, its affinity is about 100-times less than that of $InsP_3$. The stability of this isomer may, therefore, contribute to its Ca^{2+} releasing ability in the intact cell.

Heparin inhibited $InsP_3$ binding to oocyte membranes at concentrations (100 μg/ml) similar to those needed to block $InsP_3$-mediated Ca^{2+} release in the intact oocyte [43], but higher than those reported for the inhibition of affinity purified oocyte $InsP_3Rs$ (5 μg/ml) [22]. This difference may simply arise from the binding of heparin to non-$InsP_3R$ proteins present in our oocyte microsomal membrane preparations, thereby decreasing its specific activity.

Caffeine acts intracellularly at millimolar concentrations to inhibit $InsP_3$-mediated Ca^{2+} release in the oocyte, via a mechanism different from that of Ca^{2+}-mediated inhibition or inhibition of phosphodiesterase enzymes [44]. In contrast, our binding data show that as much as 10 mM caffeine has little effect on the binding of $InsP_3$ to its receptor. Recent experiments on cerebellar microsomes showed a similar discrepancy, in that caffeine inhibited $InsP_3$-induced Ca^{2+} release from cerebellar microsomes, but had no effect on ligand binding [45]. Those observations may be reconciled if caffeine acts by modulating the gating of the Ca^{2+} channel rather than the $InsP_3$ receptor site.

Facilitation of $InsP_3R$ binding and channel activity by Ca^{2+}

Similar to findings in other permeabilized cells and microsomal preparations [46, 47], whole cell studies in the oocyte have shown $InsP_3$-induced Ca^{2+} release is both facilitated and inhibited by cytosolic Ca^{2+} [33, 48]. In our binding assays, only facilitation of $InsP_3$ binding by Ca^{2+} was detected, even at micromolar concentrations of Ca^{2+}. In contrast, micromolar concentrations of free Ca^{2+} have been shown to inhibit $InsP_3$-mediated release of Ca^{2+} from oocyte microsomes [22]. One explanation of these results is that Ca^{2+} may convert the $InsP_3R$

from a low affinity (active) to a high affinity (desensitized) state, thus increasing the affinity of the receptor for $InsP_3$, but decreasing its functional activity [46]. This seems unlikely since Scatchard analysis revealed that Ca^{2+} had little effect on the affinity of the receptor. Alternatively, a loosely associated protein or factor could mediate Ca^{2+} inhibition of channel activity (e.g. calmedin), the activity of which was lost during membrane isolation procedures [7, 11]. Electrophysiological experiments with affinity purified oocyte $InsP_3R$ support this hypothesis, since $InsP_3$-gated channel opening was facilitated by micromolar concentrations of Ca^{2+} (Fig. 7).

Single channel recordings from affinity purified $InsP_3Rs$ demonstrated that channel activity was dependent on both Ca^{2+} and $InsP_3$, suggesting that Ca^{2+} is a necessary co-agonist for activation of the oocyte $InsP_3R$. Free Ca^{2+} concentrations of 5 μM, which resulted in maximal binding of $InsP_3$ to its receptor, also facilitated channel opening, with no observed inhibition of activity. In contrast to our findings, bell-shaped dose-response curves for Ca^{2+} facilitation of $InsP_3$ activated channels from brain synaptosomes [49] have been reported. In those studies, non-solubilized vesicular preparations were used for reconstitution, so that, as mentioned above, an associated protein or factor could have mediated the inhibition observed at higher Ca^{2+} concentrations. In another study which employed affinity purified $InsP_3Rs$, channel activity was observed with both Ca^{2+} (100 nM) and $InsP_3$ (4.8 μM) present in the bathing medium [32]. However, the effects of a range of Ca^{2+} concentrations or $InsP_3$ alone on channel activity were not reported, though clearly a concentration of 0.1 μM Ca^{2+} did not abolish channel activity. Results from studies where Ca^{2+} release from permeabilized cells was measured suggest that changes in intra-luminal (*trans*) $[Ca^{2+}]$ might affect $InsP_3R_I$ channel kinetics [50], however this mechanism would not be apparent in our studies since a constant level of *trans* Ba^{2+} was maintained.

