The Structure and Localization of $InsP_3$ Receptor in Oocytes and Eggs of *Xenopus* and its function in egg activation

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by
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Abbreviations:

$InsP_3$, inositol 1,4,5-trisphosphate;

$InsP_3R$, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum;

kb, kilobase(s);

SDS, sodium dodecyl sulfate;

PAGE, polyacryoamide gel electrophoresis;

GTP-γ-S, guanosine 5'-γ-thiotriphosphate;

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid,

BES, [N, N-bis[2-Hydroxyethl]-2-aminoethanesulfonic acid],

PEG, polyethylene glycol.

CONTENTS

Summary

A transient increase in the intracellular calcium (Ca^{2+}) concentration has been shown to play a key role at fertilization. Injection of inositol 1,4,5-trisphosphate ($InsP_3$) is known to cause a response that mimics the fertilization (egg activation). As a first step to investigate the involvement of InsP3 receptor mediated Ca++ release upon egg activation, the primary structure of the InsP3 receptor expressed in *Xenopus* oocytes was determined by cDNA cloning and its spatial and temporal expression patterns were characterized. The predicted *Xenopus* $InsP_3$ receptor (2693 amino acids) had extensive sequence similarity to the mouse $InsP_3$ receptor. The amino-terminal 1990 amino acids encoded by the cDNA is expressed in NG108-15 cells and showed characteristic $InsP_3$-binding activity. Analysis of developmental expression of the receptor suggested that its mRNA was maternally transcribed and translated, and it was expressed as early as stage II during oogenesis. Immunolocalization techniques by using the antibody against the $XInsP_3R$ fusion protein revealed that $XInsP_3R$ was localized to a well organized ER-like reticular networkin both the cortex and cytoplasm and was enriched in the animal half in full-grown stage VI ovarian oocytes and ovulated eggs, implying its potential roles in the Ca^{2+} wave formation from the sperm entry site. A dramatic redistribution of the $XInsP_3R$ took place during meiotic maturation with a relevance to the reorganizations of organella. These results imply the contribution of the $XInsP_3R$ to the formation and propagation of Ca^{2+} wave. To clarify the function of the receptor in egg activation, functional ablation of the receptor was performed. Microinjection of antisense phosphorothioate-oligodeoxyribonucleotides complmentary to $XInsP_3R$ mRNA blocked $InsP_3$ induced egg activation, indicating that the $XInsP_3R$ plays essential roles upon egg activation through the $InsP_3$-induced Ca^{2+} release.

INTRODUCTION

Inositol 1,4,5-trisphosphate ($InsP_3$) is the key second messenger that mediates cellular functions in a variety of cells by mobilizing calcium (Ca^{2+}) from intracellular stores. In all eggs investigated so far, a transient increase in Ca^{2+} that occurs at fertilization and propagates in the form of Ca^{2+} waves is required to prevent polyspermy and for cell cycle progression (Jaffe 1980, 1983; Winkler et al. 1980). The increase in intracellular Ca^{2+} has been observed during the activation of a wide variety of eggs such as sea urchin (Steinhard et al. 1977; Stricker et al., 1992), medaka fish (Yoshimoto et al., 1986), starfish (Eisen et al., 1984), frog (Busa and Nuccitelli, 1985; Kubota et al., 1987), mouse (Cuthertson and Cobbold, 1985) and hamster (Miyazaki et al., 1986). Sperm-egg interaction in the plasma membrane has been shown to cause Ca^{2+} release through activating phosphoinositide (PI) turnover system in eggs of various animals. It has been hypothesized that a binding of sperm to its proposed receptor at the egg surface activates PI-linked phospholipase C enzyme (Tuner et al., 1986, 1987), which then hydrolyses the plasma membrane lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), and releases the water soluble product inositol 1,4,5-trisphosphate ($InsP_3$), which can stimulate the release of Ca^{2+} from intracellular stores (Kline et al., 1988, 1991; Miyazaki et al., 1989; Jaffe et al., 1988). Microinjection of $InsP_3$ into frog, sea urchin, medaka, fish and starfish eggs and hamster activates early developmental events such as membrane depolarization, cortical granule exocytosis, cortical contraction and abortive cleavage furrow formation (Whitaker and Irvine, 1984; Busa et al., 1985; Picard et al., 1985; Slack et al., 1986; Miyazaki, 1988; Iwamatsu, 1989). It was demonstrated that in some cells the release of Ca^{2+} induced by $InsP_3$ ($InsP_3$-induced Ca^{2+} release: IICR) is mediated by binding to its receptor (Berridge and Irvine, 1989; Supattapone et al., 1988b) on the Ca^{2+} storage sites. Previously, our group and others purified the $InsP_3R$ from the mouse cerebellum and isolated its cDNA and determined its primary structure as an $InsP_3$-gated Ca^{2+} channel (Maeda et al., 1990; Furuichi et al., 1989). By the immunogold electron microscopy, it was shown that the $InsP_3R$ is abundantly localized on the smooth ER, especially

on the stacked smooth ER of mouse cerebellar Purkinje cells (Otsu et al., 1990; Satoh et al., 1990). The $InsP_3R$ is shown to be expressed ubiquitously in various tissues (Maeda et al., 1988; Nakagawa et al., 1991a) including matured ovarian oocytes (Furuichi et al., 1990). Recently, our group demonstrated that Ca^{2+} release in fertilized hamster eggs is mediated solely by the $InsP_3R$ and Ca^{2+}-sensitized IICR (Miyazaki et al., 1992) by injecting a blocking monoclonal antibody (Nakade et al., 1991) to the mouse $InsP_3R$. Some lines of evidence indicated that activation of *Xenopus* eggs is mediated by Ca^{2+} release through the activation of the putative $InsP_3$ receptor ($InsP_3R$) on the ER (Busa et al., 1985; Kline et al., 1988; Han and Nuccitelli, 1990; DeLisle and Welch, 1992). Although it has been suggested that a putative $InsP_3R$ is present in *Xenopus* oocytes only based on the $InsP_3$-responsive egg activation (Busa et al., 1985), very little is known about its molecular properties and intracellular localization in the *Xenopus* oocytes and eggs.

The *Xenopus* eggs provides us an excellent model system not only to study Ca^{2+} excitability and the molecular mechanism of fertilization, but also to analyze the early development of vertebrate embryos. Another advantage of the *Xenopus* system is that ablation experiments by antisense oligonucleotide are easily available (Sagata et al., 1988; Baker et al., 1990; Woolf et al., 1990). In *Xenopus*, it is also possible to characterize the role of a gene product upon egg activation conveniently by observing cortical contraction (Elinson, 1975; Palecek et al., 1978; Hara and Tydeman, 1979; Kline, 1988; Bement and Capco, 1989)in combination with the ablation experiments.

To investigate the role of $InsP_3R$ in the activation of *Xenopus* egg, I have isolated cDNA clones of $InsP_3R$ from *Xenopus* oocytes ($XInsP_3R$) and performed a functional expression of the $InsP_3$-binding activity. The comparison between the mouse and *Xenopus* $InsP_3R$ has revealed extensive homology, which has allowed us to elucidate the functional domains for $InsP_3$-binding as well as the Ca^{2+} channel activity of the $InsP_3R$ function. The expression pattern of $XInsP_3R$ mRNA and protein revealed that $XInsP_3R$ was transcribed and translated in the early stage of oogenesis. $InsP_3R$ in immatured fully-grown stage VI oocytes and ovulated eggs was studied by

using immunocytochemical techniques with specific antibody against the fusion protein. The $XInsP_3R$ was localized in the cytoplasm in the animal hemisphere as well as in the cortex of both hemispheres in the *Xenopus* unfertilized eggs, in consistent with the fact that the Ca^{2+} wave is initiated at the sperm entry site and propagate through the egg (Busa et al., 1985). The physiological role of the localization of the receptor upon egg activation is also discussed. Ablation of $XInsP_3R$ by using sequence-specific phosphorothioate antisense oligo-deoxyribonucleotides blocked $InsP_3$-induced egg activation indicate that the $XInsP_3R$ plays an important role upon egg activation possibly through the IICR.

Experimental Procedures

Construction and Screening of cDNA library

Oligo-dT primed cDNA library prepared from defolliculated *Xenopus* oocytes poly(dA)+RNA constructed in λgt10 vector (a kind gift of Dr. D.Melton, Harvard University; Rebagliati, 1985) was used in the initial screening. Random primed cDNA library was constructed with poly(A)$^+$RNA isolated from *Xenopus* oocytes by the guanidium isothiocyanate-CsCl method followed by purification by Oligotex-dT30 (Roche), and was synthesized using a random haxamer as primers using the cDNA synthesis system (Amersham). The cDNA synthesis was end filled with *E.coli* DNA polymerase I (Amersham) and ligated with an *Eco*RI adaptor (Pharmarcia) and then ligated into λgt11 or λZAPII (Stratagene). Oligo-dT primed λgt10cDNA library was first screened with *Eco*RI/*Bgl*II 1.2kb cDNA fragment containing the transmembrane region of the previously described mouse cerebellum $InsP_3$ receptor (Furuichi et al., 1989b). Plaque hybridization was performed by incubating filters with $2x10^7$ cpm α^{32}P-dATP labelled prpbes, 50% formamied,1% SDS, 1M NaCl, 200 μg/ml boiled single-strand herring sperm DNA, and 10% dextran sulfate at 42°C for 16 hours and washed in 2xSSC at room

temperature.

Approximately 50 out of $1x10^7$ clones screened were positive in the first screening. Seven out of the ten clones chosen for further analysis were positive independent clones. In order to obtain 5'adjacent cDNAs, random hexamer primed λgt11 and λZAPcDNA libraries were screened with the positive cDNA clones. Washes were done with 0.1xSSC at 42°C. Finally, the full-length $XInsP_3R$ cDNA was isolated in overlapping clones (Figure 1).

Expression of Ligand Binding Sites of *Xenopus* $InsP_3R$ by Transfection

A plasmid pX5'6K was constructed by inserting: a) the *Pst*I to *Esp*I fragment from XZ5 (base pairs 146-2974, Figure 1); b) the *Esp*I to *Apa*I fragment from X13 (base pairs 3115-3329); c) the *Apa*I to the 3'*Not*I-*Eco*RI linker of X14 (3329-6122) into *Pst*I site and *Not*I site of pBluescriptII KS(+) vector, and the *Cla*I site in the multicloning site was filled-in and ligated with *Not*I linker. The *Sal*I site of expression vector pβacts (Furuichi et al., 1989b) was converted to a blunt-end by using DNA polymerase I *Klenow* fragment and ligated with *Not*I linker to make pβactsN. *Not*I fragment of pX5'6K was inserted into *Not*I site of pβactsN. The resultant plasmid, pβactX5'6K(146-6122 bp), was constructed in this manner to exclude the ATG codon that is found in the 5' untranslated region of the receptor message and that is not used for initiation. This plasmid contains a part of the $XInsP_3R$ cDNA encoding the N-terminal 1990 amino acids of the $XInsP_3R$ cDNA between a β-actin promoter and a simian virus 40 polyadenylation sequence. The structures of the resultant plasmid contructs were confirmed by a partial nucleotide sequencing.

The pβactX5'6K and pβactsN as control were transiently transfected into NG108-15 cells using the calcium-phosphate precipitation method as described (Chen and Okayama, 1987). Briefly, NG108-15 cells were grown in DMEM and transfered to a 10 mmΦ dish on the previous day for transfection. After incubation at 37°C, 5% CO_2 for 20-24 hours, 20 μg of pβactX6k or pβactN was mixed with 0.5ml of 2x BBS buffer(50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.90) and 0.5 ml of 0.25M $CaCl_2$. After incubation for 10-20 minutes at room

temperature,the mixture were added to each 10 mmΦ dish of NG108-15 cells, cells were allowed to incubate at 37°C, 5% CO_2 for 20-24 hours. The medium were discarded and fresh DMEM were added to the transfected cells. Cells were harvested 96 hours after transfection, the cytosol was prepared as described previously (Miyawaki et al., 1991) with some modications (see the later section), the expression was analyzed by immunoblot studies using the monoclonal antibody 4C11 against the mouse $InsP_3R$ (Maeda et al., 1989), and the $InsP_3$ binding activity was measured by the PEG precipitation method according to Miyawaki et al. (1991) with modification as described in the later section.

