Calcium Signaling by Cyclic ADP-ribose, NAADP, and Inositol Trisphosphate Are Involved in Distinct Functions in Ascidian Oocytes*

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ADP-ribosyl cyclase catalyzes the synthesis of two structurally and functionally different Ca\(^{2+}\) releasing molecules, cyclic ADP-ribose (cADPR) from \(\beta\)-NAD and nicotinic acid-adenine dinucleotide phosphate (NAADP) from \(\beta\)-NADP. Their Ca\(^{2+}\)-mobilizing effects in ascidian oocytes were characterized in connection with that induced by inositol 1,4,5-trisphosphate (InsP\(_3\)). Fertilization of the oocyte is accompanied by a decrease in the oocyte Ca\(^{2+}\) current and an increase in membrane capacitance due to the addition of membrane to the cell surface. Both of these electrical changes could be induced by perfusion, through a patch pipette, of nanomolar concentrations of cADPR or its precursor, \(\beta\)-NAD, into unfertilized oocytes. The changes induced by \(\beta\)-NAD showed a distinctive delay consistent with its enzymatic conversion to cADPR. The cADPR-induced changes were inhibited by preloading the oocytes with a Ca\(^{2+}\) chelator, indicating the effects were due to Ca\(^{2+}\) release induced by cADPR. Consistently, ryanodine (at high concentration) or 8-amino-cADPR, a specific antagonist of cADPR, but not heparin, inhibited the cADPR-induced changes. Both inhibitors likewise blocked the membrane insertion that normally occurred at fertilization consistent with it being mediated by a ryanodine receptor. The effects of NAADP were different from those of cADPR. Although NAADP induced a similar decrease in the Ca\(^{2+}\) current, no membrane insertion occurred. Moreover, pretreatment of the oocytes with NAADP inhibited the post-fertilization Ca\(^{2+}\) oscillation while cADPR did not. A similar Ca\(^{2+}\) oscillation could be artificially induced by perfusing into the oocytes a high concentration of InsP\(_3\) and NAADP could likewise inhibit such an InsP\(_3\)-induced oscillation. This work shows that three independent Ca\(^{2+}\) signaling pathways are present in the oocytes and that each is involved in mediating distinct changes associated with fertilization. The results are consistent with a hierarchical organization of Ca\(^{2+}\) stores in the oocyte.

Ca\(^{2+}\) signaling in cells generally involves both its influx from the extracellular medium and its release from intracellular stores. Two families of intracellular Ca\(^{2+}\) release channels have been characterized, namely, the inositol 1,4,5-

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The abbreviations used are: InsP\(_3\), inositol 1,4,5-trisphosphate receptor; NAADP, nicotinic acid-adenine dinucleotide phosphate; cADPR, cyclic ADP-ribose; InsP\(_3\), inositol 1,4,5-trisphosphate; ADPR, ADP-ribose; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N\(_2\),N\(_4\),N\(_6\),N\(_8\)-tetraacetic acid; RyR, ryanodine receptor; ASW, artificial sea water; pF, picofarad.

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resulting Ca$^{2+}$ mobilization was monitored by two independent methods, by electrical measurements to sense the localized Ca$^{2+}$ changes near the plasma membrane and by fluorescent indicator to sample the cytoplasmic Ca$^{2+}$ changes. Results show that all three Ca$^{2+}$ signaling mechanisms are present in the oocytes and are involved not only in mediating the early electrical changes at the cell surface associated with fertilization but also in modulating the subsequent Ca$^{2+}$ oscillation.

**EXPERIMENTAL PROCEDURES**

**Ascidian Eggs**—As described previously (24), specimens of the hermaphroditic ascidian *Phallusia mammillata* were collected near Sète on the French Mediterranean coast. Mature oocytes were extracted from the oviduct and kept in artificial sea water (ASW). Sperm was drawn directly from the spermiduct. Chorions and follicle cells surrounding the oocyte were removed either manually, using fine sharpened tungsten needles, or enzymatically (26). Fertilization was induced by inseminating dechorionated eggs with a dilute suspension of sperm.

