in circulating B cells (*21*), and neural cells expressing $p75^{NGFR}$ survive in media containing serum (Fig. 2). The mechanism by which unbound $p75^{NGFR}$ or other members of this receptor superfamily lead to neural cell death is unknown. However, the structural and functional relation between $p75^{NGFR}$ and TNFR I and II suggests that they may have similar mechanisms of action.

The highest level of expression of $p75^{NGFR}$ in the central nervous system occurs in cholinergic neurons of the nucleus basalis of Meynert, the cells most severely affected in Alzheimer's disease. These cells continue to express normal (*22*) or supranormal (*23*) amounts of $p75^{NGFR}$ mRNA and protein during the neuronal degeneration associated with Alzheimer's disease. In contrast, cholinergic cells of the brainstem that resemble those of the nucleus basalis morphologically, but do not express $p75^{NGFR}$ (*24*), do not degenerate in Alzheimer's disease (*25*).

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Redundant Mechanisms of Calcium-Induced Calcium Release Underlying Calcium Waves During Fertilization of Sea Urchin Eggs

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Propagating Ca^{2+} waves are a characteristic feature of Ca^{2+}-linked signal transduction pathways. Intracellular Ca^{2+} waves are formed by regenerative stimulation of Ca^{2+} release from intracellular stores by Ca^{2+} itself. Mechanisms that rely on either inositol trisphosphate or ryanodine receptor channels have been proposed to account for Ca^{2+} waves in various cell types. Both channel types contributed to the Ca^{2+} wave during fertilization of sea urchin eggs. Alternative mechanisms of Ca^{2+} release imply redundancy but may also allow for modulation and diversity in the generation of Ca^{2+} waves.

Transient increases in the concentration of calcium ions ($[Ca^{2+}]_i$) act as cell signals. In general, the signal shows spatial and temporal inhomogeneity and takes the form of waves or oscillations within the cell (*1*). Several mechanisms have been proposed to account for regenerative Ca^{2+} release (*2*). Release of Ca^{2+} from internal stores can be stimulated by an increase in $[Ca^{2+}]_i$; this process is termed Ca^{2+}-induced Ca^{2+} release (CICR) (*3*). This Ca^{2+} release appears to be mediated by Ca^{2+} channels in the endoplasmic reticulum (ER) that are sensitive to cytoplasmic agonists, to $[Ca^{2+}]_i$, and to the amount of Ca^{2+} in the lumen of the ER (*4*). Two closely related Ca^{2+} channels with these properties are the inositol trisphosphate (IP_3) receptor (IP_3R) (5) and the

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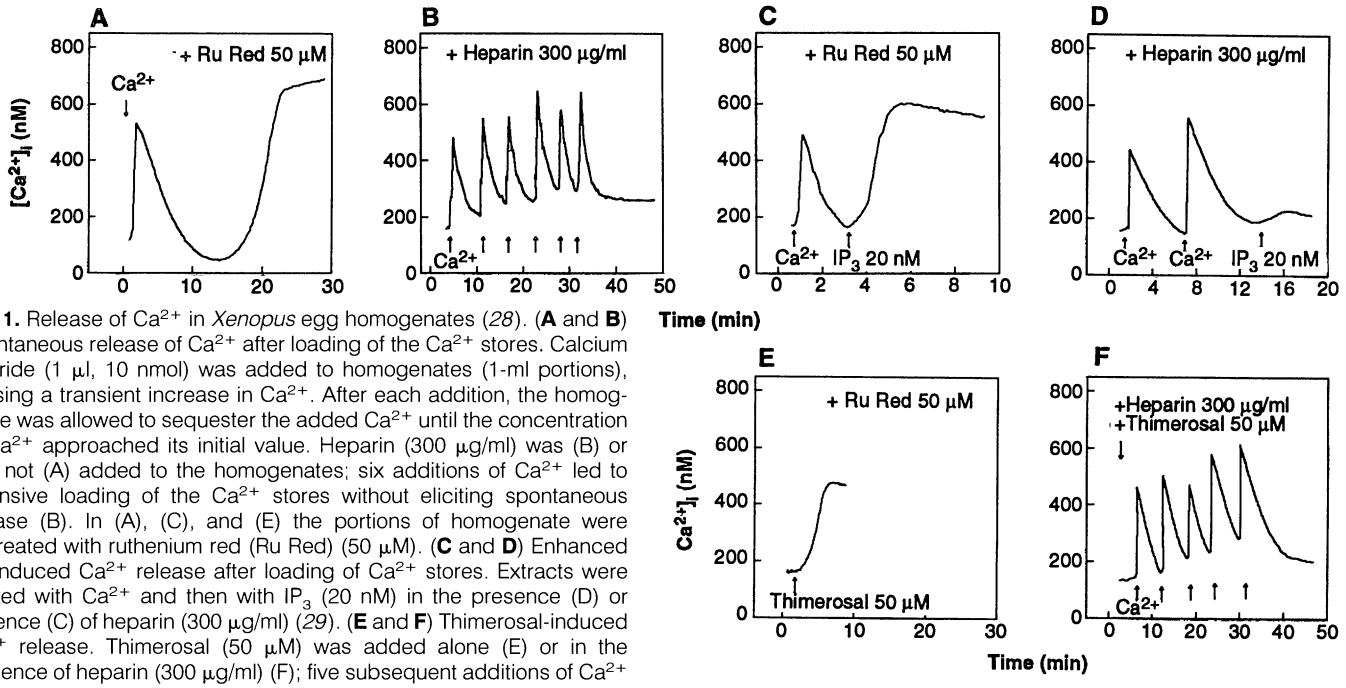