DNA Sequencing

DNA sequencing was determined on pBluescript vectors (Stratagene) using the dideoxynucleotide-chain termination method (Sanger et al., 1977).

Preparation of Cytosol Fraction from transfected NG108-15 Cells

Transfected NG108-15 cells were collected and washed twice with ice cold PBS and were mixed with 9 time volume of the solution containing 0.25M sucrose, 10 mM Tris-HCl(pH8.0, at room temperature), 0.1 mM EDTA, 1mM phenylmethyl sulphonyl fluoride, , 1 μM leupeptin, and 10 μM pepstatin A, and were homogenized in a glass-Teflon Potter homogenizer with 10 strokes at 1,000 r.p.m. The homogenate was centrifuged at 1000g for 5 min at 2°C to remove nuclei. The supernatants were centrifuged at 105,000g in a Beckman TLA100-3 rotor for 1 hour. The supernatant was used as the cytosol fraction.

[^{3}H]$InsP_3$ Binding Assay with Cytosol Fractions

Soluble protein (50 μg) was incubated with 20 nM of [^{3}H]$InsP_3$ (NEN) in 100 μl of binding buffer (50 mM Tris-HCl, pH 8.0 at room temperature/1 mM 2-mercaptoethnol/1 mM EDTA) for 10 min at 4°C. The sample was then mixed with 4 μl of γ-globulin (50 mg/ml) and 70 μl of a solution containing 30% (wt/vol) PEG 6000, 1mM 2-mercaptoethanol, and 50mM Tris-HCl (pH

8.0, at room temperature). After incubation on ice for 5 min, the protein-PEG complex was collected by centrifugation twice at 10,000 x g for 5 min at 2°C, the pellet was solublized in Solvable (NEN/DuPont), and the radioactivity was measured in Aquasol 2 (NEN/DuPont) by using a scintillation counter.

Egg Procurement

Fully-grown oocytes and meiotically mature eggs were obtained from adult female *Xenopus* laevis purchased from Hamamatsuseibutsu Co.(Shizuoka, Japan). Ovarian fragments were surgically removed from females anesthetized by hypothermia. Ovarian fragments were washed and placed in modified Barth solution (MBS; Colman, 1984; 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes [pH 7.5], 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$), and fully-grown stage VI oocytes (Dumont 1972; diameter 1.2-1.3) were obtained by manual defolliculation using wathchmaker's forceps, after 1 hour collagenase treatment (2 mg/ml type I collagenase; Sigma Chemical). Oocytes were maintained in MBS at 18°C until further use. Maturation was induced by overnight incubation in MBS containing 5 μg/ml progesterone (Sigma Chemical), usualy for 10 hours, and oocytes were judged to be meiotically mature by the appearance of a white spot on the animal pole that signifies the release of the first polar body (germinal vesicle breakdown,GVBD).

Unfertilized, ovulated eggs were obtained by injecting adult females with 800 IU human chorionic gonadotropin (Sigma Chemical) and stripping the eggs from the animals 8-10 hours later into MBS. Fertilization was performed as previously described (Newport and Kirschner, 1984); breifly, the MBS were removed and the eggs were rubbed with a testis teased open with sharp forceps just before use, and then flood with distilled water to activate the sperm .

RNases Protection Analysis

RNA was extracted from stage I-VI oocytes by vanadyl-complex method. Total RNA was hybridized with an RNA probe for $InsP_3$ receptor and analyzed by RNase protection studies

basically according to the method described previously (Melton et al., 1984; Okano et al., 1988). cDNA *Puv*II 0.66kb fragment (5233-5906bp) was subcloned into *Eco*RI site of pBluscript KS(-) vector (Stratagene) to give XpB14*pvu*II and the labeled anti-sense RNA probes was synthesized by adding T7 RNA polymerase (Boehringer Mannheim) to XpB14*pvu*II linearized with *Sal*I, in the presence of ^{32}P-UTP (800 Ci/mmol, Amersham). Aliquots of $2x10^6$ c.p.m. of the probe was hybridized to various concentrations of the total oocyte (stage I-VI) RNA (up to 20 μg at 42°C for 12 hr) and yeast tRNA controls in a solution containing 80% formamide, 40 mM PIPES, 0.4 M NaCl, and 1mM EDTA. The unhybridized regions of the probe was digested with 100ng of RNase A and 20U of RNase T1 at 17°C for 30 min. in a total volume of 0.35 ml, followed by phenol chloroform extraction and ethanol precipitation. The length of the RNase-resistant ^{32}P-RNA-RNA hybrids was analyzed by electrophoresis on denaturing 5% polyacrylamide-8M urea gel. In addition, unprotected RNA probe was applied to the gel electrophoresis as a reference.

Preparation and Immunoblot Analysis of Crude membrane Proteins from *Xenopus* Oocytes

Crude membrane proteins from *Xenopus* oocytes were prepared as followings: *Xenopus* oocytes was mixed with 9 time volume of the solution containing 0.32M sucrose in buffer A (1 mM EDTA, 0.1 mM phenylmethyl sulphonyl fluoride. 10 μM leupeptin, 10 μM pepstatin A, 1mM-2-mercaptoethanol and 50 mM Tris-HCl(pH8.0)), and were homogenized in a glass-Teflon Potter homogenizer with 10 strokes at 850 r.p.m. The homogenate was centrifuged at 1000g for 15 min at 2°C to remove nuclei. The supernatants were centrifuged at 105,000g for 15 min. at 2°C. Precipitates were used as the crude membrane fraction.

Crude membrane protein equivalent to one oocyte (for stage I to VI) were eletrophoresed onto 5%-SDS-polyacrylamide gel electrophoresis according to the method by Laemmli (1970) and transferred to a nitrocellulose sheet. The blots were stained with amidoblack or immunostained with monoclonal antibodies 4C11 (culture supernatant of hybridoma, 1 μg/ml of IgG), raised against the mouse cerebellum $InsP_3$ receptor (Maeda et al., 1989)) by using Vectastain ABC rat

IgG kit according to the manufacturer's protocol, and finallyvisualized with 1 mg/ml 3,3'-diaminobenzidine, tetrahydrochloride, 0.02% H_2O_2. Under the condition described here, almost all of the protein reacting the mouse antibodies were recovered in the P2+P3 fraction.

In Situ Hybridization

Oocytes were fixed in a mixture of ethanol: acetic acid: chromium trioxide (95:5:0.25;v:v:v) for 1 hour at 4°C, dehydration in a graded series of ethanol and embedded in paraffin as described (Melton, 1987). Antisense or sense RNA probe were prepared by *in vitro* transcription using T7 or T3 DNA dependent RNA polymerase (Boehringer Mannheim) added to templete plasmid XpB14*pvu*II (described above) linearlized by *Sal*I or *Eco*RI respectively in the presence of [a-^{35}S]UTP (NEN; 46.4TBq). *In situ* hybridization was performed as previously described (Nakagawa et al., 1991b). Briefly, before hybridization, sections were deparaffinized with xylene, rehydrated through graded ethanol, incubated with proteinase K, and dehydrated. Sections were then hybridized with ^{32}P-labelled RNA probes at 4x10^4 cpm/μl in a 100 μl volume of a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM dithiothreitol, 1xDenhart's solution, 0.25% sodium dodecyl sulfate, 10% dextran sulfate, 25 μg/ml yeast tRNA, 200 μg/ml yeast total RNA, and 200 μg/ml denatured salmon testis DNA. After hybridization for 16 h at 50°C, sections were washed in 6x standard saline citrate buffer (SSC), 100 mM 2-mercaptoethanol at 50°C; in 50% formamide, 2 X SSC at 50°C for 30 min, sections were treated with RNase A at 20 μg/ml for 30 min at 37°C in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 500 mM NaCl; and followed by washes twice in 2xSSC, 14 mM 2-mercaptoethanol for 20 min at 50°C; and in 0.1x SSC, 14 mM 2-mercaptoethanol for 20 min at 50°C. After dehydration, sections were coated with Kodak NTB-2 emulsion, stored at 4°C, developed with Kodak D-19 developer after exposure for 7 days and stained with Giemsa dye and photographed under darkfield optics.

Preparation of Fusion Proteins

A cDNA fragment (7764-8261bp) encoding 155 amino-acids of the C-terminal (2539-2693a.a) was amplified by polymerase chain reaction (PCR) using a pair of primer constructed on the basis of the sequence of $XInsP_3R$ according to the standard method (Saiki et al., 1988). The amplified fragment was subcloned into 1) pGEX-2T vector (Smith and Johnson, 1986) to give pGEX-XIP3RC in order to generate GST-fusion protein or 2) pATH vector to give pATH-XIP3RC in order to generate *Trp*E-fusion protein. *E.coli* JM109 cells carrying the pGEX-XIP3RC were grown at 37°C to logarythmic phase and treated with IPTG for 4-6 hours. Cells were collected. Crude extracts were electrophoresis on a 10% SDS-polyacrylamide gel and the band correspond to GST-XIP3RC or *Trp*E-XIP3R fusion proteins were cut out and eluted from the gel. Purified GST-XIP3RC or *Trp*E-XIP3RC were analyzed by electrophoresis on SDS-polyacryamide gels and appears as a major band. GST-XIP3RC was used for the generation of anti-$XInsP_3R$ polyclonal antisera and *Trp*E-XIP3RC was used as competition antigen in immunoprecipitation assays.

Rabbits were injected subcutaneously with 170 μg of the purified GST-XIP3RC fusion protein in Freund's complete adjuvant (1:1) for an interval of 2 weeks. After the 4th booster, sera were collected and examined for immunoreactivity to $XInsP_3R$. A major band corresponding to the $XInsP_3R$ was detected by western blot analysis.

Immunohistochemistry

Fully-grown oocytes, *in vitro* matured eggs, ovulated eggs and fertilized eggs were fixed by immersion in 4% paraformaldehyde in phosphate buffer (pH7.4), at 4°C for 2 hours. After dehydration in a graded alcohol series, paraffin-embedded samples were cut into 6 μm thick sections, which were then deparaffinized and equilibrated in phosphate buffered saline (PBS). The sections were treated washed in PBS and then with 0.5%(w/v) skim milk (DIFCO), 0.2%(v/v) Triton X-100/PBS as blocking agent for 1 hour at room temperature, then washed and incubated overnight (16-20 hours) with anti-$XInsP_3R$ polyclonal antisera diluted 1:100 with

blocking solution at 4°C. Sections incubated in preimmune-sera 1:100 dilution were used as controls. Sections were washed in PBS followed by 1 hour incubation in FITC-conjugated anti-rabbit antibody (Vector) 1:200 diluted with blocking solution at room temperature. After wash in PBS, the sections were mounted in 1% *p*-phenylene diamine in 80% glycerol, 20% PBS, examined with an inverted microscope (BH-2, Olympus Inc.) equipped with fluorescence optics and photographed with Fujicolor Super G.

Microinjection and Egg Activation

Microinjection was performed using a nitrogen air pressure microinjector IM-200 (Narishige). The micropipets for injection were pulled on a puller from GD-1 pyrex tubing (Narishige Co., LTD.). The tip of the injection pipet was broken to give a final diameter of 15-20 μm for microinjection of sense- or antisense oligonucleotides, or a final diameter of 5-8 μm for microinjection of $InsP_3$. The injection volume was calibrated by expelling the aqueous solution into air and measuring the radius of the drop. Stage VI oocytes were microinjected with 20 nl of 1 mg/ml sense or anti-sense oligonucleotides mixture, and incubated in MBS before further treatment. When the immunoprecipitation was to be carried out, oocytes were incubated in MBS for 2 hours after microinjection, and 1 μl of L-[^{35}S] methionine (Amersham; 15 mCi/ml) was added to batches of 10 oocytes in 50 μl MBS and allowed a period of 6 hours preincubation before progesterone was added. 8 hrs after microinjection, progesterone (Sigma Chemical) was added to a final concentration of 5 μg/ml to induce meiotic resumption, After a total incubation time of about 10 hours in the presence of progesterone, matured eggs which showed germinal vesicle breakdown (GVBD) by the appearance of a white spot on the animal pole, were assayed for sensitivity to $InsP_3$ to undergo activation or b) were pooled for further analysis by immunoprecipitation.