**Solutions**—Similar media were described in a previous study (24). They included artificial sea water (ASW) containing NaCl, 400 mM; KCl, 10 mM; MgCl$_2$, 50 mM; CaCl$_2$, 10 mM; Hepes, 10 mM, pH 8.0. The pipette solution was composed of sucrose, 400 mM; KCl, 200 mM; NaCl, 10 mM; MgCl$_2$, 1 mM; EGTA, 1 mM; Hepes, 20 mM, pH 7.2. Ryanodine (Calbiochem) is prepared as a stock solution at 13.5 mM in ethanol. Stock solutions of Fura-2 dextran 10,000 (Molecular Probes), InsP$_3$ (Calbiochem), cADPR (Amersham Pharmacia Biotech), β-NAD (Sigma), β-NADP (Sigma), ADPR (Sigma), NAADP (Molecular Probes), 8-amino-cADPR (Molecular Probes), BAPTA (Sigma), and heparin (Sigma) were each prepared in pipette solution. The concentration values of the compounds used in this study refer to their concentrations in the pipettes. The oocytes (~130 μm diameter) were filled by diffusion through the whole-cell patch pipette within minutes. The concentrations of the stock solution of cADPR and NAADP were verified by absorbance measurements at 260 nm and by using the published values for extinction coefficients (6, 19).

**Electrophysiological Recordings**—Dechorionated eggs in ASW were patch-clamped in the whole-cell configuration using pipettes pulled to resistances of 1.5–3 megohms with a Mecanex BB-CH puller (Geneva, Switzerland). Currents were recorded under voltage-clamp conditions.
with an RK300 amplifier (BioLogic, Clai, France) and pCLAMP software (Axon Instruments, Foster City, CA). Series resistance was compensated electronically (0.5–1.5 megohms). Eggs were successively patched with different pipettes, allowing for changes in pipette solutions as described previously (24, 25). Eggs were patch-clamped in the whole-cell configuration and recorded for hours, with the same pipette or with different pipettes, without disturbing the cell integrity (24, 25, 27, 28). Capacitance of the oocyte membrane was evaluated from the steps of current induced under triangle-wave voltage command, as previously explained (29, 30).

Calcium Imaging—Most of the procedure was performed as described previously (24). Dechorionated eggs were loaded with 1 mM Fura-2 dextran applied during a 10-min period by diffusion from the patch pipette. Eggs were observed using an epifluorescence Nikon Diaphot 300 microscope through a CF-Fluor 320 objective (numerical aperture 0.75). Fluorescence was collected from the entire cell. Eggs were illuminated at 340 and 380 nm successively using a Lambda 10 optical filter changer (Sutter Instrument Company) and a Technical Video epifluorescence DX-5 device (Woods Hole, MA). Fluorescence emission was recorded at 510 nm using an Extended Isis CCD camera (Photonic Science, Robertsbridge, UK). Data acquisition and calcium measurement were performed with the Starwise Fluo 220 system (Instar, Paris). During the acquisition process, at each sampling time, the two source intensity images (excited at 340 and 380 nm) were stored on a hard disk. The calculations of calcium concentration were done according to the formula of Grynkiewicz (31) with predefined values of calibration parameters to provide a pixel by pixel ratiometric image of \([\text{Ca}^{2+}]_i\) as in ref. (24). In the results that are shown below, \([\text{Ca}^{2+}]_i\) were averaged over the apparent whole egg diameter. Data were sampled at 4-s intervals.