Fig. 1. Release of Ca^{2+} in *Xenopus* egg homogenates (*28*). (**A** and **B**) Spontaneous release of Ca^{2+} after loading of the Ca^{2+} stores. Calcium chloride (1 μl, 10 nmol) was added to homogenates (1-ml portions), causing a transient increase in Ca^{2+}. After each addition, the homogenate was allowed to sequester the added Ca^{2+} until the concentration of Ca^{2+} approached its initial value. Heparin (300 μg/ml) was (B) or was not (A) added to the homogenates; six additions of Ca^{2+} led to extensive loading of the Ca^{2+} stores without eliciting spontaneous release (B). In (A), (C), and (E) the portions of homogenate were pretreated with ruthenium red (Ru Red) (50 μM). (**C** and **D**) Enhanced IP_3-induced Ca^{2+} release after loading of Ca^{2+} stores. Extracts were treated with Ca^{2+} and then with IP_3 (20 nM) in the presence (D) or absence (C) of heparin (300 μg/ml) (*29*). (**E** and **F**) Thimerosal-induced Ca^{2+} release. Thimerosal (50 μM) was added alone (E) or in the presence of heparin (300 μg/ml) (F); five subsequent additions of Ca^{2+} failed to elicit an overload response (F).

ryanodine receptor (RyR) (*6*), which appears to be regulated by cyclic adenosine diphosphate–ribose (cADPR) (*7, 8*).

Activating eggs have prominent spatial and temporal Ca^{2+} waves (*9*). We have pharmacologically dissected the waves of $[Ca^{2+}]_i$ in both intact eggs and homogenates. We found that in frog and sea urchin eggs Ca^{2+} waves indeed have the same basis in CICR, but the mechanisms that have been put forward to account individually for Ca^{2+} wave propagation in various cell types were found to be acting together in sea urchin eggs. Simple egg homogenates sequester Ca^{2+} into vesicular stores and release it in response to Ca^{2+}-releasing messengers (*7, 10*). We used homogenates to define the basic properties of CICR and to identify the release channel responsible.

Adenosine triphosphate (ATP)–dependent sequestration of added Ca^{2+} (*7*) overloaded the Ca^{2+} stores and led to spontaneous release of Ca^{2+} in frog egg homogenates after sequestration (Fig. 1A). Frog egg homogenates treated in this way were 50 times as sensitive to IP_3 as untreated homogenates (Fig. 1, C and D) because

