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Isolation, Characterization, and Localization of the Inositol 1,4,5-Trisphosphate Receptor Protein in *Xenopus laevis* Oocytes*

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Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) induces Ca^{2+} oscillations and waves in *Xenopus laevis* oocytes. Microsomes from oocytes exhibit high-affinity binding for $Ins(1,4,5)P_3$, and demonstrate $Ins(1,4,5)P_3$-induced Ca^{2+} release. The $Ins(1,4,5)P_3$ receptor ($InsP_3R$) was purified from oocyte microsomes as a large tetrameric complex and shown to have a monomer molecular mass of 256 kDa, compared with 273 kDa for the brain $InsP_3R$. Binding to the oocyte receptor is highly specific for $Ins(1,4,5)P_3$ and is inhibited by heparin (IC_{50}, 2 μg/ml). Immunoblot analysis revealed that an antibody against the C-terminal sequence of the brain receptor recognized the oocyte receptor. These results, in addition to the difference in pattern obtained after limited proteolysis, suggest that the oocyte $InsP_3R$ is a new shorter isoform of the mammalian brain type I $InsP_3R$. Immunofluorescence experiments indicated the presence of the $InsP_3R$ in the cortical layer and the perinuclear endoplasmic reticulum of the oocyte. However, immunological and biochemical experiments did not reveal the presence of the ryanodine receptor. The presence of an $InsP_3R$ and the absence of a ryanodine receptor support the importance of $Ins(1,4,5)P_3$ in Ca^{2+} handling by oocytes and particularly in the induction of Ca^{2+} oscillations and waves.

Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)[1] is a well described second messenger which induces Ca^{2+} release from intracellular stores in various cell types and can generate Ca^{2+} oscillations and waves (1, 2). The $Ins(1,4,5)P_3$ receptor ($InsP_3R$) was purified from mammalian brain and smooth muscle (3–5) and shown to be a Ca^{2+} release channel (6). Cloning data indicated the existence of at least two different genes coding for the $InsP_3R$ (7–8) and the existence of multiple isoforms obtained by alternative splicing (9–11). A second intracellular Ca^{2+} channel, the ryanodine receptor, was also described in several mammalian tissues and is thought to be involved in the Ca^{2+}-induced Ca^{2+} release phenomenon (12–14).

The *Xenopus laevis* oocyte is an important model for the study of Ca^{2+} oscillations and waves. Ca^{2+} oscillations can be induced in *Xenopus* oocytes under various conditions, including injection of $Ins(1,4,5)P_3$ or $Ins(1,4,5)P_3$-analogs (reviewed in Ref. 15). Another dynamic feature of Ca^{2+} in oocytes and eggs of various species is the propagation of Ca^{2+} waves (16). A Ca^{2+} wave, travelling from the animal through the vegetal pole of the *Xenopus* egg, is typically initiated at fertilization (17) or by injection of $Ins(1,4,5)P_3$ (18). Ca^{2+} waves are also expressed in *Xenopus* oocytes by stimulation of receptors linked to the inositol phosphate pathway (19, 20) or directly by injection of $Ins(1,4,5)P_3$ or $Ins(1,4,5)P_3S_3$ (21, 22). Moreover, Ca^{2+}, ionomycin, thapsigargin, caffeine, or ryanodine were unable to induce Ca^{2+} waves, suggesting that Ca^{2+} waves propagate through the cell by a serial release of Ca^{2+} from $Ins(1,4,5)P_3$-sensitive stores (21, 22).

To further define the *Xenopus* oocytes model, and to better understand the role of $Ins(1,4,5)P_3$ in the origin of complex spatiotemporal Ca^{2+} signals, like oscillations and waves, we investigated the presence of the $InsP_3R$, the ryanodine receptor, and calcium binding proteins in *Xenopus* oocytes. The $InsP_3R$ was purified, characterized, and its localization determined. No evidence for a ryanodine receptor was found. The presence of an $InsP_3R$ and the absence of a ryanodine receptor in oocytes support the role of $Ins(1,4,5)P_3$ in the induction of Ca^{2+} oscillations and waves in *Xenopus* oocytes.

EXPERIMENTAL PROCEDURES

Membrane Preparations—Oocytes were removed from *X. laevis* animals and enzymatically defolliculated (23). The cells were suspended in homogenization buffer (50 mM Tris-HCl, pH 7.25, 250 mM sucrose, 0.8 mM benzamidine, 0.2 mM PMSF, 10 μM leupeptin, 1 μM pepstatin A, 75 nM aprotinin) and homogenized with a glass-Teflon homogenizer. The homogenate was centrifuged (15 min, 4500 × *g*), and the supernatant was recovered. The pellet contained the yolk granules and the melanosomes. The supernatant was centrifuged again (35 min, 142,000 × *g*), and the microsomal pellet was resuspended in end medium (20 mM Tris-HCl, pH 7.25, 300 mM sucrose, 0.8 mM benzamidine, 0.2 mM PMSF), frozen in liquid N_2, and stored at −135 °C. Eggs were obtained by injecting *X. laevis* females with 1000 units of chorionic gonadotropin. The eggs were recovered 10 h after injection. The jelly layer was removed by incubating the eggs for 30 min in 50 mM HEPES, pH 7.4, 5 mM dithiothreitol. Microsomes were prepared as described for oocytes, with the exception that for binding studies, an additional centrifugation step (90 min, 142,000 × *g*) through a layer of 15% sucrose in 20 mM Tris-HCl, pH 7.4, 0.8 mM benzamidine, 0.2 mM PMSF was included in order to remove any remaining lipid material. The following microsomal fractions were

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[1] The abbreviations used are: $Ins(1,4,5)P_3$, D-*myo*-inositol 1,4,5-trisphosphate; $InsP_3R$, $Ins(1,4,5)P_3$ receptor; PMSF, phenylmethylsulfonyl fluoride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; $Ins(1,3,4,5)P_4$, D-*myo*-inositol 1,3,4,5-tetrakisphosphate; EGTA [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; $Ins(1,3,4)P_3$, D-*myo*-inositol 1,3,4-trisphosphate; $Ins(2,4,5)P_3$, D-*myo*-inositol 2,4,5-trisphosphate; $Ins(1,4,5)P_3S_3$, DL-*myo*-inositol 1,4,5-trisphosphorothioate; $Ins(1,3,4,5,6)P_5$, D-*myo*-inositol 1,3,4,5,6-pentakisphosphate; $InsP_6$, *myo*-inositol hexakisphosphate.

obtained by previously published methods: rabbit and frog brain microsomes (24), frog KCl-washed skeletal muscle microsomes (25), rabbit light sarcoplasmic reticulum (26), and rabbit isolated triads from skeletal muscle (27). Protein was determined by a modified Lowry procedure (28, 29), using bovine serum albumin as standard.