Positive feedback by Ca^{2+}, following receptor occupancy by $InsP_3$, has been proposed as a variant of calcium-induced Ca^{2+} release, to account for the generation of Ca^{2+} spikes, oscillations and travelling waves [18, 51, 52]. In the oocyte, the pronounced facilitation of $InsP_3$ binding and channel activity by Ca^{2+} is likely to be of physiological relevance, since it is half-maximal at a concentration (60 nM) approximately the same as the resting level of free intracellular Ca^{2+} [Yao Y., Parker I. unpublished data]. In addition, our electrophysiological results demonstrate a direct allosteric facilitation by Ca^{2+}, and suggest that an ancillary protein may be involved in mediating the inhibitory effects of Ca^{2+} on the $InsP_3R$. Activation of this protein could impose time delays on negative feedback, facilitating oscillatory behaviour of the system, and could itself be modulated by endogenous factors. Further characterization of the physiological and pharmacological properties of these $InsP_3$-activated Ca^{2+} dependent channels should provide a clearer understanding of the mechanisms underlying the macroscopic behaviour of $InsP_3$-mediated intracellular Ca^{2+} release in the oocyte.

Acknowledgements

We would like to thank Dr T.C. Sudhof for the generous gift of anti-$InsP_3R$ antibody, Dr John Dempster for providing us with the Strathclyde Electrophysiological Software, Dr Robin Irvine for providing $Ins(2,4,5)P_3$, and Dr J. Kahle for her extensive assistance in the preparation of this manuscript. This work was supported by an NIMH training fellowship (MH 101045) to N. Callamaras and by grant GM 39831 from the Public Health Service.

References

1. Berridge MJ. Irvine RF. (1993) Inositol trisphosphates and calcium signalling. Nature, 361, 315-325.
2. Ferris CD. Snyder SH. (1992) Inositol phosphate receptors and calcium disposition in the brain. J. Neurosci., 12(5), 1567-1574.
3. Supattapone S. Worley PF. Baraban JM. Snyder SH. (1988) Solubilization, purification, and characterization of an inositol trisphosphate receptor. J. Biol. Chem., 263, 1530-1534.
4. Mignery GA. Newton CL. Archer BT. Sudhof TC. (1990) Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. J. Biol. Chem., 265, 12679-12685.
5. Worley PF. Baraban JM. Snyder SH. (1989) Inositol 1,4,5-trisphosphate receptor binding: autoradiographic localization in rat brain. Biochem. Biophys. Res. Commun., 159, 976-982.
6. Maeda N. Niinobe M. Inoue Y. Mikoshiba K. (1989)

Developmental expression and intracellular location of P400 protein characteristic of Purkinje cells in the mouse cerebellum. Dev. Biol., 133, 67-76.