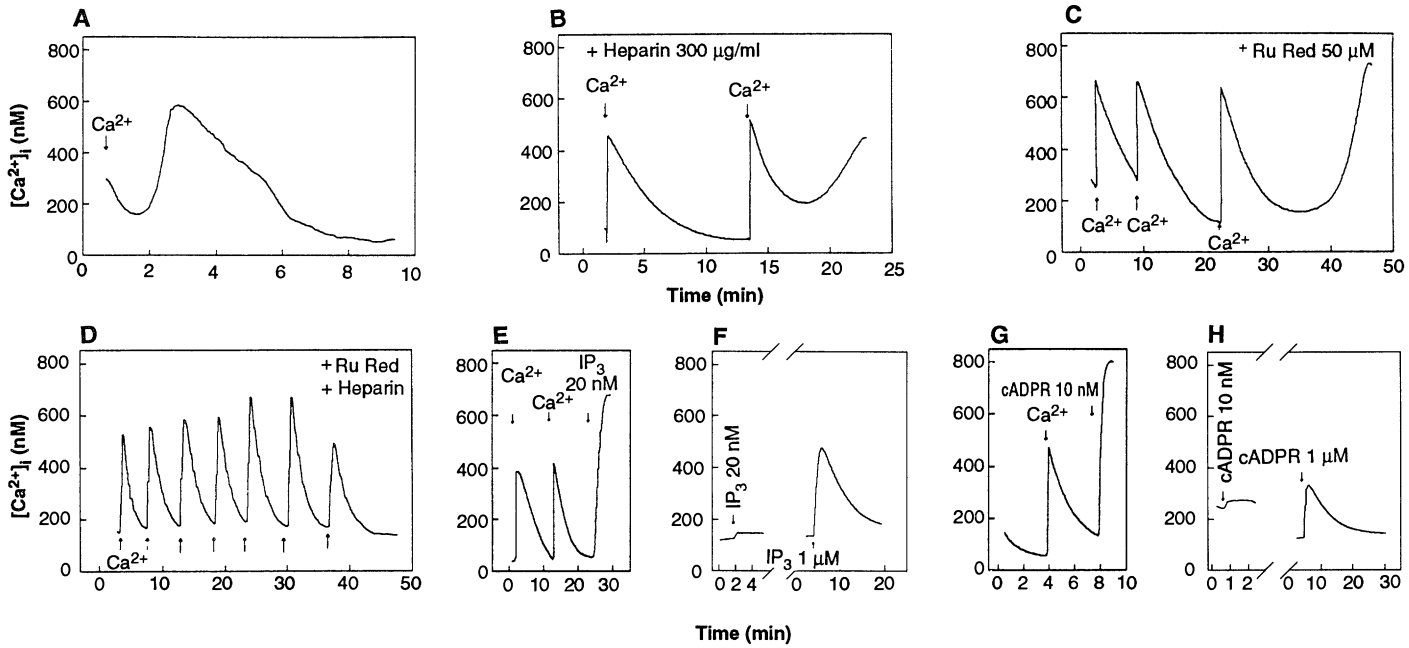

Fig. 2. Release of Ca^{2+} in sea urchin egg homogenates. (**A** through **D**) Spontaneous Ca^{2+} release after the addition of Ca^{2+}. Portions of Ca^{2+} were added to a sea urchin egg homogenate (as in Fig. 1) without other additions (A) or in the presence of heparin (300 μg/ml) (B), or ruthenium red (50 μM) (C), or both agents together (D). (**E** through **H**) After Ca^{2+} addition, 20 nM IP_3 (E) or 10 nM cADPR (H) was added. Homogenates without any added Ca^{2+} were also exposed to IP_3 (F and G) or to cADPR (H) at the indicated concentrations. Methods were similar to those described in (*7, 10*).

maximal Ca^{2+} release in untreated homogenates required 1.1 ± 0.2 μM (mean ± SEM, n = 4) IP_3. The spontaneous release may result from the sensitization of the preparation to IP_3 in the homogenate (*11*) because spontaneous release is blocked by the IP_3R antagonist heparin (Fig. 1B). The IP_3R is also sensitized by treatment with oxidizing sulfhydryl reagents (*12, 13*). Addition of the sulfhydryl reagent thimerosal to a frog egg homogenate led to a large but initially slow Ca^{2+} release, the kinetics of which were similar to those of the Ca^{2+} release prompted by the addition of Ca^{2+} (Fig. 1E). The effect of thimerosal was also enhanced by Ca^{2+} loading (*14*). This effect appeared to result from sensitization of IP_3R because thimerosal caused no Ca^{2+} release in the presence of heparin, even after repeated additions of Ca^{2+} (Fig. 1F).