Five minutes prior to microinjection, matured eggs previously microinjected with sense- or antisense oligonucleotides were transfered to Ca^{2+} free F1 solution (+EGTA 1 mM)(Hollinger and Corton; 1980; 31.25 mM NaCl, 1.75 mM KCl, 0.5 mM Na_2HPO_4, 1.9 mM NaOH, 0.06 mM

$MgCl_2$, and 10.0 mM Tricine at pH 7.8), to avoid activation upon impalement. 2 nl of 100 μM $InsP_3$ in 0.1 mM Hepes [pH 7.8], 10 μM EGTA was microinjected. Eggs microinjected with 0.1 mM Hepes pH 7.8, 10 μM EGTA was used as control. Eggs were transfered back to Ca^{2+} free F1 solution (+ 1 mM EGTA) and scored for cortical contraction occurred within 10 minutes under a steromicroscope.

Immunoprecipitation Assays for Detection of $XInsP_3R$

Crude membrane proteins were prepared from aliqotes of 3 oocytes microinjected with sense- or antisense oligonucleotides and *in vitro* matured by progesterone as described above, and were suspended in 45 μl of 50 mM Tris-HCl (pH 8.0), 1mM EDTA, 1mM 2-mercaptoethanol, 1%(v/v) Triton X-100. After 30 min incubation at 4°C, the solublized supernatant was recovered by centrifugation at 10,000 x g for 10 min at 4°C. 85 μl of buffer B (0.02%(v/v) Triton X-100, 0.2M NaCl, 10 mM sodium phosphate buffer pH 7.6) was added to 15 μl (correspondent to 1 egg) of the recovered supernatant with or without the presence of competiting antigen (0.4 mg/ml purified *Trp*E-XIP3RC fusion protein or *Trp*E as control). After 30 min preabsorption with 10 μl 10%(w/v) suspension of Pansorbin (Calbiochem), supernatant was recovered and 1 μl of anti-$XInsP_3R$ antisera was added to the samples, which were then incubated for 1 hour at 4°C. 10 μl of 10%(w/v) suspension of Pansorbinbin was added and the sample tubes were rotated for 1 h at 4°C and then the Pansorbin particles were washed three times with 500 μl of buffer B. The Pansorbin pellets were mixed with 20 μl of SDS sample buffer and then were heated in a boiling water bath for 3 min, After centrifugation the supernatants were subjected to 5% SDS-polyacrylamide gel electrophresis in the buffer systen of Laemmli (1970). The gel was fixed, enhanced in Enlightning (NEN/DuPont), dried and exposed to Kodat XAR film.

Materials

$Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ were from Dojin, $Ins(2,4,5)P_3$, $Ins(1)P_1$ and $Ins(1,4)P_2$ were from Boehringer Mannheim. All other chemicals were of reagent grade.

Results

The Identification of the $InsP_3R$ in *Xenopus* Oocytes and its cDNA Cloning and Sequencing Analysis

The presence of a protein immunologically related to the mouse $InsP_3R$ in *Xenopus* oocytes was confirmed by the immunoblot analysis of crude membrane preparation of *Xenopus laevis* oocytes by using various antibodies against the mouse $InsP_3R$. A single protein band slightly smaller than the mouse $InsP_3R$ was detected with 4C11 and 10A6 monoclonal antibodies (Maeda et al., 1989) and polyclonal antibodies (Nakade et al., unpublished results) against the mouse $InsP_3R$ (Muto, 1991) which is estimated to be about 300kd by using the mouse $InsP_3R$, 313kd on the basis of the cDNA sequence (Furuichi et al., 1989a,b), as a molecular weight marker. The present results are consistent with previous observation of the $InsP_3$-induced egg activation, which implied a putative $InsP_3R$ is present in *Xenopus* oocytes (Busa et al., 1985). In order to characterize more about the molecular nature of this protein, we isolated cDNA clones encoding the $InsP_3R$ from *Xenopus* oocytes cDNA libraries with the mouse $InsP_3R$ cDNA probe and determined its primary structure.

The oligo-dT primed *Xenopus* oocytes cDNA library constructed in phage λgt10 (Rabagliati et al., 1985) was screened at a low stringency with a ^{32}P-labeled cDNA fragment containing the proposed transmembrane region of the $InsP_3$ receptor from mouse cerebellum (Furuichi et al., 1989b). To isolate more complete overlapping clones, random hexamer primed *Xenopus* oocytes cDNA libraries in a λgt11 and a λZAPII were screened at a high stringency with the largest cDNA insert of ten positive clones obtained. The cDNA clones were subcloned into pBluescriptIIKS+ (Stratagene) and the complete cDNA sequence was determined by dideoxy-chain termination method (Sanger et al., 1977) using clones XZ5, X14, X13, X10, and X3C (Figure 1). The cDNA sequence had a single long open reading frame of 2693 amino acids

preceded by an untranslated leader sequence of 148 nucleotides (Muto, 1991).

The XInsP$_3$R has a striking similarity in its overall structure to the mouse InsP$_3$R. The amino acid sequence of the XInsP$_3$R shares 90% identity with that of the mouse InsP$_3$R, although these homologous sequences are interspersed by some stretches of inserted, deleted and diverged sequences (Figure 2). Among the sequence differences, it is notable that two regions that can be deleted by alternative splicing from the mouse sequence (SI: residues 318-332 ,SII: residues 1692-1731; Danoff et al., 1991; Nakagawa et al., 1991a) were missing in the present *Xenopus* sequence.

The structure and function analyses of the rodent InsP$_3$Rs (Migrery and Sudhof, 1990; Miyawaki et al., 1991) suggested the receptor consists of three main domains: 1. an N-terminal InsP$_3$-binding domain, 2. a putative Ca^{2+} channel domain near the C-terminus, and 3. a long intermediate domain containing phosphorylation sites by c-AMP dependent protein kinase (PKA) and putative ATP-binding sites. The intermediate domain is likely to be involved in the coupling of InsP$_3$-binding and channel opening, and also in the modulation of the channel activity (Suppatopone et al., 1988a; Mignery and Sudhof, 1990; Ferris et al., 1990; Maeda et al., 1991; Miyawaki et al., 1991). We compared the primary structures of the *Xenopus* and mouse InsP$_3$Rs by focusing these three functional domains.

1. An N-terminal InsP$_3$-binding domain: Previous studies showed that the InsP$_3$ binding domain exists within 650 residues of the N-terminus of the rodent receptor (Mignery and Smith, 1990; Miyawaki et al., 1991). The primary structure of this region was well conserved between mouse and *Xenopus* InsP$_3$Rs, although the present *Xenopus* sequence did not contain SI domain (Figure 2). Regarding the mouse InsP$_3$-binding region of 650 amino acids, the N-terminal 635 amino acids of the *Xenopus* counterpart was composed of 96% identical and 2% functionally similar amino acids, except for the diverged 24 amino acids (residues 318-341 in the XInsP$_3$R), suggesting this region is responsible for the InsP$_3$-binding also in the XInsP$_3$R. I confirmed that the InsP$_3$-binding activity is encoded by the present *Xenopus* clone by the functional expression of the cDNA (Figure 3, 4; see next paragragp). Interestingly, the diverged 24 residues were

localized just on the C-terminal side of mouse receptor SI domain (Figure 2).

2. A putative Ca^{2+} channel domain near the C-terminus: Various lines of evidence indicated that the rodent $InsP_3R$ has clusters of transmembrane regions and a putative Ca^{2+} channel activity near the C-terminus region (Furuichi et al., 1989b; Ferris et al., 1989; Mignery et al., 1990; Miyawaki et al., 1990, 1991; Maeda et al., 1991). The deduced amino acid sequence of the $XInsP_3R$ was analyzed for hydrophobicity by the method of Kyte and Doolittle (1982) (data not shown, see Muto, 1991). The profile indicated the presence of at least 6 main peaks near the C terminus. Each of these 6 highly hydrophobic segments (residues 2223-2241, 2255-2273, 2299-2319, 2338-23154, 2387-2409, 2514-2533) comprising about 20 amino-acids. Most of these hydrophobic domains were flanked by charged residues. Therefore, this region could represent multiple membrane-spanning sequences (residues 2223-2533). The hydrophobicity profile of this region was extremely conserved between the mouse and *Xenopus* receptors and an overall of 95% identical amino acid were detected in the putative membrane-spanning sequences (Figure 2). They were also well conserved even in the *Drosophila* counterpart (Miyawaki et al., 1991; Yoshikawa et al., 1992). Particularly striking is the complete conservation of amino-acid sequences of M5 and M6 between the *Xenopus* and the mouse $InsP_3R$ which resemble the corresponding sequences of M3 and M4 of the rabbit ryanodine receptor (Takeshima et al., 1989). An overall resemblance of the amino-acid sequence between the *Xenopus* $InsP_3R$ and the ryanodine receptor was observed (Figure 2) as previously described (Furuichi et al., 1989b). The ryanodine receptor mediates Ca^{2+} release from the sarcoplasmic reticulum (SR) of skeletal muscle. Furthermore, Miyawaki et al.(1990) demonstrated the mouse $InsP_3R$ cDNA encoded a Ca^{2+} channel activity by the transfection analyses. Therefore, it is likely that the well conserved C-teminus region (2223-2533) of the $XInsP_3R$ forms a Ca^{2+} channel.

3. An intermediate domain: This domain contains modulatory sites for the channel activity in the rodent receptors. The rodent $InsP_3R$ is phosphorylated (Mikoshiba et al., 1985) in a c-AMP dependent manner (Supattapone et al., 1988; Yamamoto et al., 1989). Phosphorylation by PKA was reported to modulate the release of Ca^{2+} by $InsP_3R$ (Suppattapone et al., 1988a; Nakade et

al., submitted). The mouse receptor has two PKA phosphorylation sites (Huganir and Greengard, 1983; R/K-R/K-X-S/T; serine residues 1588 and 1755) in the intermediate domain. These PKA sites are also present in similar positions of the *Xenopus* sequence (serine residues 1575, 1702).

ATP promotes an IICR in a concentration-dependent manner in the reconstitution experiments with purified rodent $InsP_3R$ and a lipid bilayer (Ferris et al., 1990; Maeda et al., 1991). Both the mouse and *Xenopus* receptors contain potential ATP-binding sites. Normally, the consensus sequences for nucleotide-binding sites (G-X-G-X-X-G-(nX)-K; Wierenga and Hol, 1983) have a 17-21 residue spacer between the glycine-rich sequence and the common lysine residue. These consensus nucleotide binding sites were present in similar positions in the mouse and *Xenopus* sequences (residues 1775-1781 and 2016-2022 in mouse; residues 1720-1726 and 1962-1967 in *Xenopus*) with an additional site in the *Xenopus* $InsP_3R$ (1674-1679) corresponding to the junction of SII domain of the mouse $InsP_3$ although the common lysine residue is apart from the sequence by 39 residues. The striking similarities in the potential modulatory sites between mouse and *Xenopus* receptors indicate that the channel activities of these receptors are similarly regulated.