Purification of the $InsP_3R$—We purified the $InsP_3R$ from rabbit brain, *Xenopus* oocytes, and *Xenopus* eggs. The method was based on that described by Chadwick *et al.* (4). In short, microsomes were solubilized (90 min, 4 °C) in buffer A (50 mM Tris-HCl, pH 8.3, 10 mM β-mercaptoethanol, 1 mM EDTA) containing in addition 1 M NaCl, 2.5% CHAPS, 10 mg/ml L-α-phosphatidylcholine, 0.8 mM benzamidine, 0.2 mM PMSF, 1 μM leupeptin, 1 μM pepstatin A, and 75 nM aprotinin A. After centrifugation, the supernatant was diluted 3.3-fold and put on a heparin-agarose column. The 0.6 M NaCl eluate was incubated with wheat germ agglutinin-Sepharose, and after wash steps in high (0.6 M) and low (0.1 M) salt conditions, the specifically bound proteins were eluted in low salt conditions with 0.3 M *N*-acetyl-D-glucosamine. This eluate is centrifuged for 140 min on a linear sucrose density gradient (VTi50 rotor, Beckman; 238,000 × *g*). The gradients were composed of 10–30% sucrose in buffer A containing 0.5 M NaCl, 0.36% CHAPS, and 1.5 mg/ml L-α-phosphatidylcholine. In experiments concerning the presence or absence of the ryanodine receptor, the procedure of McPherson and Campbell (24) was used.

Binding Assays—For $Ins(1,4,5)P_3$ binding, samples were incubated for 30 min at 0 °C in buffer A containing 5–10 nM [^{3}H]$Ins(1,4,5)P_3$. Nonspecific binding was assayed in the additional presence of 5 μM unlabeled $Ins(1,4,5)P_3$. Incubations were terminated by vacuum filtration through glass fiber filters and rapid washing with buffer A. For detergent-solubilized samples, proteins were precipitated with 2 mg/ml γ-globulins and 12.5% polyethylene glycol before filtration. $Ins(1,3,4,5)P_4$ binding was performed with a similar procedure, except that buffer B (25 mM sodium acetate, 25 mM KH_2PO_4, pH 5.1, 1 mM EDTA) was used throughout the assay. The assay for [^{3}H]ryanodine binding was as described earlier (24).

Calcium Flux Experiments—Ca^{2+} uptake in microsomes was performed in 25 mM HEPES-KOH, pH 7.2, 108 mM KCl, 10 mM creatine phosphate, 2.6 mM $MgCl_2$, 2.5 mM benzamidine, 2 mM ATP, and 1 mM PMSF. $^{45}Ca^{2+}$ was added at a concentration of 8 μCi/ml. Ca^{2+} efflux was initiated by diluting the vesicles (1:50) in efflux medium (25 mM HEPES-KOH, 141 mM KCl, 5 mM NaCl, 1 mM EGTA, and 0.6 mM $MgCl_2$). Free Ca^{2+} in uptake and efflux medium was buffered with EGTA (30). Modifications to the basic uptake and efflux media are described in the figure legends. $^{45}Ca^{2+}$ in the vesicles was assayed at various time points by filtration of aliquots through 0.45-μm filters and scintillation counting.

Antibodies against the $InsP_3R$—An antibody against the chemically synthesized 15 C-terminal amino acids of the brain $InsP_3R$ (LGHPPHMNVNPQQPA) was raised in New Zealand White rabbits and affinity-purified against the bovine serum albumin-coupled peptide as described previously (29). A polyclonal antibody against brain $InsP_3R$ was raised in a goat. The goat was injected at multiple sites with 150 μg of purified rabbit brain $InsP_3R$ in Freund's complete adjuvant and boosted 7 weeks later. The antiserum was collected 2 weeks after the booster injection and affinity-purified against purified brain receptor.

SDS-PAGE and Immunoblotting—Samples were analyzed on 3–12% linear gradient SDS-PAGE (31). The gels were stained with Coomassie Blue or transferred to nitrocellulose (32). Transfers were incubated overnight with antibodies and stained with peroxidase-conjugated secondary antibodies. Alternatively, transfers were stained with peroxidase-conjugated concanavalin A.

Deglycosylation—The purified $InsP_3R$ molecules were denatured in 0.1% SDS and diluted 2-fold in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2% octyl-β-glucoside. *N*-Glycosidase F was added at a final concentration of 20 units/ml. Incubation was for 90 min at 37 °C and stopped by boiling for 4 min in sample buffer for SDS-PAGE.

Proteolytic Digestion—Limited digestion of the purified receptor was done with α-chymotrypsin at a protease/receptor ratio of 1/80 in buffer A. Incubation was on ice, for up to 1 h, and was stopped by addition of 10 mM PMSF and 100 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone and boiling for 2 min in sample buffer for SDS-PAGE.

Phosphorylation Studies—Phosphorylation was done in buffer A containing 11 mM $MgCl_2$, 35 μM [γ-^{32}P]ATP (40 μCi/ml), and 7 μg/ml of the catalytic subunit of the cAMP-dependent protein kinase. Incubation was for 5 min at 30 °C and was stopped by boiling for 2 min in sample buffer for SDS-PAGE.

Immunofluorescence Microscopy—Oocytes were fixed in 3% paraformaldehyde in AC_{320} (33) for 2 h at 4 °C, washed, embedded in Tissue-Tek® O.C.T. Compound, and frozen at −80 °C. Sections (10 μm) were blocked with 1% bovine serum albumin in 50 mM sodium phosphate, 154 mM NaCl for 30 min at 37 °C. The sections were subsequentially incubated with affinity-purified antibody against the C-terminal of the $InsP_3R$ (4 °C, 2 h), washed, and incubated with affinity-purified fluorescein isothiocyanate-labeled goat anti rabbit IgG antibody (20 °C, 30 min). Sections were examined with a Zeiss-Axioplan fluorescence microscope.

Materials—Polyclonal antibodies against the ryanodine receptor (14, 24), chicken and dog cardiac calsequestrin (34), and against calreticulin (35), were as described. [^{3}H]$Ins(1,4,5)P_3$, [^{3}H]$Ins(1,3,4,5)P_4$, [^{3}H]ryanodine, and $^{45}Ca^{2+}$ were from Du Pont-New England Nuclear. [γ-^{32}P]ATP and $Ins(1,4,5)P_3$ were from Amersham Corp. $Ins(1,3,4)P_3$, $Ins(1,3,4,5)P_4$, and $InsP_6$ were from Calbiochem, and $Ins(2,4,5)P_3$, $Ins(1,3,4,5,6)P_5$, and *N*-glycosidase F were from Boehringer Mannheim. All other chemicals were of reagent grade.