We also added the RyR agonists cADPR, caffeine, and ryanodine to frog egg homogenates. They did not cause Ca^{2+} release, nor did pretreatment of homogenate portions with the RyR antagonist ruthenium red (*14*) affect CICR (Fig. 1, A, C, and E). Egg homogenates therefore resemble intact frog oocytes in showing regenerative Ca^{2+} release through IP_3R (*15*).

Sea urchin egg homogenates behaved differently. They too showed spontaneous release of Ca^{2+} and sensitization to IP_3 after Ca^{2+} loading (Fig. 2, A and E), but the spontaneous release was not abolished by heparin (Fig. 2B). This spontaneous release might occur through RyR (*16*); caffeine and ryanodine caused Ca^{2+} release in sea urchin eggs (*7, 17*) and in homogenates (*7*), whereas ruthenium red blocked caffeine-induced Ca^{2+} release. Spontaneous release after addition of Ca^{2+} was detected in the presence of ruthenium red (Fig. 2C). However, spontaneous release was abolished if both ruthenium red and heparin were present (Fig. 2D), implying that both RyR and IP_3R contribute to CICR in sea urchin egg homogenates. Loading stores in the sea urchin egg homogenate with Ca^{2+} sensitized IP_3R to IP_3 and RyR to cADPR by a factor of 50 or more (Fig. 2, E through H). A similar sensitization of RyR occurred with caffeine, whereby loading stores with Ca^{2+} allowed a maximal Ca^{2+} release at 0.2 to 0.5 mM caffeine (*14*), whereas 10 mM caffeine gave a maximal response in untreated homogenates (Table 1) (*7*). It has been suggested that thimerosal acts solely on IP_3R (*12*) and also solely on RyR (*18*). Thimerosal caused release of Ca^{2+} in sea urchin egg homogenates (Fig. 3A). However, in the sea urchin egg homogenates, neither ruthenium red nor heparin alone prevented thimerosal's effects (Fig. 3, B and C). Together, ruthenium red and heparin did inhibit Ca^{2+} release in response to thimerosal (Fig. 3D). Thimerosal also