Functional Expression of Ligand Binding Site of $XInsP_3R$ cDNA in NG108-15 cells

In order to confirm that the $InsP_3$ binding activity is encoded by the cDNA we cloned in the present study, we performed a functional expression of the $XInsP_3R$ cDNA in the NG108-15 cells by the transient transfection experiment. With an analogy to the mouse $InsP_3R$, of which the $InsP_3$ binding domain was shown to be present within 650 residues of the N-terminus of the mouse receptor (Miyawaki et al., 1991), the $InsP_3$ binding site of $XInsP_3R$ was also suggested to lie in the N-terminal. An expression vector pBactX6k was constructed to express the $XInsP_3R$ cDNA, including the cDNA insert encoding the truncated protein corresponding to the N-

terminal 1990 amino acids of the XInsP$_3$R under the control of the β-actin promoter (Figure 3). This plasmid was introduced into NG108-15 cells by the standard calcium-phosphate precipitation method (Chen and Okayama 1987). Three days after transfection, cells were collected and InsP$_3$ binding activities were assayed as described (Miyawaki et al., 1990). Immunoblot analysis of cytosol fractions using a monoclonal antibody 4C11 against the mouse InsP$_3$R (Maeda et al., 1989) revealed that the 4C11 reacted with the soluble truncated protein expressed in the transfected cells (Figure 4a). The InsP$_3$ binding assay revealed that the truncated protein had an InsP$_3$-binding activity; no InsP$_3$ binding was found for control DNA (vector which did not include the cDNA insert) transfected NG108-15 cells. Scatchard analysis of the expressed protein displays an apparent K_D of 66±14nM (n=5). The specificity for inositol phosphates was tested in a competition assay of inositol phosphates against [^{3}H]Ins(1,4,5)P$_3$. The competition potencies were in the following orders: Ins(1,4,5)P$_3$ > Ins(1,3,4,5)P$_4$, Ins(2,4,5)P$_3$ > Ins(1,4)P$_2$, Ins(1)P$_1$ (Figure 4b). This inhibition pattern was similar to that of mouse InsP$_3$R (Miyawaki et al., 1990) and the truncate *Drosophila* InsP$_3$R expressed in NG108-15 cells (Yoshikawa et al., 1992). Thus, the cloned cDNA was shown to encode an InsP$_3$ binding activity with a high homology with the previously cloned mouse InsP$_3$ receptor cDNA (Furuichi et al. 1989a,b).

The mRNA and protein level of XInsP$_3$R during *Xenopus* oogenesis

To understand biological roles of the InsP$_3$R during *Xenopus* development, we analyzed the temporal pattern of the XInsP$_3$R gene expression throughout oogenesis. Quantitative RNase protection analysis revealed that the XInsP$_3$R mRNA was present in the smallest oocytes (stage I) which accumulated to a large amount as early as stage III (Dumont, 1972) and gradually decreased and reached its steady state with the oocyte growth and showed no significant change in *in vitro* maturation by progesterone (Figure 5). The temporal and spatial expression of

$XInsP_3R$ mRNA throughout oogenesis was confirmed by *in situ* hybridization studies. Signals in both hemispheres of oocytes in stage I-IV, with more grains in the smallest oocytes of stage I-II than larger oocytes of stage III-IV were detected. Signals were weak in fully-grown oocytes of stage V-VI(Figure 6), possibly due to a large increase in the volume of the oocytes accompanied with growth.

The protein level of the $XInsP_3R$ during oogenesis was examined by immunoblot analysis. During oogenesis, the protein was also detected in as early as stage I-II oocytes and its level gradually increased and reached its plateau at stage IV on a per oocyte base (Figure 7). This discrepancy between profiles of steady state level of the mRNA and protein during the oocyte growth was likely due to the difference of the stability of the mRNA and protein or due to differential translational regulation of $XInsP_3R$ mRNA during these developmental stages. RNase protection and immunoblot analyses of developmental expression of the $XInsP_3R$ during oogenesis suggested that its mRNA was maternally transcribed and translated, and both mRNA and protein the $XInsP_3R$ accumulate to a considerable amount toward fertilization suggested that the $XInsP_3R$ might play an important role in the event of the fertilization or egg activation.

Immunolocalization of the $InsP_3R$ in *Xenopus* oocytes and eggs

Immunolocalization of the $XInsP_3R$ was examined by using specific antisera (anti-$XInsP_3R$) raised against the fusion protein of glutathion-s-transferase and the C-terminus region of $XInsP_3R$ (GST-XIP3R fusion proteins) expressed in bacterial cells (see Experimental Procedures). The specificities of the antisera to the $XInsP_3R$ was confirmed by immunoblot analyses (data not shown). The immunostaining results by using anti-$XInsP_3R$ showed strong stainings in the interior cytoplasm especially enriched in the animal hemisphere as well as the cortical layer near the cortex (Figure 8A-E) throughout the entire oocyte of the immature fully-grown stage VI oocytes. The staining in the interior of the immature fully-grown stage VI

oocytes showed a characteristic ER-like reticular network with an especially stronger stainings in the cytoplasmic areas free of yolk platelets in the animal hemisphere and the perinuclear region than that in the vegetal hemisphere.

When oocytes were meiotically matured by the progesterone treatment, the staining pattern showed a drastic change. Positive stainings in the cortex and the subcortical region were observed in both hemispheres (Figure 8F). The strong positive signals detected arround the nucleus of the immatured fully-grown oocytes were absent, while yolk free patches with strong stainings enriched in the animal hemisphere were observed. Smaller patches of positive stainings can be detected in the vegital hemisphere. The characteristic staining of the ER-like reticular network was detected in the cortical layer in both hemispheres of the eggs, while the signals in the deep cytoplasm were much weaker (Figure 8F).

In ovulated unfertilized eggs, enriched signals in the animal hemisphere were observed and patches of positive stainings enriched in the animal half that were observed in *in vitro* mature eggs were detected in some batches, though not all of the ovulated unfertilized eggs (Figure 9 A). Strong cortical stainings consist of interconnected small circles lay in arrays were observed in the periphery of the egg in both hemispheres (Figure 9 A,C), which were absent in *in vitro* matured eggs. The well organized structure of the ER-like reticular network of positive stainings observed in meiotically matured eggs were also detected in the cortical layer of ovulated unfertilized eggs (Figure 9 A,C). The signal in the cortex appeared to encircle nonstaining areas (Figure 9 C, arrow) and thus suggest that the $InsP_3$-dependent Ca^{2+} channel might be organized around individual cortical granules. This possibility must be further confirmed by electron-microscopic studies.

In the fertilized eggs, a redistribution of the $XInsP_3R$ took place: the sharp strong staining signals in the cortex detected before fertilization became fuzzy (Figure 9 B, D, arrow), suggesting the desruption of the $XInsP_3R$ consisting structure. In some batches of eggs, a broader subcortical layer was positively stained in addition to the cortical layer (Figure 10 B,E). Considering that fertilization is followed by cortical granule exocytosis, the desruption of the

structure surrounding the cortical granules is reasonable and this agrees well with the staining pattern of the $XInsP_3R$. The exact nature of the dramatic reorganization of the $XInsP_3R$ during oocyte maturation and egg activation remains largely unknown and which still awaits further studies by immuno-electron-microscopy. Since sperm enters in the animal half, and that Ca^{2+} wave occurs from the site where sperm enters, the physiological role of this subcellular localization may be closely related to the fertilization-induced transient Ca^{2+} increase.

Fuctional ablation of the $XInsP_3R$ by anti-sense oligonucleotide injection: its effects upon egg activation

To examine the role of the $XInsP_3R$ upon egg activation, the functional ablation of the receptor was performed by injection of antisense oligonucleotide. Recently, it has been shown that microinjection of antisense oligonucleotides into *Xenopus* oocytes leads to degradation of both endogenous and exogenous mRNA species and prevents synthesis of specific protein products (Melton 1985; Woolf et al., 1990). In the present study, phosphorothioate oligonucleotides derivatives which are more stable and are more effective in degrading mRNAs than unmodified oligonucleotideswere used (Woolf et al., 1990; Baker et al., 1990). Antisense oligonucleotides complementary to the 30 nucleotide sequences ofthe 5'flanking and translation start site (-8 to +14, 5'-AACTAGACATCTTGTCTGACATTGCTGCAG-3') or the corresponding sense oligonucleotides (5'-CTGCAGCAATGTCAGACAAGATGTCTAGTT-3') were microinjected into fully-grown stage VI oocytes. The oocytes microinjected with 20 nl of 1mg/ml were incubated for 4hours and were meiotically matured by overnight incubation with 5 ug/ml progesterone. Matured eggs were then assayed for their $InsP_3$ responsive "cortical contraction" that is thought to represent egg activation (Elinson, 1975; Palecek et al., 1978; Hara and Tydeman, 1979; Bement and Capco, 1989a). Figure 10A showed the experimental design.

Oocytes microinjected with sense- or antisense-oligonucleotides were meiotically matured *in*

vitro by the addition of progesterone, 2 nl of 100 μM $InsP_3$ was then microinjected into matured eggs, and cortical contraction (the movement of the margin toward the animal pole) was observed under a stereomicroscope. Figure 10 B (a,b) showed the normal response upon egg activation by $InsP_3$ in oligonucleotides uninjected eggs. Note that the pigmented area got smaller in activated eggs (Figure 10 B(b)) compared to the eggs before the $InsP_3$ injection (Figure 10 B (a)). While in control eggs microinjected with a buffer including 0.1 mM HEPES pH 7.8/10 μM EGTA, no cortical contraction was observed (data not shown). Eggs microinjected with antisense-oligonucleotides showed lower responses to $InsP_3$ compared to those microinjected with sense oligonucleotides. Figure 10 B (c),(d) showed typical response to $InsP_3$ in matured eggs microinjected with sense (Figure 10B (c)) or antisense oligonucleotides (Figure 10B (d)). Cortical contraction was observed in 25.2% of eggs microinjected with antisense-oligonucleotides, in 79.2% of eggs with sense-oligonucleotides (Figure 10C).

To examine whether the $XInsP_3R$ protein level is reduced by antisense oligonucleotide injection, immunoprecipitation of the $XInsP_3R$ in the crude membrane preparation of the *in vitro* matured eggs previously microinjected witholigonucleotides in the above assays was performed by using the polyclonal anti-$XInsP_3R$ antisera. The steady state level of the $XInsP_3R$ protein was measured by labelling the oocytes continuously for a period of 16 hours with [^{35}S]-methionine (Amersham), which were simultaneusly *in vitro* matured in the presence of progesterone (see experimental procedures). In this condition of labelling by [^{35}S]-methionine, the steady state level rather than the rate of synthesis can be measured. The *in vitro* matured eggs were collected and crude membrane fractions were prepared and were subjected to immunoprecipitation with anti-$XInsP_3R$. The band corresponding to $XInsP_3R$ was confirmed by competition with the *Trp*E-XIP3RC fusion protein (Figure 11A), and its intensity is much decreased in crude membrane fraction of eggs previously microinjected with antisense-oligonucleotides than with sense-oligonucleotides (Figure 11 B). Thus, the reduced level of the $XInsP_3R$ protein by the injection of the antisense oligonucleotides was considered to be responsible for the decreased reactivity to $InsP_3$ for egg activation.

Discussion

Phosphoinositide-signalling cascades are known to have pleiotropic effects on *Xenopus* development, such as maturation of oocytes (Sandberg et al., 1990), egg activation on fertilization (Busa et al., 1985; Busa and Nuctitelli, 1985; Kubota et al., 1987) and dorsal-ventral axis formation of embryo (Busa, 1988; Busa and Gimlich, 1989; Maslanshi et al., 1992). In the present study, we characterized the structure, function, expression profiles of the XInsP$_3$R during *Xenopus* oogenesis, which is likely to play key roles in these phosphoinositide signalling cascades. The spatio-temporal pattern of localization revealed by immunochemical studies together with the results of functional ablation by specific antisense oligonucleotides strongly suggest the involvement of the XInsP$_3$R in the formation and the propagation of Ca^{2+} wave upon egg activation.