RESULTS

Presence of $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ Binding Proteins in Xenopus Oocytes and Eggs—Microsomes from *Xenopus* oocytes and eggs express a low but significant amount of specific $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ binding activity (Table I). The $Ins(1,4,5)P_3$ binding was 10–20 times lower than in rabbit brain microsomes. The $Ins(1,3,4,5)P_4$ binding was 2–5 times lower in oocytes and eggs than in rabbit brain membranes.

$Ins(1,4,5)P_3$-induced Ca^{2+} Release—The binding of $Ins(1,4,5)P_3$ suggests that the microsomes contain an $InsP_3R$. To determine if the membranes contain a functional $InsP_3R$, we tested for the ability to release Ca^{2+} in response to $Ins(1,4,5)P_3$. Microsomal vesicles isolated from *Xenopus* oocytes accumulated Ca^{2+} in an ATP-dependent manner (Fig. 1*A*). The uptake was insensitive to the mitochondrial inhibitors oligomycin and antimycin A, suggesting uptake in a non-mitochondrial compartment. Addition of $Ins(1,4,5)P_3$ induced a rapid dose-dependent release of Ca^{2+} from the vesicles (Fig. 1*B*). The release of Ca^{2+} by $Ins(1,4,5)P_3$ was dependent on the extravesicular Ca^{2+} concentration. Maximal release (>80%) occurred at Ca^{2+} concentrations of 0.5 μM, and decreased at both lower and higher concentrations (Fig. 1*C*). Based on these results, we concluded that oocyte microsomes contain a functional $InsP_3R$.

Purification of the $InsP_3R$ from Xenopus Oocytes—We purified the oocyte/egg $InsP_3R$ to obtain further insight into its properties. A summary of the purification of the $InsP_3R$ from rabbit brain and *Xenopus* oocytes is given in Table II. Purification level was based on comparison of the $Ins(1,4,5)P_3$ binding activity detectable in each fraction, compared with the binding in the CHAPS-solubilized supernatant. Between 77% (brain) and 87% (oocytes) of the membrane proteins were solubilized. The $Ins(1,4,5)P_3$ binding activity is roughly equivalent in the brain or oocytes solubilized material, indicating that the binding activity observed in oocyte and egg microsomes was underestimated. This could be due to vesicle

TABLE I

Specific $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$, and ryanodine binding activity in microsomes from Xenopus oocytes, eggs, and rabbit brain

[^{3}H]$Ins(1,4,5)P_3$, [^{3}H]$Ins(1,3,4,5)P_4$, and [^{3}H]ryanodine binding were measured at a concentration of 10 nM, as indicated under "Experimental Procedures." Values are expressed in femtomoles/mg protein. Mean ± S.E. is given with the number of observations in parentheses.

	[^{3}H]$Ins(1,4,5)P_3$	[^{3}H]$Ins(1,3,4,5)P_4$	[^{3}H]Ryanodine
Oocytes	2.5 ± 0.9 (6)	14.1 ± 2.0 (5)	ND[a]
Eggs	4.4 ± 2.6 (4)	51.4 ± 13.9 (4)	ND
Rabbit brain	52.8 ± 11.7 (7)	82.4 ± 16.2 (6)	59.4 ± 6.8 (3)

[a] ND, not detectable.

aggregation, interference of yolk proteins, or to the presence of receptors in a nonaccessible form. The further purification procedure for the $InsP_3R$ involved three steps, heparin-agarose chromatography, wheat germ agglutinin-Sepharose chromatography, and sucrose density gradient centrifugation. The rabbit brain receptor was purified up to a specific binding activity of 19 pmol/mg and the *Xenopus* oocyte receptor up to 44 pmol/mg. In either case, we achieved a purification of about 1000-fold compared with the solubilized material. This purification level is similar to that obtained in brain (3) and non-brain tissues (4, 5). Fig. 2 (*A* and *B*) shows the final purification step on the linear sucrose density gradient and the recovery of the $Ins(1,4,5)P_3$ binding activity from rabbit brain or *Xenopus* oocytes in the same fraction of the gradient (fraction 9). This position corresponds to a density of 1.094 g/ml. Using the position of the molecular mass markers catalase (11 S), thyroglobulin (19.2 S), and the ryanodine receptor (30 S), an S value of 23 S was calculated for the $InsP_3R$ (Fig. 2*C*). Similar results were also obtained using egg microsomes as starting material (data not shown).

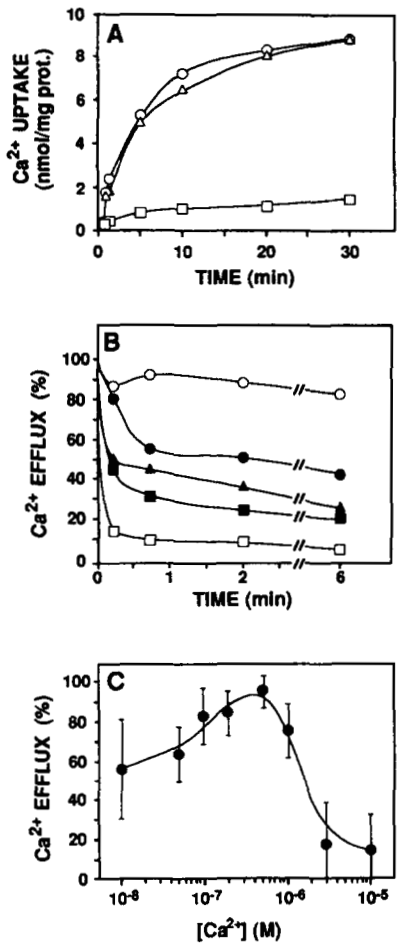

FIG. 1. **Ca^{2+} uptake and release in *Xenopus* oocyte microsomes.** Ca^{2+} uptake and Ca^{2+} efflux were assayed as described under "Experimental Procedures." Each experiment was performed 6 to 14 times with different batches of oocytes. Typical experiments are shown in *A* and *B*. Mean ± S.E. are presented in *C*. *A*, Ca^{2+} uptake in the absence of ATP (□), in the presence of 2 mM ATP (○), and in the presence of 2 mM ATP, supplemented with 5 μM oligomycin and 10 μM antimycin A (△). Free Ca^{2+} was buffered to 0.3 μM. *B*, Ca^{2+} release in control conditions (○), in the presence of 0.03 (●), 0.1 (▲), or 1 (■) μM $Ins(1,4,5)P_3$ or in the presence of 5 μM A23187 (□). Free Ca^{2+} was buffered to 0.1 μM. *C*, Ca^{2+} release by 0.25 μM $Ins(1,4,5)P_3$, at varying extravesicular $[Ca^{2+}]$.

Structural Analysis of the Purified Oocyte $InsP_3R$—After staining with Coomassie Blue the $InsP_3R$ from brain and oocyte appeared essentially pure (Fig. 3). The rabbit brain $InsP_3R$ migrates on SDS-PAGE as a characteristic doublet with an apparent molecular mass of 273 ± 3 (n = 13) kDa, whereas the $InsP_3R$ from *Xenopus* oocytes migrates with the distinctly lower molecular mass of 256 ± 4 kDa (n = 13) (paired Student's t test indicate a significant difference with $p < 0.005$). Preliminary data indicated that the $InsP_3R$ from brain of *X. laevis* has the same molecular weight as the rabbit brain $InsP_3R$ (data not shown).