RESULTS
cADPR and \(\beta\)-NAD Activate Calcium Release and Induce Changes in the Oocyte Calcium Current and Membrane Capacitance—Perfusion of cADPR into an unfertilized ascidian egg through a whole-cell patch pipette elicited two electrophysiological effects, a rapid decrease in the depolarization-activated \(\text{Ca}^{2+}\) current and a concomitant but slower increase in membrane capacitance (Fig. 1A). The latter change is indicative of membrane insertion into the cell surface (24). The magnitude of these changes were highly significant, amounting to about 60% decrease in the \(\text{Ca}^{2+}\) current and 20% increase in capacitance. In control experiments, perfusion with standard pipette solution without cADPR produced no observable electrical changes (not shown). The measured amplitude of the \(\text{Ca}^{2+}\) current remained constant even when the same oocyte was repeatedly patched by different pipettes (24, 25, 27, 28). If the oocyte was preloaded by perfusion with a high concentration of ryanodine (100 \(\mu\)M), a RyR blocker, application of cADPR subsequently was ineffective in eliciting any changes (24). In contrast, an inhibitor of the InsP₃R, heparin, at up to 3 mg/ml, did not prevent the cADPR effects (not shown). These results indicate that cADPR-induced changes may be mediated by a RyR-like \(\text{Ca}^{2+}\) release channel. That \(\text{Ca}^{2+}\) release indeed was involved was further demonstrated by preloading the oocytes with a \(\text{Ca}^{2+}\) chelator, BAPTA. As illustrated in Fig. 1B, subse-
quent application of cADPR produced no change in either the Ca$^{2+}$ current or the membrane capacitance. These electrical measurements thus provide a very sensitive assay for monitoring Ca$^{2+}$ release especially in the localized regions adjacent to the plasma membrane. A concentration-response curve summarizing 37 recordings of the Ca$^{2+}$ current is shown in Fig. 1C. The half-maximal effective concentration of cADPR was about 0.1 nM, among the lowest values ever reported (32).

b-NAD, the precursor of cADPR, was similarly effective in inducing the two electrical changes as shown in Fig. 2A. However, the kinetics were significantly slower, especially the decrease of the Ca$^{2+}$ current. The half-time for the current decrease induced by b-NAD was 170 s, 3–4 times longer than that induced by cADPR (Fig. 2B). The rate of the capacitance increase induced by b-NAD was similarly slower, with an initial rate of about 20 pF/min (cf. Fig. 2A) as compared with about 50 pF/min in the case of cADPR (cf. Fig. 1A). This time lag is consistent with the presence of an ADP-ribosyl cyclase in the oocytes converting b-NAD to cADPR, which is then responsible for inducing the observed electrical changes. It would thus be expected that 8-amino-cADPR, a specific antagonist of cADPR (33), should block the effects of b-NAD. This was found to be the case as shown in Fig. 3. Perfusion of a high concentration (1 µM) of 8-amino-cADPR by itself did not elicit any current or capacitance changes but totally blocked the activating effects of both b-NAD and its product, cADPR (Fig. 3, A and B).

Fertilization of ascidian oocytes is accompanied by a large increase in membrane capacitance similar to that induced by cADPR (24, 27, 30). Evidence suggests that the fertilization-induced membrane insertion is mediated by Ca$^{2+}$ released via the RyR (24). As will be shown later (cf. Fig. 9B), this membrane insertion was totally abolished by 8-amino-cADPR.

**NAADP Activates Calcium Release and Decreases the Oocyte Calcium Current without Affecting Membrane Capacitance**—Results above suggest that an ADP-ribosyl cyclase is present and operative in ascidian oocytes. The cyclase is known to be a multifunctional enzyme capable of catalyzing not only the cyclization of b-NAD to produce cADPR but also an exchange reaction to produce yet another Ca$^{2+}$ release metabolite from b-NADP, NAADP (Ref. 18, and reviewed in Ref. 12). The Ca$^{2+}$ stores sensitive to NAADP in sea urchin eggs homogenates can be separated from those sensitive to cADPR and InsP$_3$ by density centrifugation (19) and appear to possess a Ca$^{2+}$ transport system that is insensitive to thapsigargin (34). The effects of NAADP in ascidian oocytes are likewise very different from those of cADPR. Although perfusion of nanomolar concentrations of NAADP into the oocytes induced a decrease in Ca$^{2+}$ current, the change was much slower than that induced by cADPR (compare Figs. 1A and 4A). The concentration-response curve of NAADP is shown Fig. 4B, summarizing 24 assays with NAADP concentrations ranging from nanomolar to micromolar. The half-maximal effective concentration was about 3 nM, 30-fold higher than that of cADPR (Fig. 1C).

The most dramatic difference between the effects of NAADP and cADPR was, however, in the capacitance measurements.
Contrary to that observed with cADPR, NAADP never elicited any capacitance changes as shown in Figs. 4A and 5A. This is further emphasized in Fig. 5A. Of the 19 oocytes perfused with NAADP, none of them showed any increase in membrane capacitance. In contrast, all 31 oocytes responded to cADPR with a capacitance increase averaging to about 40 pF after 5 min. As a control, ADP-ribose (ADPR), the hydrolysis product of cADPR, was also tested, and it too did not induce any capacitance changes (Fig. 5A).