7. Ferris CD. Huganir RL. Supattapone S. Snyder SH. (1989) Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. Nature, 342, 87-89.
8. Maeda N., Niinobe M. Mikoshiba K. (1990) A cerebellar Purkinje cell marker P400 protein is an inositol 1,4,5-trisphosphate ($InsP_3$) receptor protein. Purification and characterization of $InsP_3$ receptor complex. EMBO J., 9, 61-67.
9. Ross CA. Meldolesi J. Milner TA. Satoh T. Supattapone S. Snyder SH. (1989) Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje neurons, Nature, 339, 468-70.
10. Sharp AH. Snyder SH. Nigam SK. (1992) Inositol 1,4,5-triphosphate receptors. J. Biol. Chem., 267, 7444-7449.
11. Worley PF. Baraban JM. Supattapone S. Wilson VS. Snyder SH. (1987) Characterization of inositol trisphosphate receptor binding in brain. Regulation by pH and calcium. J. Biol. Chem., 262, 12132-12136.
12. Ferris CD. Huganir RL. Snyder SH. (1990) Calcium flux mediated by purified inositol 1,4,5-trisphosphate receptor in reconstituted lipid vesicles is allosterically regulated by adenine nucleotides. Proc. Natl. Acad. Sci. USA, 87, 2147-2151.
13. Supattapone S. Danoff SK. Theibert A. Joseph SK. Steiner J. Snyder SH. (1988) Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. Proc. Natl. Acad. Sci. USA, 85, 8747-8750.
14. Ross CA. Danoff SK. Schell MJ. Snyder SH. Ullrich A. (1992) Three additional inositol 1,4,5-trisphosphate receptors: Molecular cloning and differential localization in brain and peripheral tissues. Proc. Natl. Acad. Sci. USA, 89, 4265-4269.
15. Khan AA. Steiner JP. Snyder SH. (1992) Plasma membrane inositol 1,4,5-trisphosphate receptor of lymphocytes: Selective enrichment in sialic acid and unique binding specificity. Proc. Natl. Acad. Sci. USA, 89, 2849-2853.
16. Danoff SK. Ferris CD. Donath C. et al. (1991) Inositol 1,4,5-trisphosphate receptors: distinct neuronal and nonneuronal forms derived by alternative splicing differ in phosphorylation. Proc. Natl. Acad. Sci. USA, 88, 2951-2955.
17. Varney MA. Rivera J. Lopez Bernal A. Watson SP. (1990) Are there subtypes of the inositol 1,4,5-trisphosphate receptor? Biochem. J., 269, 211-216.
18. Parker I. Yao Y. (1991) Regenerative release of calcium from functionally discrete subcellular stores by inositol trisphosphate. Proc. R. Soc. Lond. B, 246, 269-274.
19. DeYoung G. Keizer J. (1992) A single pool inositol 1,4,5-trisphosphate-based model for agonist stimulated oscillations in Ca^{2+}. Proc. Natl. Acad. Sci. USA, 89, 9895-9899.
20. Parker I. Miledi R. (1986) Changes in intracellular calcium and in membrane currents evoked by injection of inositol trisphosphate into *Xenopus* oocytes. Proc. R. Soc. Lond. B, 228, 307-315.
21. Dascal N. (1987) The use of *Xenopus* oocytes for the study of ion channels. CRC Crit. Rev. Biochem., 22, 317-387.
22. Parys JB. Sernett SW. DeLisle S. Snyder PM. Welsh MJ. Campbell KP. (1992) Isolation, characterization, and localization of the inositol 1,4,5-trisphosphate receptor protein in *Xenopus laevis* oocytes. J. Biol. Chem., 267, 18776-18782.
23. Parker I. Miledi R. (1989) Nonlinearity and facilitation in phosphoinositide signalling studied by the use of caged inositol trisphosphate in *Xenopus* oocytes. J. Neurosci., 9, 4068-4077.
24. Parker I. Ivorra I. (1990) Localized all-or-none calcium liberation by inositol trisphosphate. Science, 250, 977-979.
25. Lechleiter J. Giarard S. Peralta E. Clapham D. (1991) Spiral calcium wave propagation and annihilation in *Xenopus laevis* oocytes. Science, 252, 123-126.
26. Mignery GA. Sudhof TC. Takei K. De Camilli P. (1989) Putative receptor for inositol 1,4,5-trisphosphate is similar to the ryanodine receptor. Nature, 342, 192-195.
27. Miledi R. Parker I. (1984) Chloride current induced by injection of calcium into *Xenopus* oocytes. J. Physiol. (Lond.), 357, 173-183.
28. Pietri F. Hilly M. Claret M. Mauger JP. (1990) Characterization of two forms of inositol 1,4,5-trisphosphate receptor in rat liver. Cell. Signal., 2, 253-263.
29. Tsien R. Pozzan T. (1989) Measurement of cytosolic free Ca^{2+} with quin-2. Meth. Enzymol., 172. 230-262.
30. Suarez-Isla B. Wan K. Lindstrom J. Montal M. (1983) Single-channel recordings from purified acetylcholine receptors reconstituted in bilayers formed at the tip of patch pipets. Biochemistry, 22, 2319-2323.
31. Sambrock J. Fritsch EF. Maniatis T. (1989) Molecular cloning. A laboratory manual., Cold Spring Harbor, Cold Spring Harbor Laboratory Press, pp. 18.3-18.86.
32. Maeda N. Kawasaki T. Nakade S. et al. (1991) Structural and functional characterization of inositol 1,4,5-trisphosphate receptor channel from mouse cerebellum. J. Biol. Chem., 266, 1109-1116.
33. Yao Y. Parker I. (1992) Potentiation of inositol trisphosphate-induced Ca^{2+} mobilization in *Xenopus* oocytes by cytoplasmic Ca^{2+}. J. Physiol. (Lond.), 458, 319-328.
34. Charbonneau M. (1990) The organization of the cortical endoplasmic reticulum in *Xenopus* eggs depends on intracellular pH: artifact of fixation or not? Cell. Differ. Dev., 30, 171-179.
35. Berridge MJ. (1988) Inositol trisphosphate-induced membrane potential oscillations in *Xenopus* oocytes. J. Physiol. (Lond.), 403, 589-599.
36. Charbonneau M. (1989) Weak bases mimic the fertilization-associated chloride conductance increase and induce morphological changes in the cortex of *Xenopus laevis* eggs. Cell. Differ. Dev., 26, 39-51.
37. Lupu-Meiri M. Shapira H. Oron Y. (1988) Hemispheric asymmetry of rapid chloride responses to inositol trisphosphate and calcium in *Xenopus* oocytes. FEBS Lett., 240, 83-87.
38. Takei K. Stukenbrok H. Metcalf A. et al. (1992) Ca^{2+} stores in Purkinje neurons: Endoplasmic reticulum subcompartments demonstrated by the heterogeneous distribution of the $InsP_3$ receptor, Ca^{2+}-ATPase, and