A comparison between the $InsP_3R$ molecules purified from rabbit brain, *Xenopus* oocytes, and eggs show that all three molecules are recognized by the affinity-purified C-terminal-specific antibody (Fig. 4). The oocyte/egg $InsP_3R$ was not recognized by a goat polyclonal antibody raised against the pure brain $InsP_3R$ (data not shown). The absence of cross-reactivity of antibodies against brain $InsP_3R$ in *Xenopus* oocytes was already reported (36). Concanavalin A also recognizes the brain and the oocyte/egg $InsP_3R$, indicating the presence of one or more glycosylation sites.

To ascertain that the lower molecular mass of the $InsP_3R$ in oocytes is not due to a difference in glycosylation, we examined the molecular mass of the receptor, before and after treatment with *N*-glycosidase F (Fig. 5). *N*-Glycosidase F cleaves Asn-linked high mannose and hybrid and complex oligosaccharides. Treatment with *N*-glycosidase F removed the sugar groups, as shown by the failure of the $InsP_3R$ to bind concanavalin A after incubation with *N*-glycosidase F. The observed shift in molecular mass was in both cases small, about 5 kDa, indicating that in both receptors the number of sites which are glycosylated are limited and cannot account for the observed difference in molecular mass. The limited extent of glycosylation of the $InsP_3R$ was previously shown in brain (37).

TABLE II

Summary of the $InsP_3R$ purification from rabbit brain and from Xenopus oocytes

The purification procedure is indicated under "Experimental Procedures." Protein recovery (in percent), total $Ins(1,4,5)P_3$ binding activity (picomoles) for 1000 mg of starting material, specific $Ins(1,4,5)P_3$ binding activity (pmol/mg protein), and purification factor (compared with supernatant) are given for the different fractions (CHAPS-solubilized supernatant, heparin-agarose eluate, wheat germ agglutinin-Sepharose eluate, and purified receptor after density gradient centrifugation). Each purification was done at least in 5-fold, and mean values are given.

	Rabbit brain				Xenopus oocytes			
	Protein	Total activity	Specific activity	Purification	Proteins	Total activity	Specific activity	Purification
	%	*pmol*	*pmol/mg*	*-fold*	%	*pmol*	*pmol/mg*	*-fold*
Supernatant	76.6	23.0	0.02	1 ×	86.7	26.5	0.04	1 ×
Heparin-agarose eluate	5.5	20.3	0.39	20 ×	6.0	77.5	1.38	35 ×
WGA-Sepharose eluate	0.20	12.4	6.41	321 ×	0.12	22.2	18.68	467 ×
Purified receptor	0.02	4.0	18.54	927 ×	0.03	11.4	43.74	1094 ×

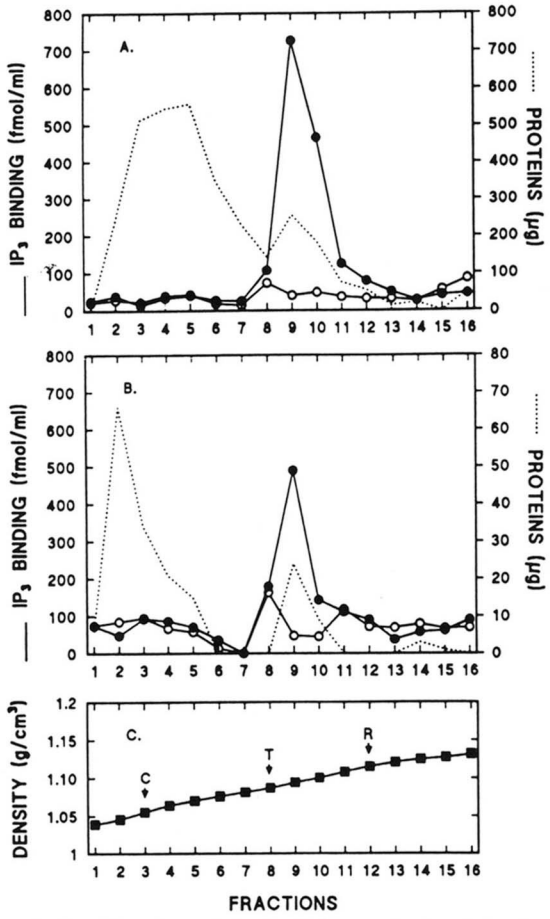

FIG. 2. **Purification of the $InsP_3R$, analysis of the fractions from the sucrose density gradient.** A typical purification is shown for rabbit brain (*A*) and for *Xenopus* oocytes (*B*). The density in each fraction, as measured with a refractometer, and the localization of the molecular mass markers catalase (*C*), thyroglobulin (*T*), and ryanodine receptor (*R*) is shown in *C*. $Ins(1,4,5)P_3$ binding was done as described under "Experimental Procedures" at a concentration of 6 nM [^{3}H]$Ins(1,4,5)P_3$. Total (●) and nonspecific (○) $Ins(1,4,5)P_3$ binding is indicated in femtomoles/ml. Protein amount in each fraction is also indicated (note the presence of different *y* scales in *A* and *B*).

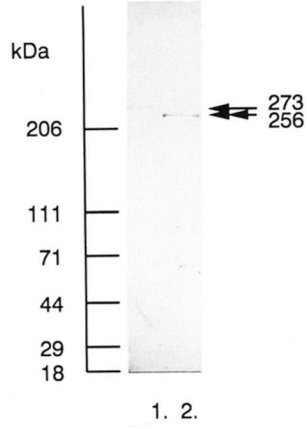

FIG. 3. **Comparison of rabbit brain $InsP_3R$ and *Xenopus* oocyte $InsP_3R$ on SDS-PAGE.** Receptors were purified as described under "Experimental Procedures." *Lane 1* contains rabbit brain $InsP_3R$ and *lane 2 Xenopus* oocyte $InsP_3R$. Gels were stained with Coomassie Blue. The *arrow* indicates the position of the rabbit brain $InsP_3R$ and the *double arrow* the position of the *Xenopus* oocyte $InsP_3R$.