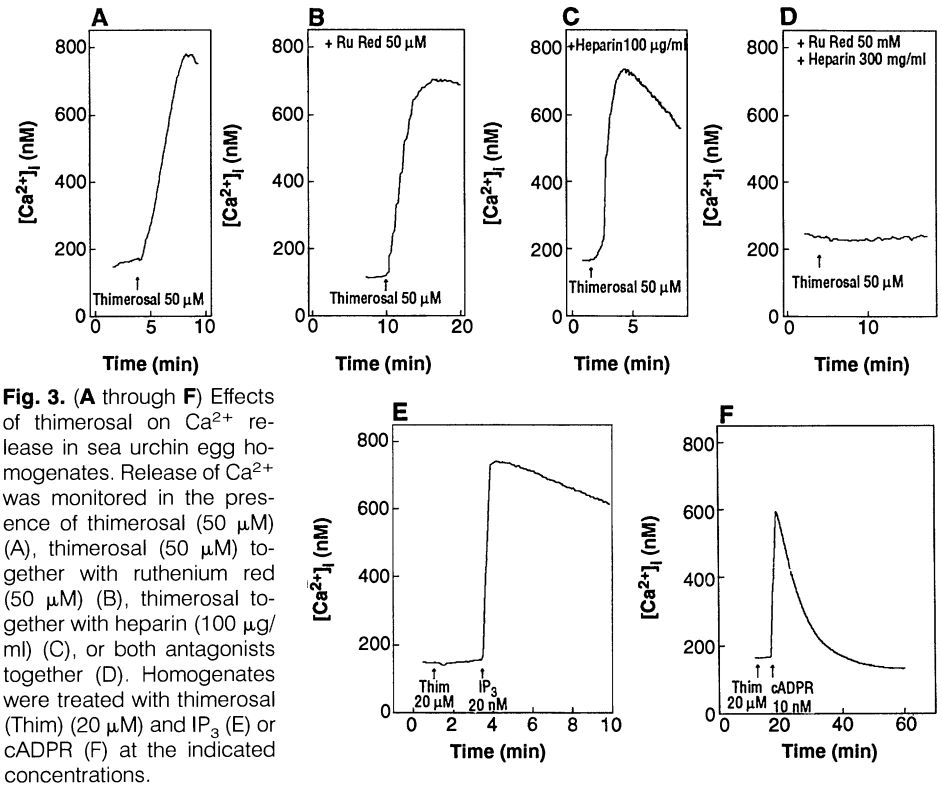

Fig. 3. (**A** through **F**) Effects of thimerosal on Ca^{2+} release in sea urchin egg homogenates. Release of Ca^{2+} was monitored in the presence of thimerosal (50 μM) (A), thimerosal (50 μM) together with ruthenium red (50 μM) (B), thimerosal together with heparin (100 μg/ml) (C), or both antagonists together (D). Homogenates were treated with thimerosal (Thim) (20 μM) and IP_3 (E) or cADPR (F) at the indicated concentrations.

Table 1. The inhibition of Ca^{2+} release in sea urchin eggs and egg homogenates measured with fura 2 in response to the addition or microinjection of IP_3, cADPR, caffeine, or ryanodine (to the final concentration shown) was measured as described (*7, 32*). The Ca^{2+} transients are quantified as the peak height attained (nM). Mean and SEM are shown, with the number of measurements in parentheses.

Calcium-releasing agent	Heparin (300 μg/ml)	Ruthenium red (50 μM)
	Homogenates	
IP_3 (1 μM)	25 ± 8 (4)	510 ± 38 (4)
cADPR (0.1 μM)	905 ± 58 (4)	53 ± 21 (4)
Caffeine (10 mM)	726 ± 103 (4)	15 ± 7 (4)
Ryanodine (600 μM)	569 ± 95 (4)	25 ± 9 (4)
	Eggs	
IP_3 (20 nM)	190 ± 30 (3)	1290 ± 320 (5)
cADPR (40 nM)	1030 ± 180 (3)	250 ± 40 (4)

sensitized the homogenates to both IP_3 (Fig. 3E) and cADPR (Fig. 3F). Thus, the effects of thimerosal and Ca^{2+} loading are similar, and thimerosal appears to affect both RyR and IP_3R (*13*). The sea urchin egg appears to have both a functional IP_3R and a functional RyR, whereas the frog egg has only a functional IP_3R.

In the frog egg Ca^{2+} waves can be induced by pricking the egg or by local application of a Ca^{2+} ionophore (*19*). The response to a Ca^{2+} ionophore indicates that a CICR mechanism is involved. We measured the Ca^{2+} waves after local application of the Ca^{2+} ionophore ionomycin (Fig. 4A, column D). Because we used a Ca^{2+}-sensitive fluorescent dye and a confocal fluorescent microscope, we were able to visualize the Ca^{2+} wave in an equatorial section through the egg. The wave crossed the cytoplasm and was not confined to the cortex. The wavefront was roughly spherical and traveled at 5 μm/s (*20*). The $[Ca^{2+}]_i$ was relatively uniform across the cytoplasm behind the wave. The Ca^{2+} wave at fertilization had very similar characteristics (Fig. 4A, column A). The wave started at the presumed site of sperm-egg interaction (sperm were added locally at the 10 o'clock position or upper left portion of the egg) and crossed the egg over a period of 5 to 6 min. Inseminated eggs that had been microinjected with heparin showed only a local increase in $[Ca^{2+}]_i$, presumably at the site of sperm-egg interaction, indicating that heparin had completely blocked the regenerative wave mechanism