Structural homology with the mouse InsP$_3$R

The nucleotide sequence of the XInsP$_3$R indicated a long open reading frame of 2693 amino acids (Muto, 1992). The nucleotide sequence surrounding the first initiation codon (ATG) of the open reading frame, GCAATGT agreed reasonably with the consensus sequence of the eukaryotic translational start site (CCA/GCCATG(G); Kozak, 1984). The deduced coding sequence extended from this initiation codon to an inframe termination codon (TAG) at nucleotide position 8079. The polyadenylation signal AATAAA (residues 8769-8774) was found 13 nucleotides upstream of the poly(dA) tract (data not shown, see Muto, 1991). The size of the XInsP$_3$R mRNA was estimated to be 10kb according to results of the Northern blot hybridization analysis using *Xenopus* oocytes total RNA with the cDNA probe (data not shown). This agreed with our assignment of the mRNA start site and the polyadenylation site. The *Xenopus* InsP3R deduced from the present cDNA sequence was composed of 2693 amino acids with a calculated molecular mass of 307 kDa. This also agreed with the result from the immunoblot analysis (Muto, 1992) in which a band slightly smaller than mouse InsP$_3$R (313kd, estimated by the cDNA sequence) was detected.The *Xenopus* InsP$_3$R deduced from the cDNA sequence was

remarkably similar to the mouse cerebellar $InsP_3R$, not only in the primary structure but also in proposed transmembrane topology (Figure 3). The sequence homology was high especially in the putative Ca^{2+} channel forming region, $InsP_3$-binding region and potential modulatory sites of the channel activity. The functional expression of the N-terminal domains of the $XInsP_3R$ in NG108-15 cell lines confirmed that the present clone showed $InsP_3$-binding activities with a high affinity (Figure 4b). Therefore, it was suggested that our cDNA molecule encodes the *Xenopus* $InsP_3R$ which has both an $InsP_3$-binding site and a Ca^{2+} channel activity. Sequence data indicated that the $XInsP_3R$ was likely to be modulated by phosphorylation by PKA and binding of ATP in a similar fashion to the mouse receptor, while the *Drosophila* $InsP_3R$ has no potential phosphorylation sites by PKA (Yoshikawa et al., 1992). The existence of consensus sequence for N-glycosylation in the loop between M5 and M6 at similar positions as the mouse $InsP_3R$ suggests that $XInsP_3R$ is possibly N-glycosylated similarly with the mouse $InsP_3R$ (Furuichi et al., 1989b; Maeda et al., 1988). This sugar chain containing domain lies in the lumen side of the ER and which agrees well with our model of 6 transmembrane domai. This is consistent with results of recent studies that identified and characterized an $InsP_3R$ in *Xenopus* oocytes, which have functional phosphorylation site(s) and glycosylation sit(s) localized near the C-terminal portion of the receptor (Parys et al., 1992). The overall homology of the *Xenopus* $InsP_3R$ with the mouse $InsP_3R$ revealed by the sequence study confirmed the crossreactivity of mouse $InsP_3R$ polyclonal antibody and monoclonal antibodies observed in the immunoblot analyses (Muto, 1991). The sequence of $XInsP_3R$ we presently cloned resembles an isoform of the mouse $InsP_3R$ that expresses dominantly in peripheral tissues, in which two regions (SI and S II; Nakagawa et al., 1991a) are deleted by the alternative splicing, and thus gave a lower molecular weight than the non-spliced isoform of the mouse $InsP_3R$.

Ca^{2+} wave and the $XInsP_3R$

We characterized the intracellular localization of the $InsP_3R$ in the *Xenopus* oocytes and eggs with specific antisera, anti-$XInsP_3R$ which were raised against GST-XIP3RC fusion protein (see

experimental procedure). Interestingly, the localization of the XInsP$_3$R in the cytoplasm in addition to the cortical layer throughout immature oocyte (Figure 8A-e)is consistent with the previous confocal studies that observed spiral waves of intracellular Ca^{2+} release following exogenouly expressed receptor activation (Lechleiter et al., 1991a,b) or spiral wave by injection of GTP-γ-S and InsP$_3$ that were indistinguishable from receptor-induced Ca^{2+} patterns (Leichleiter and Clapham, 1992). In the recent study by Parys et al.(1992) using an affinity purified polyclonal antibody directed against the C-terminal 15 amino acid of the rat brain InsP$_3$R showed a restricted localization to the cortical layer with essentially no stainings in the interior of the oocytes, whereas we could detected positive staining signals in the fully-grown oocytes, matured eggs and ovulated eggs. This difference could be explained by the nature of the epitope of their antibody, since they used a relatively less conserved region between the mouse and *Xenopus* as an epitope (Parys et al., 1992). The primary structure of the rat (Mignery et al., 1990) and *Xenopus* InsP$_3$Rs revealed that only 11 out of C-terminal 15 amino acids were identical between them. Therefore, the sensitivity and the specificity of the immunolocalization analysis by Parys et al. (1992) may not be high enough to detect all the InsP$_3$R in oocytes due to the possible poor cross reactivity of their antibody to the *Xenopus* InsP$_3$R. Moreover, the results of Parys (1992) indicating the absence of XInsP$_3$R in the interrior of the oocytes do not agree with the previously proposed model of the existence of excitable media throughout the whole oocytes (Leichleiter and Clapham, 1992). Therefore, the present result is the first fine immunolocalization data of the XInsP$_3$R in oocytes and eggs.

The localization of the XInsP$_3$R underwent a drastic reorganization during the course of maturation and fertilization. Enrichment of XInsP$_3$R to the cortex and the subcortical layer, and the formation of yolk free patches with dense stainings which localized to the animal half were characteristic of both the *in vitro* matured and ovulated eggs (Figure 8F, 9A). The staining signals enriched in the animal hemisphere (Figure 8, 9) in the oocytes and eggs suggested that the XInsP$_3$R plays an essential role in propagation of Ca^{2+} wave from the sperm entry site at the animal pole and travel through the entire eggs toward vegetal pole (Busa and Nuccitelli, 1985).

Yolk free patches with strong stainings appeared coincidently with germinal vesicle breakdown (GVBD) and with the disappearance of the strong staining signals in the perinuclear region observed in immatured fully-grown oocytes (unpublished data), suggests the stainings in the perinuclear region are the precursor. The yolk free patches seemed to move from the center of the oocytes to the animal half from immunocytochemical analyses of *in vitro* matured eggs treated with progesterone for different periods (unpublished data). In some batches of ovulated unfertilized eggs these patches were also observed. The exact nature of the patches observed is still obscure and is now under investigation by means of electron-microscopy. In ovulated eggs, enriched localizations to the cortex with a well organized arrays of stainings were observed. Previous studies demonstrated that during *Xenopus* oocyte maturation, the annulate lamellae disaggregates and the cortical endoplasmic reticulum is formed (Campanella and Andreuccetti, 1977; Gardiner and Grey, 1983; Campanella et al., 1984; Larabell and Chandler, 1988; Bement and Capco, 1989b). The cisternae of the cortical endoplamic reticulum form a distinctive junction with the plasma membrane in *Xenopus* eggs. It was hypothesized that these plasma membrane-ER junctions in Xenopus eggs are the sites that transduce extracellular events into intracellular calcium release during fertilization and activation of development. This hypothesis was based on the fact that these plasma membrane-ER junctions show a striking similarity with plasma membrane-sarcoplasmic reticulum junctions in muscle cells (Gardiner and Grey, 1983). In muscle cells, these are considered essential for triggering intracellulare Ca^{2+} release from the sarcoplasmic reticulum. The formation of the cortical endoplasmic reticulum coincides well with the onset of activation responsiveness to ionophore and pricking and was speculated to be the storage sites of the Ca^{2+} that propagate upon activation (Charbonneau and Grey, 1984). Since the $XInsP_3R$ showed no drastic change in the level of $XInsP_3R$ protein expression during maturation revealed by the immunoblot analyses (Figure 7) while a drastic change of localization was observed by immunocytochemical analyses (Figure 8,9), the redistribution of the $XInsP_3R$ was suggested strongly to contribute to the onset of the responsiveness to ionophore and pricking in the course of maturation. Moreover the present data of localization and redistribution of the

$InsP_3R$ had striking similarities with the formation of cortical endoplasmic reticulum, supports their hypothesis and suggested strongly that the $XInsP_3R$ might localized in the cortical ER in the *Xenopus* matured oocytes and eggs which agrees well with a transient Ca^{2+} release upon fertilization and the propagation of Ca^{2+} wave from the sperm entry site at the animal pole and travels through the entire eggs toward vegital pole (Busa and Nuccitelli, 1985).

Another striking feature is that the positive signals seemed to surround the cortical granules correlates well with the hypothesis of an involvement of the cortical endoplasmic reticulum in the propagation of cortical granule exocytosis favored by many electron microscopic studies of the cortical endoplasmic reticulum (Campanella et al., 1984; Larabell and Chandler, 1988). Surprisingly, the elaborate structure at the cortex and subcortical region observed in ovulated unfertilized eggs was disrupted by the fertilization (Figure 9) and a dramatic reorganization of the $XInsP_3R$ localization occured. The increase of intracellular Ca^{2+} at fertilization is followed by the wave of cortical granule exocytosis, and previous studies observed an striking reorganization of the ER at fertilization of sea urchin (Terasaki and Jaffe, 1991) and an altered ryanodine-sensitive Ca^{2+} release channel (ryanodine receptor; RyR) staining pattern upon sea urchin egg activation (Macpherson et al., 1992). These findings observed in sea urchin eggs are relevant to the results of the present studies indicating that the localization of the $XInsP_3R$ is reorganized putatively by the cortical granule exocytosis upon the fertilization, since the $XInsP_3R$ as well as the sea urchin ryanodine receptor are localized to the cortical endoplasmic reticulum surrounding the cortical granule. These conserved localization and reorganization of the intracellular Ca^{2+} channels in eggs may suggest their important roles upon fertilization. Interestingly, despite similar reorganizations of the ER and intracellular Ca^{2+} channels (e.g. $InsP_3R$ and RyR) are observed at fertilizations of sea urchin and *Xenopus*, no RyR existed in *Xenopus* oocytes/eggs (Parys et al., 1992) in consistent with the results obtained in hamster (Miyazaki et al., 1992). These results imply the specific role of the $InsP_3R$ in the fertilization of vertebrates, which is likely to be different from that of sea urchin. Then, what is the difference? In the sea urchin, activation of the $InsP_3R$ is not sufficient for causing Ca^{2+} wave. Both the

$InsP_3$-dependent and -independent release of Ca^{2+} from separate intracellular stores conribute to the Ca^{2+} wave during fertilization (Rakow and Shen, 1990). RyR is present in sea urchin eggs and involved in egg activation (Rakow and Shen, 1990; Fujiwara et al., 1990) possibly through mediating CICR. Our group, however, demonstrated that the IICR plays a predominant role not only in the formation but also in the propagation of Ca^{2+} wave in hamster eggs (Miyazaki et al., 1992) by injecting the 18A10 monoclonal antibody which blocks channel activity of $InsP_3R$ (Nakade et al., 1991). The localization of $XInsP_3R$ to the cytoplasm throughout the egg as well as to the cortex in both the hamster (Miyazaki et al., 1992) and *Xenopus* could explain that Ca^{2+}-sensitized IICR generates Ca^{2+} wave and Ca^{2+} ossilation, indicating that the vertebrate-specific role of $InsP_3$ upon fertilization (discussed later).

Functional ablation of the $InsP_3R$ and block of egg activations

Microinjection of antisense oligonucleotides complementary to the cloned $XInsP_3R$ mRNA blocked $InsP_3$-induced activation of *in vitro* matured eggs. In vertebrates, matured eggs are arrested at the second meiotic metaphase by the cytostatic factor (CSF), now known to be the c-*mos* proto-oncogene product (Mos, Sagata et al., 1989). I observed the proteolysis of c-mos upon $InsP_3$-induced activation of *in vitro* matured eggs, after a time lag of about 40 minutes after $InsP_3$ injection (unpublished data). These results showed that the $XInsP_3R$ cDNA cloned in this study is important for the egg activation possibly through the IICR, and the transient Ca^{2+} rise upon egg activation is followed by a cascade of metabolic activation, such as activation of phosphatase and activation of the ubiquitin pathway which in turn inactivate maturation promoting factor (MPF) and c-mos (or CSF) (Lorca et al., 1991; Glotzer et al., 1991, Watanabe et al., 1991), thus finally release the meiotic arrest and enable the egg to enter the succesive embryonic cell cycle. The results of immunolocalization in the present study and ablation

experiments of the $InsP_3R$ function by the antisense oligonucleotide (present study) as well as a monoclonal antibody (Miyazaki et al., 1992) suggest that the $InsP_3R$ plays an essential role upon egg activation and fertilization of various animals through mediating IICR propagation of Ca^{2+} wave and thereby activating subsequent metabolic chain reactions. The present data well explains the results presentented by Lechechechechleiter and Clapham (1992) for intracellar Ca^{2+} signalling and also the results by Miyazaki et al. (1992) and DeLisle and Welsh (1992) demonstrating that IICR plays a crucial role in both sperm-mediated Ca^{2+} wave initiation and propagation in hamster and frog eggs, respectively. It is proposed that Ca^{2+} wave propagation is directed by IICR, and is modulated by the cytoplasmic concentration and diffusion of Ca^{2+} (Lechleiter and Clapham, 1992) depending on a bell-shaped calcium-response curve of the $InsP_3R$ (Bezprozvanny et al., 1991; Finch et al., 1991). I am in process to characterize the mechanism Ca^{2+} transients further promotes egg activation and early embryonic development.