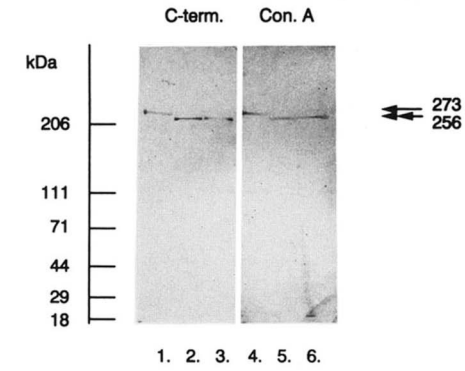

FIG. 4. **Analysis of the purified $InsP_3R$ from rabbit brain (1, 4), *Xenopus* oocytes (2, 5), and eggs (3, 6) after SDS-PAGE and transfer to nitrocellulose.** Blots were stained with an affinity-purified antibody directed against the C-terminal of brain $InsP_3R$ (1–3) or with concanavalin A (4–6). The *arrow* indicates the position of the rabbit brain $InsP_3R$ and the *double arrow* the position of the oocyte/egg $InsP_3R$.

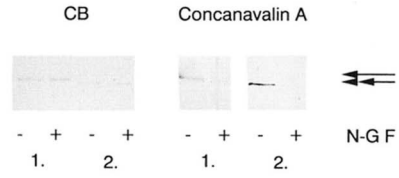

FIG. 5. **Deglycosylation of the $InsP_3R$.** Purified $InsP_3R$ from rabbit brain (1) and from *Xenopus* oocytes (2) were treated with *N*-glycosidase F, as described under "Experimental Procedures." Control samples (−) or *N*-glycosidase F-treated samples (+) were analyzed by SDS-PAGE and either stained with Coomassie Blue (*left panel*) or transferred to nitrocellulose and stained with concanavalin A (*right panel*). The *arrow* indicates the position of the rabbit brain $InsP_3R$ and the *double arrow* the position of the oocyte $InsP_3R$. Note the small shift in molecular mass and the concomitant disappearing of the concanavalin A staining by treatment with *N*-glycosidase F. Only the relevant portion of the gel is shown.

We compared the structure of the brain and oocyte $InsP_3R$, using the technique of limited proteolysis. After incubation of the receptor with α-chymotrypsin for various time periods, a number of proteolytic fragments were obtained (Table III). The molecular mass of the fragments obtained from the brain and the oocyte receptor are different, confirming structural differences between the two molecules. We used SDS-PAGE followed by Western blotting and labeling with either concanavalin A or the C-terminal-specific antibody for identification of the fragments. The specificity of the binding of concanavalin A to the fragments was ascertained by control experiments in the presence of methyl α-D-mannopyranoside. Both concanavalin A and the antibody recognized the same fragments, suggesting that the glycosylation site(s) are localized near the C-terminal portion of the receptor (Table III). The molecular mass of the C-terminal fragments are 132 ± 5 (3) and 88 ± 1 (7) kDa for the brain receptor and 114 ± 4 (4) and 84 ± 2 (5) kDa for the oocyte receptor. The 132- and 114-kDa fragments appear at early time points (<10 min), but the 88- and 84-kDa fragments appear only during more prolonged incubations with the enzyme (>30 min). This result suggests that the latter are derived from the former in a sequential way.

In experiments with cAMP-dependent protein kinase, we demonstrated that the $InsP_3R$ from *Xenopus* oocytes can be phosphorylated with the same conditions as the brain $InsP_3R$, indicating the presence of a functional phosphorylation site (Fig. 6).

Biochemical Characterization of the Purified Oocyte

TABLE III

Proteolytic fragments obtained by limited digestion with α-chymotrypsin

Pure $InsP_3R$ molecules were incubated, on ice, for up to 1 h in the presence of α-chymotrypsin. The fragments were analyzed by SDS-PAGE, followed by immunoblotting and labeling with concanavalin A or the C-terminal specific antibody. The molecular mass is expressed in kilodaltons. Mean ± S.E. is given with number of experiments in parentheses.

		Concanavalin A	C-terminal antibody
Rabbit brain			
Intact receptor	268 ± 4 (7)[a]	+++	+++
Fragments	210 ± 1 (2)[a]		
	175 ± 3 (6)[a]	+[b]	
	132 ± 5 (3)	+++	+++
	104 ± 3 (3)		
	98 ± 2 (3)		
	88 ± 1 (7)	+++	+++
	77 ± 2 (3)		
Xenopus oocytes			
Intact receptor	248 ± 9 (5)	+++	+++
Fragments	162 ± 5 (5)		
	114 ± 4 (4)	+++	+++
	101 ± 1 (5)		
	84 ± 2 (5)	+++	+++

[a] Doublet protein.
[b] Nonspecific binding.

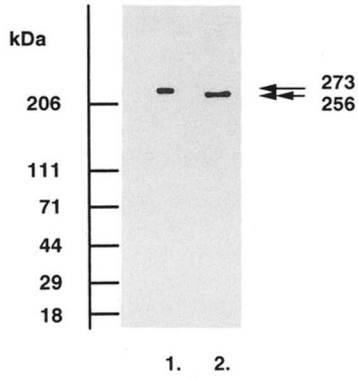

FIG. 6. **Phosphorylation of the $InsP_3R$ by cAMP-dependent protein kinase.** Phosphorylation of the rabbit brain (1) and of the *Xenopus* oocyte (2) $InsP_3R$ was as indicated under "Experimental Procedures." Results are shown after SDS-PAGE, Western blotting, and autoradiography. The *arrow* indicates the position of the rabbit brain $InsP_3R$ and the *double arrow* the position of the *Xenopus* oocyte $InsP_3R$.

$InsP_3R$—Binding of $Ins(1,4,5)P_3$ was measured as indicated under "Experimental Procedures"; 5–10 nM [^{3}H]$Ins(1,4,5)P_3$ was used. The oocyte $InsP_3R$ has a high affinity for $Ins(1,4,5)P_3$. Scatchard analysis of the $Ins(1,4,5)P_3$ binding to different batches of purified oocyte receptor gives an apparent K_d of 46 ± 17 nM (n = 3) and a B_{max} of 402 ± 188 pmol/mg protein (n = 3) (Fig. 7). The specificity of the $InsP_3R$ for inositol phosphates was tested in a competition assay of cold inositol phosphates against labeled [^{3}H]$Ins(1,4,5)P_3$. The specificity is the following: $Ins(1,4,5)P_3$ >> $Ins(2,4,5)P_3$, $Ins(1,3,4,5)P_4$ >> $Ins(1,3,4)P_3$, $InsP_6$ > $Ins(1,3,4,5,6)P_5$ (Table IV).

ATP inhibits 63% of the $Ins(1,4,5)P_3$ binding at a concentration of 1 mM (Table IV). The *Xenopus* oocyte $InsP_3R$ is pH-sensitive, with a maximal binding activity at alkaline pH (Table IV). The binding of [^{3}H]$Ins(1,4,5)P_3$ to the oocyte $InsP_3R$ was potently inhibited by heparin (IC_{50} 2 μg/ml) (Fig. 8).