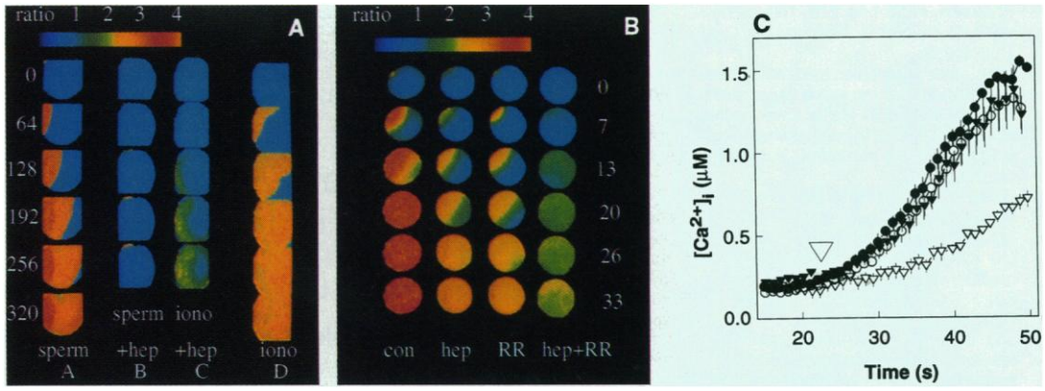

Fig. 4. (**A**) Images of Ca^{2+} waves in *Xenopus* eggs at fertilization and after parthenogenic activation (*30*). Confocal images of cytoplasmic Ca^{2+}, with the Ca^{2+}-sensitive dye calcium green. High Ca^{2+} concentrations are shown in red, and resting Ca^{2+} concentrations in blue. Successive images shown were recorded 64 s apart. The egg diameter is 1 mm, and the image in each frame represents 0.7 mm. The number column is time (in seconds). Column A: Ca^{2+} wave at fertilization. Column B: Insemination after microinjection of heparin (300 μg/ml). After 15 min, ionomycin was applied to the same egg at the 9 o'clock position (column C). Column D: Image of a different egg, to which ionomycin was applied at the 10 o'clock position. (**B**) Corresponding Ca^{2+} waves at fertilization in sea urchin eggs (*31, 32*). The images shown were recorded 6.6 s apart. The egg is 100 μm in diameter. Column 1: The fertilization wave started at the site of sperm entry (10 o'clock position) in all columns and crosses the egg over a period of 20 s. Column 2: Egg microinjected with heparin (300 μg/ml) and then fertilized. Eggs were microinjected with ruthenium red alone (RR) (50 μM) (column 3) or with ruthenium red and heparin (300 μg/ml) (column 4) and then fertilized. (**C**) Measurement of $[Ca^{2+}]_i$ in single sea urchin eggs at fertilization with fura 2. Control fertilization (●); heparin (300 μg/ml) (▼); 50 μM ruthenium red (○); heparin (300 μg/ml) + 50 μM ruthenium red (▽). The error bars represent the standard error of the mean of five experiments. The large inverted triangle indicates the time at which an increase in $[Ca^{2+}]_i$ was first detected. Because there were variable latencies for the initiation of responses, the traces were superimposed for comparison with the initiation of Ca^{2+} transients occurring at 22 s.

(Fig. 4A, column B). When ionomycin was applied locally with a micropipette to the same egg, we saw a larger, local increase in $[Ca^{2+}]_i$ but no Ca^{2+} wave (Fig. 4A, column C). Heparin at a concentration of 100 μg/ml halved the wave velocity.

We verified our measurements of Ca^{2+} concentration by scoring egg activation with contraction of the pigment as a criterion (*21*). Heparin caused 50% inhibition of egg activation at concentrations of 100 to 200 μg/ml. Egg activation was effectively blocked by a heparin concentration of 300 μg/ml with 4 out of 35 eggs activated upon insemination but was unaffected by ruthenium red at concentrations of up to 50 μM with 25 out of 29 eggs activated upon insemination (*22*). These data support our conclusion that the Ca^{2+} wave in frog eggs is governed by IP_3R.

The sea urchin egg is much smaller than the frog egg, but the fertilization Ca^{2+} wave travels at a similar velocity (*23*) (Fig. 4B, column 1). It passes as a spherical wavefront through an equatorial section. The region behind the wavefront showed no inhomogeneities in Ca^{2+} concentration, either in the egg nucleus (the egg nucleus is present in the optical section of Fig. 4B, column 1, for example) or in subcortical regions, where it has been suggested that RyR may be relatively abundant (*24*). All compartments appeared to change uniformly. Microinjection of either heparin or ruthenium red reduced the amplitude and propagation velocity slightly but did not prevent the wave from crossing the egg (Fig. 4B, columns 2 and 3). Microinjection of both antagonists together led to a loss of the Ca^{2+} wave (Fig. 4B, column 4). Because of the failure of the mechanical block to polyspermy in the absence of the Ca^{2+} wave, eggs were polyspermic under these conditions.