Of the 19 oocytes perfused with NAADP and not showing a capacitance change, all responded with an average of about 60% decrease in Ca^{2+} current after 2 min of perfusion, a magnitude similar to that induced by cADPR (Fig. 5B). The current decrease induced by either NAADP or cADPR could be blocked by preloading the oocytes with BAPTA, indicating in both cases that the effect was due to Ca^{2+} release activated by the agonists. There was, however, a major difference between these agonists with respect to the action of the antagonist, 8-amino-cADPR, which inhibited only the current decrease induced by cADPR but not that induced by NAADP (Fig. 5B). This latter result indicates that the Ca^{2+} release mechanism activated by NAADP was distinct from that activated by cADPR, consistent with that reported in sea urchin eggs (19). This difference was also supported by an experiment in which application of a high concentration of ryanodine to oocytes (100 μM) blocked the cADPR-sensitive decrease in Ca^{2+} current (24) but, similar to 8-amino-cADPR, did not inhibit the NAADP-induced changes (data not shown).

It has previously been reported that ADPR can interfere with channels and Ca^{2+} events in ascidian oocytes (35). This is confirmed in Fig. 5B. The effect of ADPR on the current, however, was not mediated by Ca^{2+} release since preloading the oocytes with BAPTA, which effectively eliminated the actions of both cADPR and NAADP, did not inhibit the effect of ADPR. The effect of ADPR appeared to be specific to that molecule since other similar compounds such as 1 mM ADP (not shown) or 8-amino-cADPR (Fig. 3A) did not cause a decrease in the current. An unexpected feature of the ADPR effect was its sensitivity to inhibition by 8-amino-cADPR (Fig. 5B). It is possible that the action of ADPR is related to its ability to covalently react with amino groups of proteins (36). Irrespective of the exact mechanism, it is clear that ADPR did not mobilize Ca^{2+} since its action was not blocked by BAPTA (Fig. 5B) and thus was not investigated further.

**The NAADP Signaling Is Independent of RyR, but Is Related to InsP_{3}R—** The effects of NAADP and cADPR on the current are independent (Fig. 6A). As usual, perfusion of NAADP into the oocyte decreased its Ca^{2+} current. Subsequent perfusion of cADPR into the same oocyte induced a further decrease in its Ca^{2+} current and an increase in membrane capacitance. As detailed above, both cADPR-induced changes can be attributed to the involvement of RyR. The magnitude of the subsequent current change was typical of that induced by cADPR alone, indicating that cADPR and NAADP act independently and that their effects on the current were essentially additive.

The inactivating effect of NAADP on the oocyte Ca^{2+} current is, in some respects, similar to that observed with InsP_{3} (24). Fig. 6B shows that perfusion with InsP_{3} induced a slow decrease in the current very much like that seen with NAADP (Fig. 4A). Also, neither NAADP nor InsP_{3} altered the membrane capacitance. However, the receptor for NAADP is distinct from that of InsP_{3} since neither heparin, an InsP_{3} receptor antagonist, nor pretreatment of the oocytes with InsP_{3} inhibited the action of NAADP (Fig. 6B, right panel). Nevertheless, the InsP_{3} and the NAADP-sensitive Ca^{2+} stores appear to be able to interact functionally. Thus, as shown in Fig. 6C, pretreatment of the oocytes with NAADP could render InsP_{3} incapable of causing a decrease in the Ca^{2+} current. Indeed, pretreatment with NAADP (n = 4, Fig. 6C, right panel) was found to be as effective as heparin in blocking the subsequent action of InsP_{3}. It thus appears that the order of addition is important. Prior activation by NAADP inhibits InsP_{3} but the converse is not true; no effect was seen on the action of NAADP by pretreatment with InsP_{3}. These intriguing and apparently paradoxical observations were investigated further.