Immunolocalization—Until now, the absence of antibodies

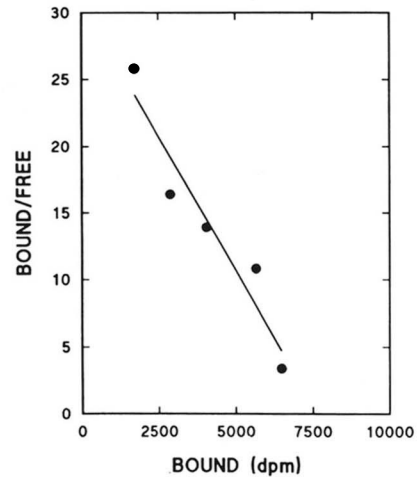

FIG. 7. **Scatchard analysis of [^{3}H]$Ins(1,4,5)P_3$ binding to the purified oocyte $InsP_3R$.** Assays were conducted with 0.1 μg $InsP_3R$, 8 nM [^{3}H]$Ins(1,4,5)P_3$, and various concentrations of nonradioactive $Ins(1,4,5)P_3$. The experiment was independently performed on three different batches of purified $InsP_3R$, each in triplicate. Analysis yielded an average apparent K_d of 46 ± 17 nM (n = 3) and an average B_{max} of 402 ± 188 pmol/mg protein (n = 3). The data of a typical experiment are shown.

TABLE IV

Inhibition of [^{3}H]$Ins(1,4,5)P_3$ binding

Substances known to modulate the brain $InsP_3R$ were examined for their effect on the $InsP_3R$ purified from *Xenopus* oocytes. [^{3}H]$Ins(1,4,5)P_3$ binding was measured at a concentration of 10 nM, as indicated under "Experimental Procedures." Each experiment was done at least twice.

		Binding
		% of control
Control		100
$Ins(1,4,5)P_3$	50 nM	49
	500 nM	28
$Ins(1,3,4)P_3$	2 μM	69
$Ins(2,4,5)P_3$	1 μM	48
	2 μM	5
$Ins(1,3,4,5)P_4$	1 μM	46
	2 μM	3
$Ins(1,3,4,5,6)P_5$	2 μM	86
$InsP_6$	2 μM	60
ATP	1 mM	37
pH	7.3	40
	9.3	179

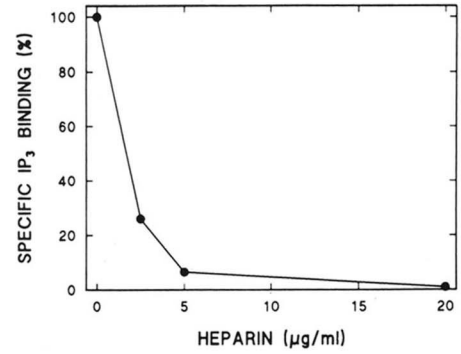

FIG. 8. **Inhibition of the $Ins(1,4,5)P_3$ binding by heparin.** The $InsP_3R$ was purified from *Xenopus* oocytes as described under "Experimental Procedures." Various concentrations of heparin were present in the assay medium.

against the oocyte $InsP_3R$ made it impossible to ascertain the intracellular localization of the receptor. Using the technique of stratified eggs the Ins(1,4,5)P_3-sensitive stores were localized to the endoplasmic reticulum (36). We used the affinity-purified C-terminal-specific antibody for immunolocalization of the $InsP_3R$. Staining was apparent throughout the cortical region and in the perinuclear endoplasmic reticulum in the animal hemisphere. Much less staining was observed in the vegetal hemisphere and was restricted to the cortical layer. No staining was observed in the germinal vesicle (Fig. 9).

Absence of a Ryanodine Receptor—The Ca^{2+} flux experiments already indicated the importance, in quantitative terms, of the Ins(1,4,5)P_3-sensitive store (Fig. 1). Furthermore, no ryanodine binding was detected in oocytes and egg microsomes (Table I). We could not elicit binding activity, despite changes in the conditions of the assay (ryanodine concentration, incubation temperature, and time). Since binding experiments on crude microsomes can underestimate the number of receptors present (see above), we tried to solubilize and partially purify any ryanodine receptor which would be present. We used, on oocyte microsomes, the procedure of McPherson and Campbell (24), which permitted the partial purification of the brain $InsP_3$ and ryanodine receptor molecules on the same density gradient. In our case, analysis of the density gradient still indicated the presence of an Ins(1,4,5)P_3 binding protein, but no ryanodine binding could be observed throughout the gradient (data not shown). Moreover, SDS-PAGE and immunoblotting did not reveal the presence of a high molecular weight protein compatible with the ryanodine receptor, although the antibodies recognized the two frog skeletal muscle ryanodine receptor isoforms described previously (38) (data not shown). Taken together, these data indicate the absence of a ryanodine receptor in *Xenopus* oocytes.

Presence of Ca^{2+} Binding Proteins—Immunoblot analysis of oocyte microsomes (Fig. 10) revealed the presence of two potential Ca^{2+}-binding proteins; one protein was recognized by an anti-calreticulin antibody (60 kDa), and one was recognized by two different anti-calsequestrin antibodies (58 kDa). The molecular mass of calreticulin and calsequestrin in rabbit skeletal muscle fractions was 56 and 60 kDa, respectively.

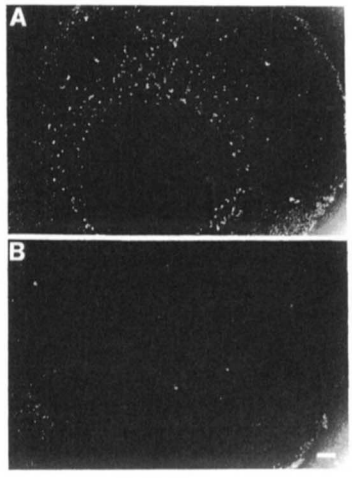

FIG. 9. **Immunolocalization of the $InsP_3R$ in a stage VI *Xenopus* oocyte.** Fixation and sectioning of the oocytes were as described under "Experimental Procedures." The section in *A* was incubated, after blocking, for 2 h with the affinity-purified antibody against the C-terminal of the brain $InsP_3R$. Incubation with affinity-purified fluorescein isothiocyanate-labeled goat anti rabbit IgG antibody (1:200) was for 30 min at room temperature. Sections were mounted in FITC-Guard and photographed. The section in *B* was not incubated with primary antibody but was otherwise treated in the same way as *A*. Orientation of both sections is as follows: animal pole *left*, vegetal pole *right*. The germinal vesicle can clearly be observed in the animal pole. *Bar*, 60 μm.