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Figure 1. Functional Domains and Restriction Map of the *Xenopus* $InsP_3$ Receptor cDNA clones.

Open box represent protein coding region, *solid box* is the transmembrane domain.
Schematic representation of the overlapping $XInsP_3R$ cDNA clones is indicated.

Restriction Map and Schematic Representation of the Xenopus IP3-R cDNA Clones

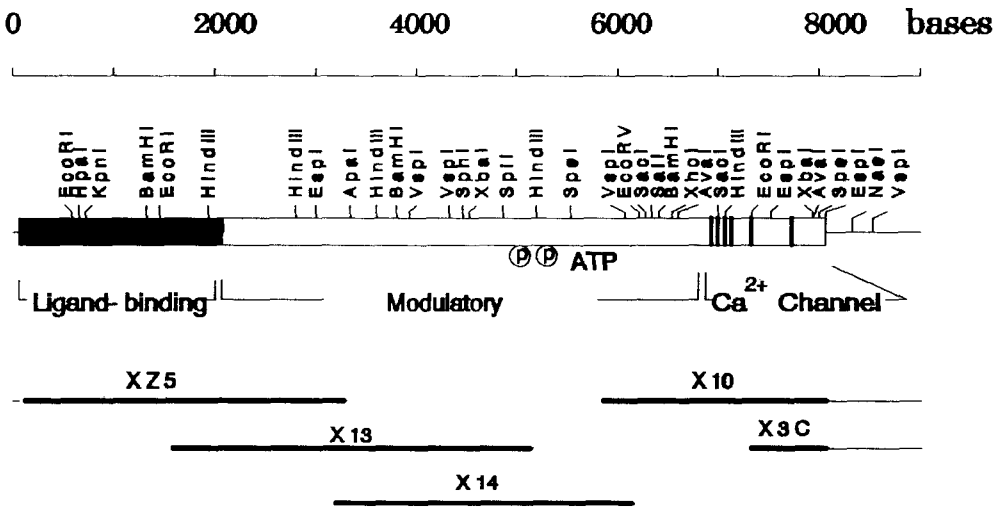


Figure 2. Alignment of the Deduced Amino Acid Sequence of the *Xenopus*, Mouse and *Drosophila melanogaster*

The amino sequences of the *Xenopus* (top lane), mouse (middle lane) and *Drosophila melanogaster* (bottom lane) are shown. Identical amino acids with in mouse or *Drosophila* $InsP_3R$ are indicate by double dots. *Dashes* indicate gaps (deletions or insertions) introduced to maximize homology. *Asterisks* indicate amino acids identical with the ryanodine receptor as previously described (Furuichi et al., 1989). Sequence data for mouse $InsP_3R$ and *Drosophila* are from Furuichi et al., (1989) and Yoshikawa et al., (1992). The hydrophobic segments with predicted secondary structure are marked with *solid lines*; the termini of each segment are tentatively assigned on the basis of the hydrophobicity profile and the amino-acid sequence. Positions corresponding to the consensus ATP binding site is marked with *broken double underlines* (Wierenga and Greengard, 1983). *Triangles* represent possible phosphorylation sites for protein kinase A. SI and SII are regions that can be deleted by alternative splicing from the mouse sequence (Nakagawa et al., 1991a).

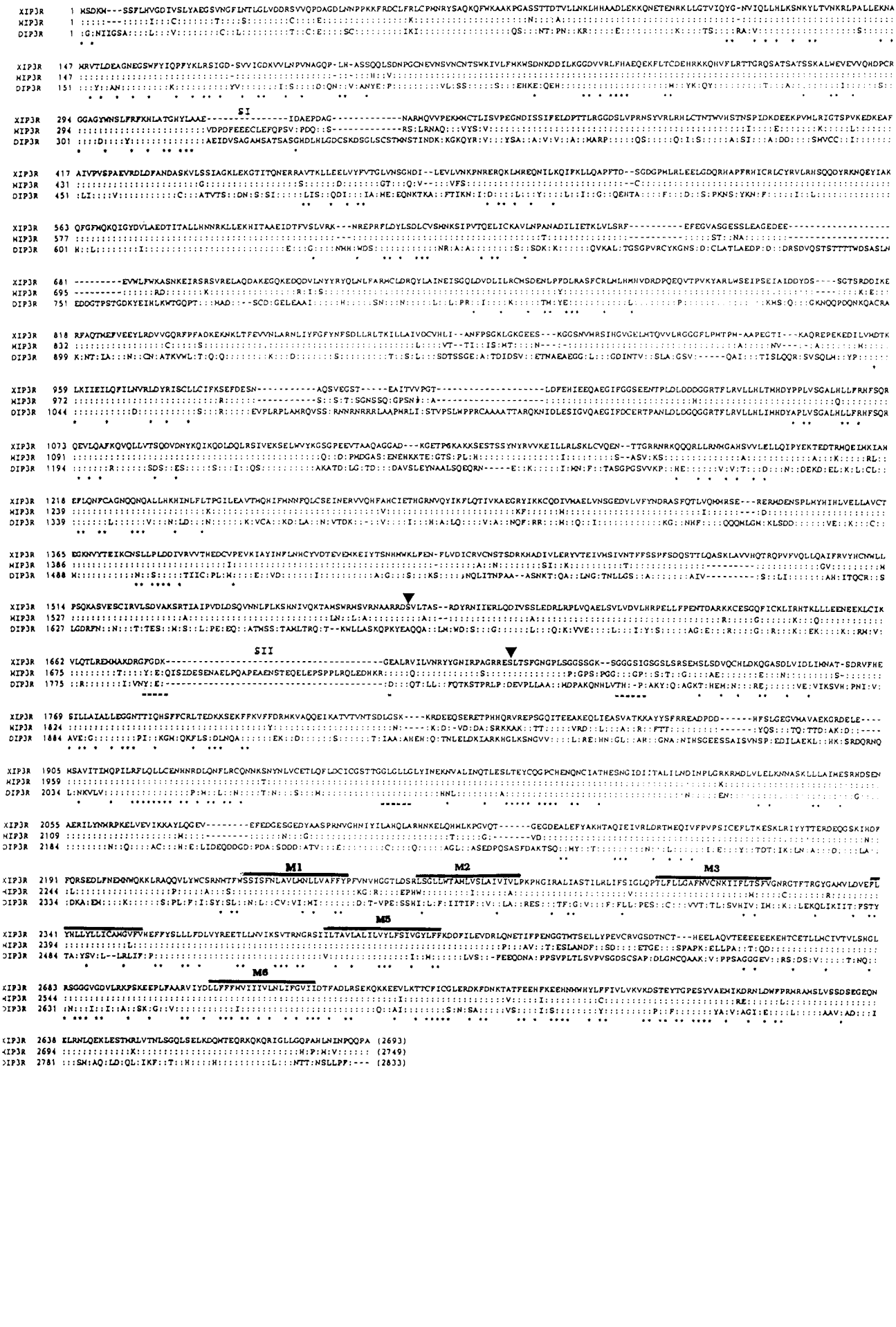

SI
SII
M1
M2
M3
M5
M6

Figure 3. Structure of the expression plasmid pβactX6K

cDNA sequence coding N-terminal 1990 amino acids of Xenopus $InsP_3R$ was subcloned under the contro of chiken β-actin promotor folowed by the SV40 poly A addition site.

pβacts'XIP3R (5'-6kb) plasmid

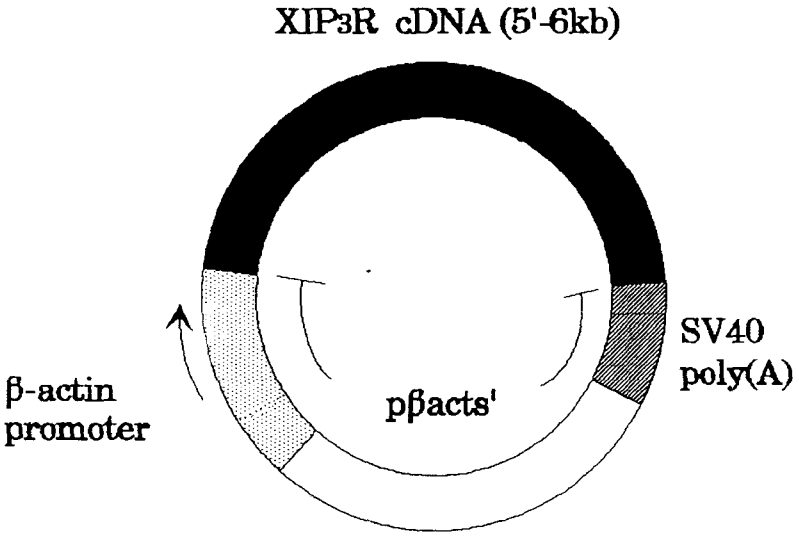
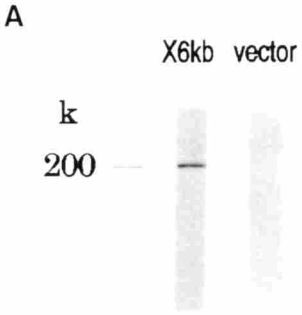


Figure 4. Immunoblot Analysis and Inhibition of Specific [^{3}H]InsP_3 binding to the Cytosol Proteins of pβactX6K Transfected NG108-15 Cells by Various Inositol Phosphates

A. Lane 1, 10 μg of the cytosol fractions from pβactX6K transfected cells; lane 2, 10 μg of the cytosol fraction from vector pβactsN transfected NG108-15 cells were loaded onto 5% SDS-PAGE and immunoblot with the anti-mouse InsP_3R monoclonal antibody 4C11 (Maeda et al., 1989) and detected with the enhanced chemiluminescence detection system (Amersham). Molecular mass at 200 kDa is shown.

B. Binding of [^{3}H]InsP_3 was measured at a concentration of 20 nM. Inhibition by Ins(1,4,5)P_3 (*filled circles*), Ins(2,4,5)P_3 (*open circles*), Ins(1,3,4,5)P_4 (*open squares*), Ins(1,4)P_2 (*open diamonds*), Ins(1,4)P_2 (*open squares*) and Ins(1)P_1 (*open triangles*) were shown.
Inhibition data for Ins(1,4,5)P_3, Ins(2,4,5)P_3, and Ins(1,3,4,5)P_4 were the average of 3 experiments.


A
X6kb vector
k
200

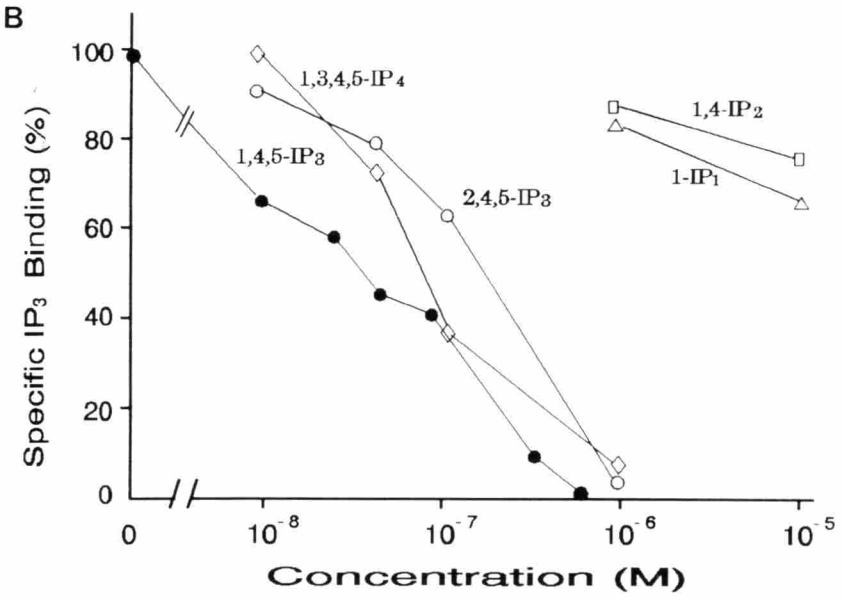

B
Specific IP3 Binding (%)
100
80
60
40
20
0
1,3,4,5-IP4
1,4,5-IP3
2,4,5-IP3
1,4-IP2
1-IP1
0
10-8
10-7
10-6
10-5
Concentration (M)

Figure 5. RNase Protection Analyses of Expression of $InsP_3R$ during Oogenesis.