The specificity of Ca^{2+} release agonists and antagonists in sea urchin eggs and homogenates is shown in Table 1. Release of Ca^{2+} by RyR agonists was blocked selectively by ruthenium red, whereas heparin selectively blocked release by IP_3. This selectivity was preserved between homogenates and eggs. Because calibration of absolute Ca^{2+} concentrations is difficult with the use of the single-wavelength confocal imaging technique, we confirmed our results with whole cell fluorescence with the Ca^{2+}-sensitive ratio-dye fura 2. The Ca^{2+} wave is represented by the rising phase of the whole cell signal, which was affected only by the simultaneous presence of heparin and ruthenium red (Fig. 4C).

The outcome of these experiments indicates that frog eggs have a single CICR mechanism that uses IP_3R, whereas in sea urchin eggs both IP_3R and RyR contribute to the formation of Ca^{2+} waves. The coexistence of the two types of Ca^{2+} release channel was a prediction of the two-store model for Ca^{2+} oscillations (*2*) that led to the search for RyR in nonmuscle cells. In the two-store model, CICR is mediated only by RyR. Our results indicate that CICR can occur through either release channel in the same cell.

This redundancy may have advantages. Redundant Ca^{2+} release mechanisms allow separate modulation of the two channels by different agonists (*25*). The two channel types have different thresholds for Ca^{2+}-dependent activation and inactivation (*26*). Differential coexpression and colocalization of IP_3R and RyR (*27*) will generate a large number of different Ca^{2+} signaling phenotypes.

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22. Mature *Xenopus* eggs were injected with heparin or ruthenium red in the presence of chlorobutanol (10 mM). The eggs were then washed in F1 medium of composition: 41.25 mM NaCl, 1.75 mM KCl, 0.063 mM $MgCl_2 \cdot 6H_2O$, 0.25 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM NaH_2PO_4, 1.9 mM NaOH, and 2.5 mM Hepes (acid); pH 7.8 (*19*), and fertilization was attempted by addition of a sperm suspension in F1. We assessed fertilization by viewing the formation of a fertilization envelope and the contraction of the pigmented hemisphere toward the animal pole (*21*).
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28. *Xenopus* egg homogenates were prepared as described for sea urchin eggs (*7*) but with the following modifications: We removed egg jelly by swirling the eggs in F1 cysteine medium (*19*), and the eggs were washed in Ca^{2+}-free F1 containing 5 mM EGTA. The eggs were homogenized in a 70% intracellular medium (IM) solution (*7*). The resulting 20% homogenates were incubated with an ATP-regenerating system, mitochondrial inhibitors, and the fluorescent dye fura 2 (*7*). Portions (1 ml) were transferred to a cuvette, and fluorescence was monitored at 510 nm, with excitation at 340 and 380 nm, in a Hitachi fluorimeter at 22°C. The ratio of the signals at the two excitation wavelengths was used to determine the concentration of free Ca^{2+}, as described in (*32*). Calibrations were done in the presence and absence of heparin or ruthenium red. Each experiment in Figs. 1 to 4 is representative of at least three determinations that gave similar results.
29. The effect is not trivially explained as a consequence of the amount of Ca^{2+} in the store because this would affect the amount of Ca^{2+} available for release but not an increased sensitivity to maximal Ca^{2+} release by an agonist (*11*).
30. One of three experiments is shown in each case (each was performed at 20°C). Mature *Xenopus* eggs were obtained as described in (*19*). Eggs were stored in modified Barth's solution (MBS) medium containing the anesthetic chlorbutanol (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 15 mM tris acid, and 10 mM chlorbutanol; pH 7.6) to reduce hydration of the jelly coat and to prevent egg activation during microinjection. Eggs were microinjected with Ca^{2+} indicator (calcium green: Molecular Probes, Junction City, OR). Just before insemination, eggs were transferred to the low-salt medium, F1. Images were obtained and processed with a Leica CLSM (Leica Laserteknik, Heidelberg, Germany) microscope with 480-nm excitation and emission filters and a ×5 objective. To obtain ratio images that eliminated dye concentration artifacts, we divided the experimental images pixel by pixel by a reference image obtained at the beginning of the experiment.
31. Eggs were obtained, microinjected, and fertilized, and the fura 2 signal was calibrated as described in (*32*). The confocal images are representative of at least five experiments for each condition. *Lytechinus pictus* eggs were maintained at 16°C.
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Calcium Mobilization by Dual Receptors During Fertilization of Sea Urchin Eggs