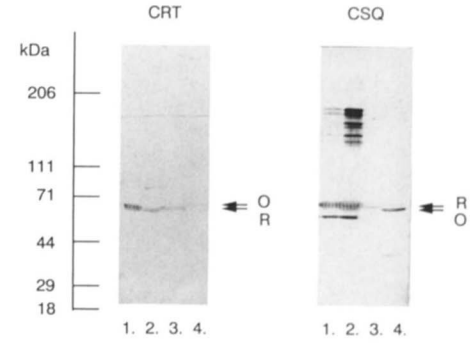

FIG. 10. **Presence of calreticulin-like and calsequestrin-like proteins in *Xenopus* oocytes.** Samples were: rabbit light sarcoplasmic reticulum vesicles, 65 μg (1); rabbit isolated triads, 65 μg (2); rabbit brain microsomes, 200 μg (3); and *Xenopus* oocyte microsomes, 300 μg (4). Staining, after SDS-PAGE and immunoblotting, was with an anti-calreticulin antibody (*left panel*) or with an antibody against chicken cardiac calsequestrin (*right panel*). The same result was obtained with an antibody against dog cardiac calsequestrin. Position of rabbit (*R*) and oocyte (*O*) reactive proteins are indicated.

DISCUSSION

This study identifies a high-affinity high-specificity $InsP_3R$ in *Xenopus* oocyte and egg microsomes. The use of the same procedure for the purification of the $InsP_3R$ from *Xenopus* oocytes and eggs and from rabbit brain indicates similarities between the $InsP_3R$ proteins from those tissues, including a highly similar molecular mass (23 S) and thus a probable similar tetrameric structure. By gel filtration chromatography (3, 5) or density gradient centrifugation combined with electron micrography (4), it was previously shown that the $InsP_3R$ from brain and smooth muscle retained their tetrameric structure during purification. Despite the similarities between the brain and the oocyte/egg receptor molecules, there were differences, and we postulate that the *Xenopus* oocyte/egg $InsP_3R$ is a new isoform of the brain type I $InsP_3R$. Indeed, the $InsP_3R$ from *Xenopus* oocytes and eggs migrates on SDS-PAGE with a molecular mass of 256 kDa, whereas the rabbit brain receptor, purified and assayed in similar conditions, migrates with a molecular mass of 273 kDa. The difference of 17 ± 2 kDa (n = 13), corresponding to a sequence about 150 amino acids shorter for the former, was consistently found. Variations in glycosylation cannot account for the molecular mass difference. Moreover, a polyclonal antibody against the brain $InsP_3R$ failed to recognize the oocyte/egg receptor, indicating sequence disparities. Limited proteolysis experiments with α-chymotrypsin also indicated differences in the structure of the two receptor molecules. Using the latter data, we can speculate on the localization of the missing segment. The alignment of the fragments on the receptor molecule, assuming identity of the C termini, points to the central part of the receptor as probable site of the deletion. This would be near one of the described sites (amino acids 1693–1731) for alternative splicing of the type I receptor (9–11). The conservation of the C-terminal extremity and the presence of glycosylation indicate at least a partial conservation of the C-terminal portion of the receptor. That portion is involved in tetramer formation and the formation of the Ca^{2+} channel (39, 40). Furthermore, the presence of a cAMP-dependent protein kinase phosphorylation site may indicate the conservation of an important regulatory function (41).

The biochemical properties of the oocyte/egg $InsP_3R$ are very similar to the properties of the brain $InsP_3R$. It is a high-affinity high-specificity receptor similar to the receptors purified from brain (3, 42) and from smooth muscle (4, 5). $Ins(1,4,5)P_3$ binding is similarly inhibited by heparin and ATP and is stimulated at alkaline pH (43).

The presence of $[^3H]Ins(1,3,4,5)P_4$ binding sites in *Xenopus* oocyte and egg microsomes can indicate the presence of an $Ins(1,3,4,5)P_4$ receptor, similar to the one described in rat brain tissue (44). $Ins(1,3,4,5)P_4$ modulates intracellular Ca^{2+} in *Xenopus* oocytes (45–47) and sea urchin eggs (48).

We did not detect a ryanodine receptor in *Xenopus* oocytes, but a calreticulin-like protein and a calsequestrin-like protein were present. A calsequestrin-like protein (49) and a ryanodine receptor-like protein (33) are present in sea urchin eggs. The exact role of those proteins in oocytes and eggs remain to be investigated.

The presence in *Xenopus* oocytes of a high-affinity high-specificity $InsP_3R$ has important implications for the understanding of the spatiotemporal aspects of Ca^{2+} signaling. The presence of both $Ins(1,4,5)P_3$-dependent and $Ins(1,4,5)P_3$-independent mechanisms were postulated for the propagation of Ca^{2+} waves (1, 2, 50). Ca^{2+} oscillations and waves were studied by electrophysiological measurements and by using Ca^{2+}-sensitive dyes (15). Those observations are restricted to the cellular domain close to the plasma membrane. Recently, arguments were given for a model describing the propagation of Ca^{2+} waves in *Xenopus* oocytes by a Ca^{2+}-modulated serial release of the $Ins(1,4,5)P_3$-sensitive stores (21, 22). The immunolocalization of the $InsP_3R$ in the cortical region of the oocyte supports the role of the $InsP_3R$ in these phenomena. The inhibition of the Ca^{2+} waves (22) and of the $Ins(1,4,5)P_3$ binding to the receptor by heparin further support the role of the $InsP_3R$. The quantitative importance of the $Ins(1,4,5)P_3$-sensitive store and the observation that no ryanodine receptor could be detected also support the model. We also observed, as was observed in other tissues (51–53), that $Ins(1,4,5)P_3$-induced Ca^{2+} release is dependent on the Ca^{2+} concentration, with maximal release between 0.3 and 0.5 μM. This can explain the fact that Ca^{2+} either inhibit (54, 55) or stimulate (22) Ca^{2+} oscillations in *Xenopus* oocytes. This effect of Ca^{2+} on $Ins(1,4,5)P_3$-induced Ca^{2+} release is central in the proposed model for Ca^{2+} wave propagation (21, 22).

In conclusion, our data indicate the presence of a high-affinity high-specificity $InsP_3R$ in *Xenopus* laevis oocytes and eggs. Although the receptor displays a similarity to the mammalian brain $InsP_3R$, the lower molecular mass, the pattern of proteolytic fragments, and the recognition by the C-terminal-specific antibody suggest that we are dealing with a new isoform of the type I receptor. The $InsP_3R$ probably plays a pivotal role in the induction of Ca^{2+} oscillations and waves in *Xenopus* oocytes and eggs.