Total RNA equivalent to five oocytes of stage I-VI (according to Dumont 1972) and *in vitro* matured (lane VI+PG) resppectively were analyzed by RNase protection for $XInsP_3R$ transcripts and subjected to 8M urea-5%PAGE. *Arrows* shows the position of the probe and the protected band.

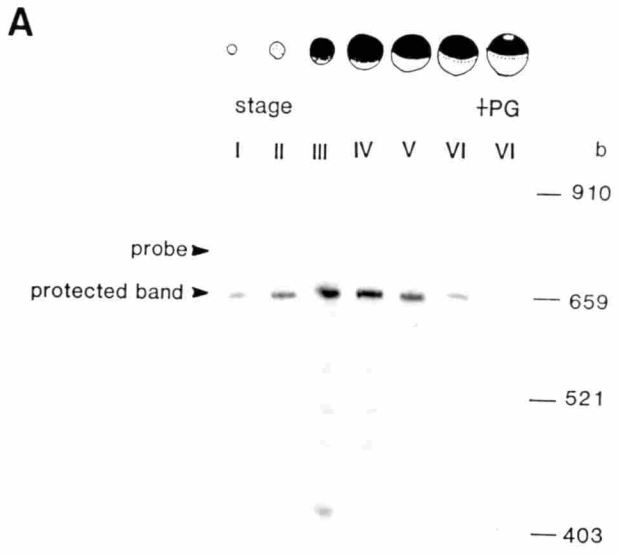
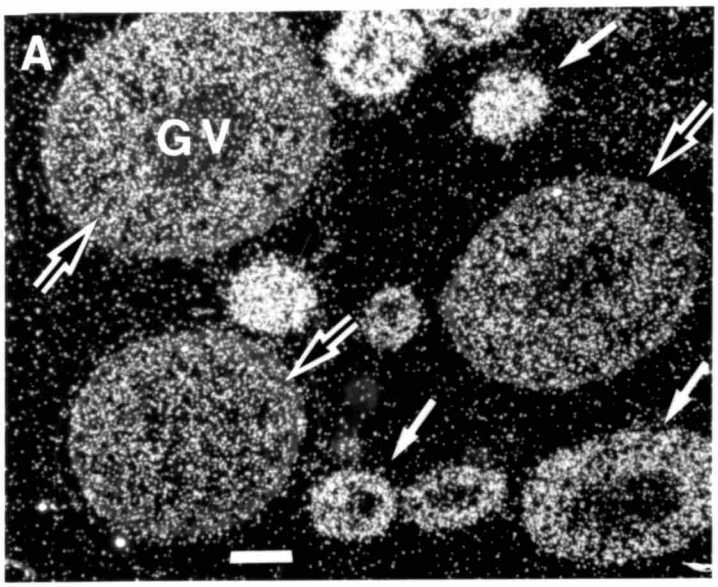

A
stage
+PG
I
II
III
IV
V
VI
VI
b
910
probe
protected band
659
521
403

Figure 6. Detection of $XInsP_3R$ in *Xenopus* Oocytes by *in Situ* Hybridization

Stage 1-IV (according to Dumont, 1972) albino oocytes were hybridized with antisense or sense strand RNA probe of $XInsP_3R$. Oocytes from albino frogs were used because the pigment granules that mark the animal hemisphere of wild-type oocytes are almost indistinguishable from autoradiograph grains. **A**, antisense strand $XInsP_3R$ RNA probe; Strong signals were detected in stage I,II oocytes (*filled arrows*). The signals decreased in stage III-IV (*open arrows*), and were weak in fully grown oocytes of stage V-VI (data not shown) possibly due to a large increase in the volume of the oocytes accompanied with growth. Controls with sense strand $XInsP_3R$ RNA probe (**S**) do not show any autoradiograph signal above background. The nucleus or germinal vesicle (*GV*) is seen as a black whole. *Scale bar*, 200 μm.


A
GV

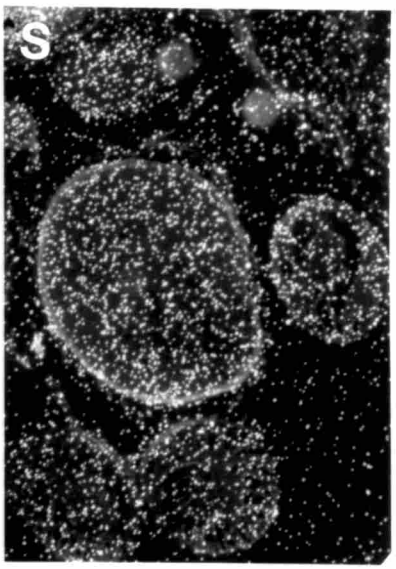

S

Figure 7. Immunoblot Analysis of Expression of $InsP_3R$ during Oogenesis.

Crude membrane protein from oocytes of stage I-VI was prepared and protein equivalent to one oocyte was loaded onto 5% SDS-PAGE and immunostained with 4C11 monoclonal antibody against mouse $InsP_3R$ (Maeda et al., 1989), immunodetection was processed with Vectastain ABC rat IgG kit according to the manufacturer's protocol.

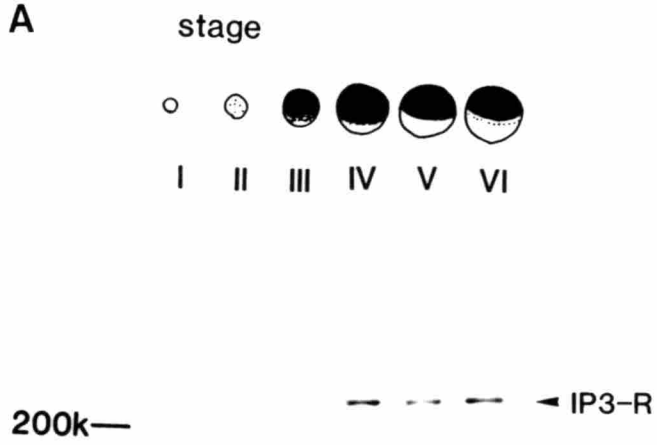
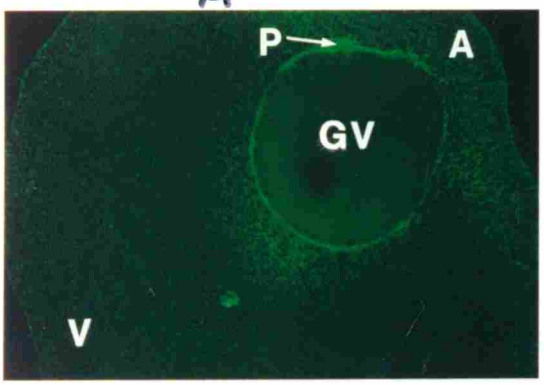

A
stage
I
II
III
IV
V
VI
200k—
IP3-R

Figure 8. Immunofluorescent Localization of $XInsP_3R$ in Oocytes and *In Vitro* Matrure Eggs of *Xenopus* Stained with Anti-$XInsP_3R$ Polyclonal Antibody

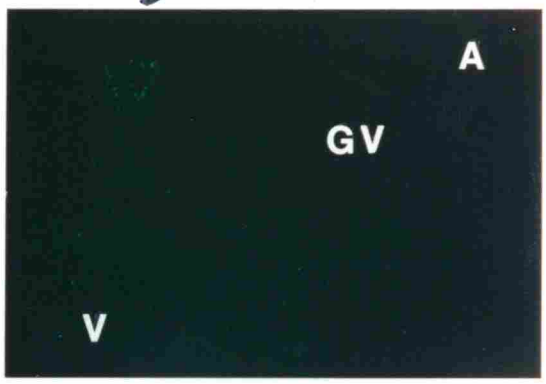
A, Staining in fully-grown stage VI oocyte demonstrate the localization of $XInsP_3R$ in the cortex, interior cytoplasm especially enriched in the animal hemisphere and the perinuclear region. **A.** *Scale bar*, 200 μm applied for **A, B. B**, Preimmune serum staining of an adjacent section of **A.** as control. No stainings are observed. **C-E**, Higher magnification of the stage VI oocytes seen in **A**, *scale bar*, 20 μm. **C**, Enriched localization of $XInsP_3R$ in the animal hemisphere of stage VI immatured oocytes. Strong staining signals are observed in yolk free cytoplasm. **D**, Strong signals observed in the perinuclear region. **E**, Signals observed in the vegetal hemisphere. **F**, Stainings in *In vitro* matured egg demonstrate localization of $XInsP_3R$ in the cortical region, york free patches in the animal hemisphere with weak stainings in the interrior deep cytoplasm.
A, animal hemisphere; **V**, vegetal hemisphere; *GV*, germinal vesicle; *Y*, Yolk platelet; *CG*, cortical granules.

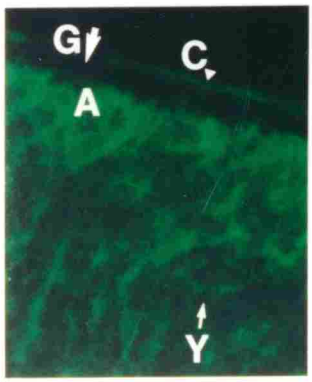
Stage VI oocyte

A

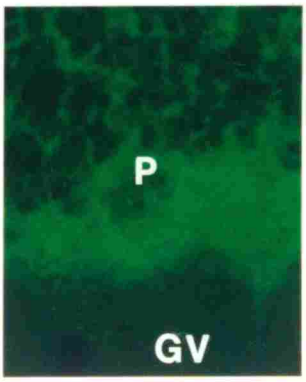


B

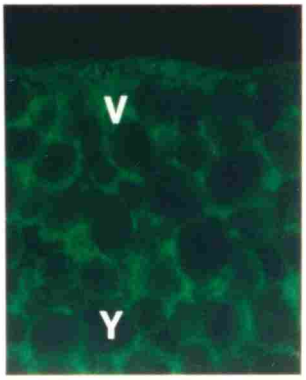




C



D



E

F In vitro matured egg

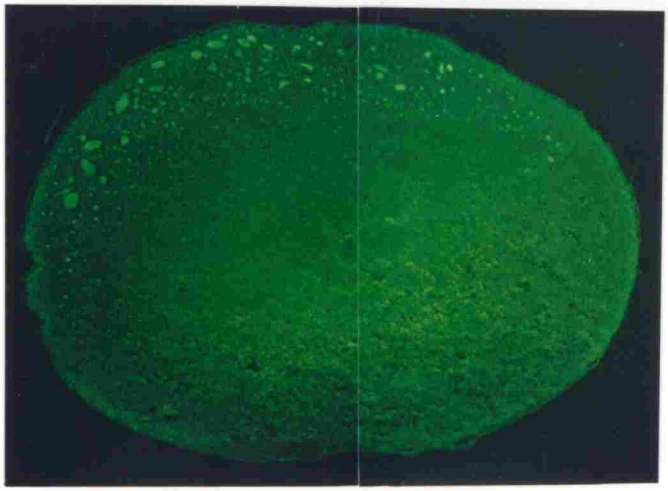


Figure 9. Immunofluorescent Localization of $XInsP_3R$ in Ovulated Unfertilized Eggs and Fertilized Eggs Stained with Anti-$XInsP_3R$ Polyclonal Antibody.