**Inhibitory Effect of NAADP on Calcium Oscillation—** It was found that the action of InsP_{3} was complex (24). As shown in Fig. 7A, it was capable of inducing not only a single event of Ca^{2+} release but prolonged Ca^{2+} oscillation lasting as long as InsP_{3} was applied through the patch pipette. Simultaneous perfusion of both NAADP and InsP_{3} did not block the first Ca^{2+} release induced by InsP_{3} but effectively and reproducibly eliminated the subsequent Ca^{2+} oscillation (n = 11), as illustrated.
in Fig. 7B. All the InsP₃-induced Ca²⁺ oscillations, including the first one, could be inhibited if the oocytes were first preincubated for minutes with NAADP before InsP₃ application (Fig. 7C). This inhibitory effect is reversible, since subsequent simultaneous application of InsP₃ and NAADP 30 min later induces a pattern of Ca²⁺ signal identical to that depicted in Fig. 7B (not shown). It thus appears that, when InsP₃ and NAADP were simultaneously applied, there was insufficient time for NAADP to effect total inhibition of the InsP₃-induced oscillation (Fig. 7B). The results obtained with NAADP preincubation (Fig. 7C) are consistent with those shown in Fig. 6C.

The InsP₃-induced Ca²⁺ oscillation was very similar to that which occurs after fertilization (24). As shown in Fig. 8A, fertilization was accompanied by an initial Ca²⁺ transient lasting more than 5 min, which was then followed by several smaller transients with a periodicity of about 2–3 min and each lasting for about 1 min. Pretreatment of the oocyte with NAADP for several minutes reproducibly eliminated these oscillations (Fig. 8B). The first Ca²⁺ transient was not inhibited by NAADP but did appear to be significantly shortened by the NAADP treatment (n = 3). This first Ca²⁺ transient has previously been shown to be contributed by both Ca²⁺ release through InsP₃R and RyR as well as by Ca²⁺ influx (24). Although NAADP did not inhibit the first Ca²⁺ transient after fertilization, it was effective in blocking the post-fertilization Ca²⁺ oscillation, which is likely to be mediated mainly by InsP₃. The inhibitory effect of NAADP on the Ca²⁺ oscillation was specific since neither pretreatment with cADPR nor 8-amino-cADPR was capable of blocking the post-fertilization Ca²⁺ oscillation (data not shown). On the other hand, the precursor of NAADP, β-NADP, had the same effects, in all respects, as NAADP itself (not shown). This is not surprising since β-NADP could have been enzymatically converted to NAADP. Also, commercial β-NADP preparations are known to be contaminated with significant amounts of NAADP. Indeed, it was the contamination that had led to the discovery of the Ca²⁺ releasing effect of NAADP (19).

Further evidence that the inhibitory effect of NAADP was restricted only to the Ca²⁺ oscillation is shown in Fig. 9A. Pretreatment of oocytes with NAADP did not abolish the fer-
Utilization-associated membrane insertion, as indicated by a normal increase in membrane capacitance, nor did it totally inhibit the first Ca\textsuperscript{2+} transient, which is consistent with that shown in Fig. 8B. In contrast, 8-amino-cADPR totally eliminated the capacitance increase as shown in Fig. 9B, whereas the Ca\textsuperscript{2+} oscillation pattern following the first transient was not affected by this cADPR antagonist (not shown). These results show that three independent Ca\textsuperscript{2+} signaling mechanisms are present and operative in ascidian oocytes. Each mechanism is activated by a different Ca\textsuperscript{2+} messenger, cADPR, NAADP and InsP\textsubscript{3}, and each appears to be involved in mediating specific changes occurring during fertilization and early development of the oocyte.