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REFERENCES

1. Berridge, M. J., and Irvine, R. F. (1989) *Nature* **341,** 197–205
2. Jacob, R. (1990) *Biochim. Biophys. Acta* **1052,** 427–438
3. Supattapone, S., Worley, P. F., Baraban, J. M., and Snyder, S. H. (1988) *J. Biol. Chem.* **263,** 1530–1534
4. Chadwick, C. C., Saito, A., and Fleischer S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2132–2136
5. Mourey, R. J., Verma, A., Supattapone, S., and Snyder, S. H. (1990) *Biochem. J.* **272,** 383–389
6. Ferris, C. D., Huganir, R. L., Supattapone, S., and Snyder, S. H. (1989) *Nature* **342,** 87–89
7. Furuichi, T., Yoshikawa S., Miyawaki, A., Wada, K., Maeda, N., and Mikoshiba, K. (1989) *Nature* **342,** 32–38
8. Südhof, T. C., Newton, C. L., Archer, B. T., III, Ushkaryov, Y. A., and Mignery, G. A. (1991) *EMBO J.* **10,** 3199–3206
9. Mignery, G. A., Newton, C. L., Archer, B. T., III, and Südhof, T. C. (1990) *J. Biol. Chem.* **265,** 12679–12685
10. Danoff, S. K., Ferris, C. D., Donath, C., Fischer, G. A., Munemitsu, S., Ullrich, A., Snyder, S. H., and Ross, C. A. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2951–2955
11. Nakagawa, T., Okano, H., Furuichi, T., Aruga, J, and Mikoshiba, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6244–6248
12. Imagawa, T., Smith, J. S., Coronado, R., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 16636–16643
13. Anderson, K., Lai, F. A., Liu, Q.-Y., Rousseau, E., Erickson, H. P., and Meissner, G. (1989) *J. Biol. Chem.* **264,** 1329–1335
14. McPherson, P. S., Kim, Y.-K., Valdivia, H., Knudson, C. M., Takekura, H., Franzini-Armstrong, C., Coronado, R., and Campbell, K. P. (1991) *Neuron* **7,** 17–25
15. DeLisle, S. (1991) *Cell Calcium* **12,** 217–227
16. Jaffe, L. F. (1983) *Dev. Biol.* **99,** 265–276
17. Busa, W. B., and Nuccitelli, R. (1985) *J. Cell Biol.* **100,** 1325–1329
18. Busa, W. B., Ferguson, J. E., Joseph S. K., Williamson J. R., and Nuccitelli, R. (1985) *J. Cell Biol.* **101,** 677–682
19. Brooker, G., Seki, T., Croll, D., and Wahlestedt, C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2813–2817
20. Lechleiter, J., Girard, S., Peralta, E., and Clapham, D. (1991) *Science* **252,** 123–126
21. Lechleiter, J. D., and Clapham, D. E. (1992) *Cell* **69,** 283–294
22. DeLisle, S., and Welsh, M. J. (1992) *J. Biol. Chem.* **267,** 7963–7966
23. Colman A. (1984) in *Transcription and Translation, A Practical Approach* (Hames, B. D., and Higgins, S. J., eds.) pp. 271–302, IRL Press, Oxford
24. McPherson, P. S., and Campbell, K. P. (1990) *J. Biol. Chem.* **265,** 18454–18460
25. Ohlendieck, K., Ervasti, J. M., Snook, J. B., and Campbell, K. P. (1991) *J. Cell Biol.* **112,** 135–148
26. Campbell K. P., Franzini-Armstrong C., and Shamoo A. E. (1980) *Biochim. Biophys. Acta* **602,** 97–116
27. Sharp, A. H., Imagawa, T., Leung, A. T., and Campbell, K. P. (1987) *J. Biol. Chem.* **262,** 12309–12315
28. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356
29. Ervasti, J. M., Kahl, S. D., and Campbell, K. P. (1991) *J. Biol. Chem.* **266,** 9161–9165
30. Fabiato, A., and Fabiato, F. (1979) *J. Physiol. (Paris)* **75,** 463–505
31. Laemmli, U. K. (1970) *Nature* **227,** 680–685
32. Tobwin, H., Staehelin, T., and Bordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
33. McPherson, S. M., McPherson, P. S., Mathews, L., Campbell, K. P., and Longo, F. J. (1992) *J. Cell Biol.* **116,** 1111–1121
34. Jorgensen, A. O., and Campbell, K. P. (1984) *J. Cell Biol.* **98,** 1597–1602
35. Milner, R. E., Baksh, S., Shemanko, C., Carpenter, M. R., Smillie, L., Vance, J. E., Opas, M., and Michalak, M. (1991) *J. Biol. Chem.* **266,** 7155–7165
36. Han, J.-K., and Nuccitelli, R. (1990) *J. Cell Biol.* **110,** 1103–1110
37. Maeda, N., Niinobe, M., Nakahira, K., and Mikoshiba, K. (1988) *J. Neurochem.* **51,** 1724–1730
38. Olivares, E. B., Tanksley, S. J., Airey, J. A., Beck, C. F., Ouyang, Y., Deerinck, T. J., Ellisman, M. H., and Sutko, J. L. (1991) *Biophys. J.* **59,** 1153–1163
39. Mignery, G. A., and Südhof, T. C. (1990) *EMBO J.* **9,** 3893–3898
40. Miyawaki, A., Furuichi, T., Ryou, Y., Yoshikawa, S., Nakagawa, T., Saitoh, T., and Mikoshiba, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4911–4915
41. Supattapone, S., Danoff, S. K., Theibert, A., Joseph, S. K., Steiner, J., and Snyder, S. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 8747–8750
42. Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M., and Mikoshiba, K. (1991) *J. Biol. Chem.* **266,** 1109–1116
43. Taylor, C. W., and Richardson, A. (1991) *Pharm. Ther.* **51,** 97–137
44. Theibert, A. B., Estevez, V. A., Ferris, C. D., Danoff, S. K., Barrow, R. K., Prestwich, G. D., and Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3165–3169
45. Ferguson, J. E., Han, J.-K., Kao, J. P. Y., and Nuccitelli, R. (1991) *Exp. Cell Res.* **192,** 352–365
46. Parker, I., and Ivorra, I. (1991) *J. Physiol. (Lond.)* **433,** 207–227
47. DeLisle, S., Pittet, D., Potter, B. V. L., Lew, P. D., and Welsh, M. J. (1992) *Am. J. Physiol.* **262,** C1456–C1463
48. Irvine, R. F., and Moor, R. M. (1986) *Biochem. J.* **240,** 917–920
49. Henson, J. H., Begg, D. A., Beaulieu, S. M., Fishkind, D. J., Bonder, E. M., Terasaki, M., Lebeche, D., and Kaminer, B. (1989) *J. Cell Biol.* **109,** 149–161
50. Busa, W. B. (1990) *J. Reprod. Fertil.* **42,** (suppl.) 155–161
51. Iino, M. (1990) *J. Gen. Physiol.* **95,** 1103–1127
52. Bezprozvanny, I., Watras, J., and Ehrlich, B. E. (1991) *Nature* **351,** 751–754
53. Finch, E. A., Turner T. J., and Goldin, S. M. (1991) *Science* **252,** 443–446
54. Parker, I., and Ivorra, I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 260–264
55. DeLisle, S., Krause, K.-H., Denning, G., Potter, B. V. L., and Welsh, M. J. (1990) *J. Biol. Chem.* **265,** 11726–11730