A. Stainings in ovulated unfertilized egg demonstrate enriched localization of $XInsP_3R$ in the cortex, and a broad cortical layer in both hemispheres. Stainings in the cytoplasm are localized to the animal half. *Scale bar*, 200 μm. **B.** Stainings in fertilized eggs demonstrate a desruption of the well organized cortical localization of $XInsP_3R$ observed in the ovulated unfertilized egg. *Scale bar*, 200 μm. **C.** Higher magnification of an ovulated unfertilized egg. Sharp staining signals lie in arrays in the cortex which seemed to surround the cortical granules (*Arrow*), and the formation of a ER-like tubular network in the subcortical layer are observed. Nonspecific stainings in the jelly layer are also observed in preimmune serum (data not shown, see **E**). *Scale bar*, 20 μm, applied for **C** and **D**. **D.** Higher magnification of a fertilized egg. The well organized stainings in unfertilized eggs are desrupted and a fuzzy staining pattern is obserbed (*arrow*). **E.** Preimmune serum staing of an adjacent section of **D**, nonspecific stainings in the jelly layer are observed sometimes.

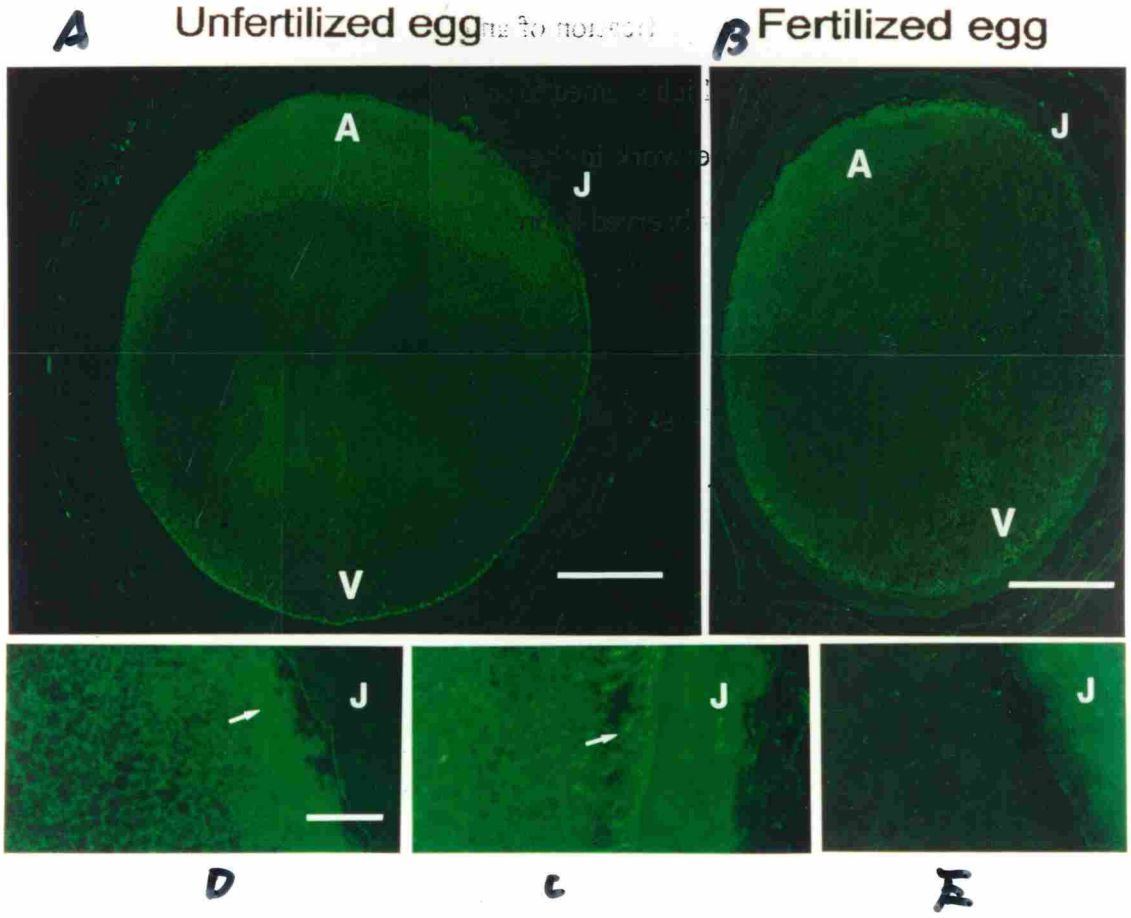

A
Unfertilized egg
B
Fertilized egg
A
J
V
J
A
V
J
J
J
D
C
E

Figure 10. Effects of Antisense Oligonucleotides upon Egg Activation

A. The experimental design is shown. The oocytes were obtained as described in experimental procedure. Antisense oligonucleotides complementary to the 30 nucleotide sequences ofthe 5'flanking and translation start site (-8 to +14, 5'-AACTAGACATCTTGTCTGACATTGCTGCAG-3') or the corresponding sense oligonucleotides (5'-CTGCAGCAATGTCAGACAAGATGTCTAGTT-3') were microinjected into fully-grown stage VI oocytes, and *in vitro* matured in the presence or 5 μg/ml profesterone, and then activated by microinjection of 2 nl of 100 μM $InsP_3$ and were assayed for cortical contraction. **B.** Responsiveness of uninjected, sense oligonucleotides injected, and antisense oligonucleotides injected eggs to $InsP_3$ injection. The pictures show the equatorial view of the eggs ten minutes after injection of $InsP_3$. Typical results were shown. *Scale bar*, 1 mm. **a**, Oligonucleotides uninjected eggs before $InsP_3$ induced activation. **b**, Olligonucleotides uninjected eggs activated by $InsP_3$ injection. Note that the decreased pigmented area in the animal hemisphere. **c**, Response of sense oligonucleotides microinjected eggs to $InsP_3$ injection. Cortical contraction occured. **d**, Response of antisense oligonucleotides microinjected eggs to $InsP_3$. Note that cortical contraction is inhibited. **C** Percentage of eggs undergo cortical contraction upon $InsP_3$ injection (numbers of eggs underwent cortical contraction/numbers of eggs injected with $InsP_3$).

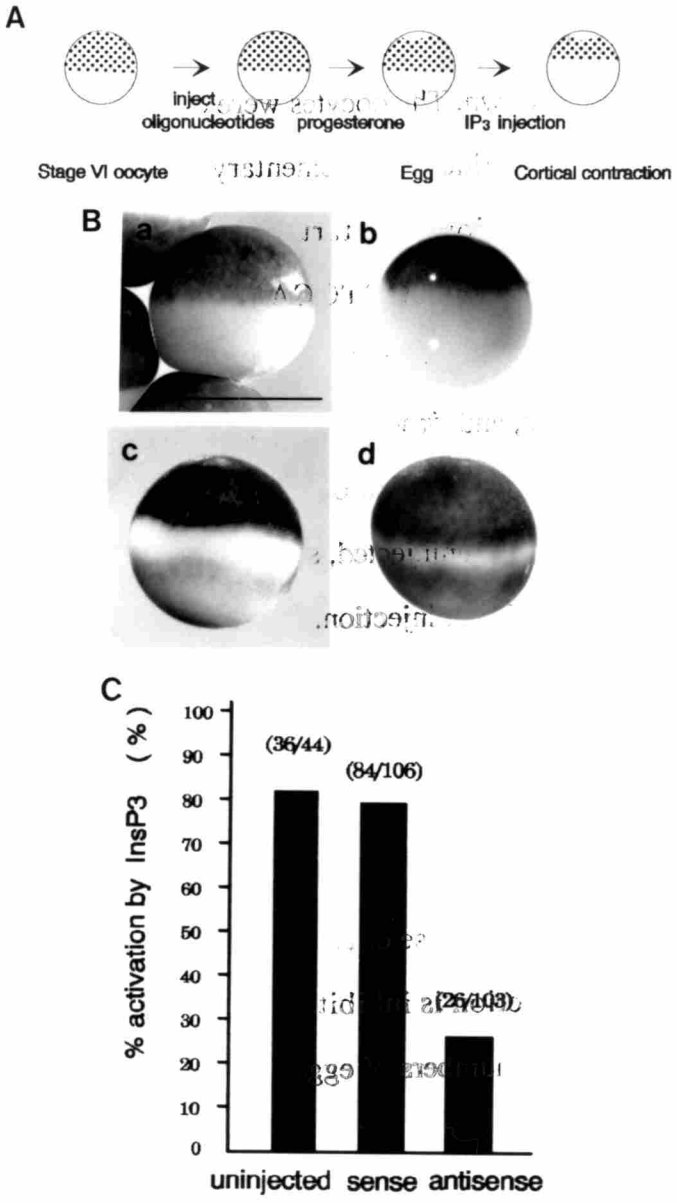
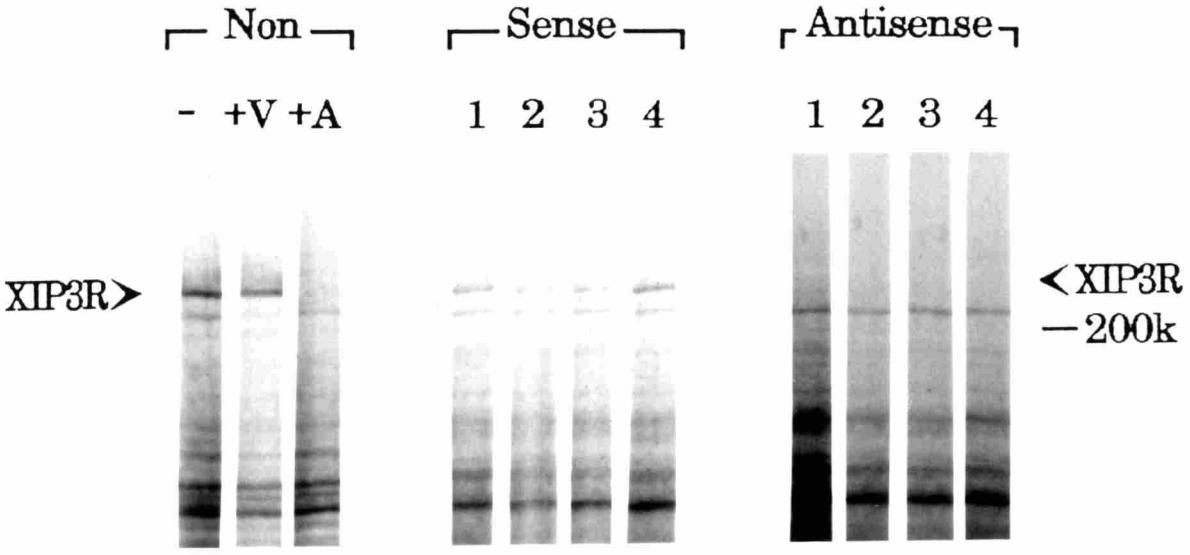

A
inject
oligonucleotides
progesterone
IP3 injection
Stage VI oocyte
Egg
Cortical contraction
B
a
b
c
d
C
% activation by InsP3 (%)
100
90
80
70
60
50
40
30
20
10
0
(36/44)
(84/106)
(26/103)
uninjected
sense
antisense

Figure 11. Immunoprecipitation Analysis of Crude Membrane Proteins in Oligonucleotides Microinjected Matured Eggs

Immunoprecipitation of crude membrane protein extracted from [^{35}S]Methionine labelled *in vitro* matured eggs by using anti-XInsP$_3$R polyclonal antibody. Lanes are as follows, non-injected: -, with the absence of competiting antigen; +V, with the presence of purified *Trp*E protein (0.4 mg/ml) as control; +antigen, with the presence of purified GST-XIP3RC fusion protein (0.4 mg/ml) as competiting antigen; antisense: antisense oligonucleotides microinjected eggs; sense: sense oligonucleotides microinjected eggs. Crude membrane proteins were prepared from batches of 3 *in vitro* matured eggs and immunoprecipitations were done with proteins equivalent to one *in vitro* matured egg. After immunoprecipitation with anti-XInsP$_3$R polyclonal antibody, with or without the presence of competiting antigen, immunoprecipitated proteins were loaded onto 5% SDS-PAGE, fixed, dried, and fluorographed as described in experimental procedures.


Non
Sense
Antisense
- +V +A
1 2 3 4
1 2 3 4
XIP3R
XIP3R
200k