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Fertilization is accompanied by a transient increase in the concentration of intracellular Ca^{2+}, which serves as a signal for initiating development. Some of the Ca^{2+} appears to be released from intracellular stores by the binding of inositol trisphosphate (IP_3) to its receptor. However, in sea urchin eggs, other mechanisms appear to participate. Cyclic adenosine diphosphate–ribose (cADPR), a naturally occurring metabolite of nicotinamide adenine dinucleotide, is as potent as IP_3 in mobilizing Ca^{2+} in sea urchin eggs. Experiments with antagonists of the cADPR and IP_3 receptors revealed that both Ca^{2+} mobilizing systems were activated during fertilization. Blockage of either of the systems alone was not sufficient to prevent the sperm-induced Ca^{2+} transient. This study provides direct evidence for a physiological role of cADPR in the Ca^{2+} signaling process.

Mobilization of intracellular Ca^{2+} occurs in a wide variety of cellular processes and is mediated by at least two major mechanisms. One messenger molecule that links surface receptor activation to the release of Ca^{2+} from internal stores is IP_3 (*1*). Another major mechanism is Ca^{2+}-induced Ca^{2+} release (CICR), which is well characterized in muscle (*2, 3*) and may function in mechanisms of Ca^{2+} oscillation and Ca^{2+} wave propagation (*4, 5*). Cyclic ADP-ribose (cADPR) is a cyclic metabolite (*6*) synthesized by a ubiquitous enzyme, ADP-ribosyl cyclase (*7, 8*), that utilizes nicotinamide adenine dinucleotide (NAD^+) as substrate. The cADPR molecule occurs naturally in mammalian tissues (*9*) and is as potent as IP_3 in mobilizing Ca^{2+} in sea urchin eggs (*10*) and vertebrate cells (*11, 12*). Some evidence indicates that cADPR may be an endogenous regulator of the CICR process (*13–15*).

The similarity between ligand binding to surface receptors and the sperm-egg interaction during fertilization has led to the proposal that Ca^{2+} mobilization associated with fertilization is similarly mediated by IP_3. Indeed, phosphoinositide metabolism increased during fertilization coincident with the increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) and the cortical exocytotic reaction (*16, 17*). Microinjection of IP_3 into eggs activates a transient increase in $[Ca^{2+}]_i$ similar to that which occurs after fertilization (*18*). In hamster eggs, injection of antibody to the IP_3 receptor can block changes in $[Ca^{2+}]_i$ induced by IP_3 or fertilization (*19*). However, in sea urchin eggs, heparin (an antagonist for the IP_3 receptor) only delays the onset of the transient increase in $[Ca^{2+}]_i$ but does not prevent it, suggesting that other mechanisms for mobilizing Ca^{2+} may exist (*20, 21*).

We investigated the effects of heparin on the IP_3-induced Ca^{2+} mobilization in intact sea urchin eggs and homogenates. In egg homogenates, Ca^{2+} release induced by IP_3 (1 μM) was completely blocked by heparin (0.2 mg/ml) (Fig. 1) (*10*). However, heparin did not affect Ca^{2+} release induced by cADPR (Fig. 1). We used the cortical exocytotic reaction in intact eggs as an index for Ca^{2+} mobilization to determine the effective concentration of heparin needed to block IP_3-induced Ca^{2+} mobilization (*22*). Microinjection of IP_3 (0.15 μM, intracellular concentration) into *Lytechinus pictus* eggs induced the cortical reaction in 100% of the injected eggs (*10*). If eggs were first microinjected with heparin [H5765 (Sigma)] at a final intracellular concentration of 1.9 mg/ml and subsequently injected with 0.12 to 0.24 μM IP_3, seven out of seven of the injected eggs did not show a cortical reaction. Increasing the concentration of IP_3 to 0.72 μM partially overcame the block by heparin, with two eggs giving a full cortical reaction and two eggs out of a total of eight eggs injected showing a partial cortical reaction (50%). To block 0.72 μM IP_3 from inducing the cortical reaction, the heparin concentration had to be increased to 4.7 mg/ml (six out of six eggs were blocked). Thus, the blockage by heparin is competitive in nature (*20, 21*). The higher effective concentration of heparin in intact eggs as compared with homogenates is likely to result

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