calsequestrin. J. Neurosci., 12, 489-505.
39. Nicotera P. Orrenius S. Nilsson T. Berggren PO. (1990) An inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool in liver nuclei. Proc. Natl. Acad. Sci. USA, 87, 6858-6862.
40. Divecha N. Banfic H. Irvine RF. (1991) The polyphosphoinositide cycle exists in the nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-1) in the plasma membrane, and the stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. EMBO J., 10, 3207-3214.
41. Parker I. Ivorra I. (1991) Inositol tetrakisphosphate liberates stored calcium in *Xenopus* oocytes and facilitates responses to inositol trisphosphate. J. Physiol. (Lond.), 433, 209-227.
42. Parker I. Miledi R. (1987) Injection of inositol 1,3,4,5-tetrakisphosphate into *Xenopus* oocytes generates a chloride current dependent upon intracellular calcium. Proc. R. Soc. Lond. B, 232, 59-70.
43. Parker I. Ivorra I. (1992) Characteristics of membrane currents evoked by photoreleased inositol trisphosphate in *Xenopus* oocytes. Am. J. Physiol., 263, 154-165.
44. Parker I. Ivorra I. (1991) Caffeine inhibits inositol trisphosphate-mediated liberation of intracellular calcium in *Xenopus* oocytes. J. Physiol. (Lond.), 433, 229-240.
45. Brown GR. Sayers LG. Kirk CJ. Mitchell RH. Michelangeli F. (1992) The opening of the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel in rat cerebellum is inhibited by caffeine. Biochem. J., 282, 309-312.
46. Pietri F. Hilly M. Mauger JP. (1990) Calcium mediates the interconversion between two states of the liver inositol 1,4,5-trisphosphate receptor. J. Biol. Chem., 265, 17478-17485.
47. Finch EA. Turner TJ. Goldin SM. (1991) Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. Science, 252, 443-446.
48. Parker I. Ivorra I. (1990) Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: a possible mechanism for oscillatory release of Ca^{2+}. Proc. Natl. Acad. Sci. USA, 87, 260-264.
49. Bezprozvanny I. Watras J. Ehrlich BE. (1991) Bell-shaped calcium response curves of Ins (1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature, 351, 751-754.
50. Missiaen L. De Smedt H. Droogmans G. Casteels R. (1992) Ca^{2+} release induced by inositol 1,4,5-trisphosphate is a steady-state phenomenon controlled by luminal Ca^{2+} in permeabilized cells. Nature, 357, 599-601.
51. DeLisle S. Welsh MJ. (1992) Inositol trisphosphate is required for the propagation of calcium waves in *Xenopus* oocytes. J. Biol. Chem., 267, 7963-7966.
52. Keizer J. DeYoung G. (1992) Two roles for Ca^{2+} in agonist stimulated Ca^{2+} oscillations. Biophys. J., 61, 649-660.

Please send reprint requests to : Dr N. Callamaras, Department of Psychobiology, Laboratory of Cellular and Molecular Neurobiology, University of California, Irvine, Irvine, CA 92717-4550, USA

Received : 26 February 1993
Revised : 2 April 1993
Accepted : 7 May 1993