**DISCUSSION**

It is generally believed that cells possess multiple types of Ca\textsuperscript{2+} stores (reviewed in Ref. 37). The recent discoveries of cADPR and NAADP (4–6, 19) in addition to InsP\textsubscript{3} provide credence to such a belief. Indeed, it has been shown that the NAADP-sensitive stores can be physically separated from those sensitive to the other two Ca\textsuperscript{2+} agonists (32, 38). Of the three, the InsP\textsubscript{3}-dependent mechanism is ubiquitous in cells (39). The cADPR-dependent mechanism is also quite widespread, being present in a variety of cells from plant to mammalian tissues (32). In contrast, the distribution of the NAADP-mechanism in cells is just beginning to be explored. This study is of notable importance since it is the first to demonstrate that the mechanism is present in a cell other than sea urchin eggs. Although both sea urchins and ascidians are marine animals, they diverged in evolution hundreds of millions of years ago. The fact that NAADP-dependent Ca\textsuperscript{2+} signaling is present in these two widely different species suggests that the mechanism may prove to be as widespread as those mediated by cADPR or InsP\textsubscript{3}. In this study, pharmacological evidence is obtained, indicating all three mechanisms may be mediated by its own independent receptor, consistent with that shown in sea urchin eggs (32). Thus, 8-amino-cADPR inhibited the effect of cADPR while heparin blocked the effect of InsP\textsubscript{3}, and neither antagonist had any effect on the action of NAADP (Figs. 5 and 6).

The intriguing question of why cells possess three different Ca\textsuperscript{2+} signaling mechanisms was explored in this study. Our results show that each of the three mechanisms has its own special function. Evidence is presented indicating the cADPR
mechanism is involved in mediating the insertion of membranes associated with fertilization. Thus, neither NAADP nor InsP3 can induce an increase in the membrane capacitance (Figs. 4 and 6). Perfusion of cADPR into oocytes mimicked the capacitance increase seen at fertilization while preapplication of 8-amino-cADPR blocked the increase naturally occurring (Figs. 1 and 9). It is likely that cADPR is also responsible for mediating the decrease in membrane Ca\(^{2+}\) current during fertilization. Although all three Ca\(^{2+}\) agonists can induce a decrease in membrane Ca\(^{2+}\) current in the oocyte, only cADPR can do so with fast enough kinetics (Figs. 1, 4, and 6) as compared with those of fertilization (24). The fast changes induced by cADPR or fertilization are consistent with the cortical localization of the Ca\(^{2+}\) stores (25).

That this effect of cADPR on the current is due to Ca\(^{2+}\) release activated by the agonist is shown by the fact that preloading the oocytes with BAPTA totally inhibited the change (Fig. 5B). However, measurements of the cytosolic Ca\(^{2+}\) using Fura-2 dextran detected no Ca\(^{2+}\) changes induced by either cADPR (24) or NAADP (Fig. 7C). This could be due to the relatively low temporal and spatial resolution of our classical fura-2 measurement. Future experiments using other probes, in conjunction with confocal microscopy may allow direct measurement of the local Ca\(^{2+}\) in the cortical region. In any case, the experiments using BAPTA presently serve to validate the current measurement as a method for monitoring localized Ca\(^{2+}\) release close to the plasma membrane. This technique has been widely used and is generally accepted (24, 25, 35). The use of the Ca\(^{2+}\) chelator to distinguish between Ca\(^{2+}\)-dependent and -independent effects is important since, in principle, factors other than Ca\(^{2+}\) may be able to induce a similar change in Ca\(^{2+}\) current. This is the case for ADPR, whose inactivating effect on the Ca\(^{2+}\) current is not inhibitable by BAPTA, indicating it is not mediated by Ca\(^{2+}\) mobilization (Fig. 5B). The ADPR-effect is likely to be due to a direct interaction of the metabolite with ion channels. Indeed, it has been reported that ADPR can directly activate a K\(^{+}\)-channel in arterial smooth muscles (40).

In contrast to cADPR and NAADP, perfusion with InsP\(_3\) induces a cytoplasmic Ca\(^{2+}\) oscillation in the oocytes similar to that observed after fertilization. Preloading the oocytes with heparin blocks the oscillation (41). Together, these results indicate that post-fertilization Ca\(^{2+}\) oscillation is mediated by InsP\(_3\). The exact mechanism of how these Ca\(^{2+}\) oscillations are generated is not known, but our results point to the critical involvement of the NAADP-sensitive Ca\(^{2+}\) stores. It has previously been shown in sea urchin eggs that NAADP itself is an inactivator of the NAADP-dependent Ca\(^{2+}\) release mechanism and can totally desensitize the release mechanism even at non-activating concentrations (38, 42). In the ascidian oocytes, our results show that pretreatment with NAADP, which presumably would discharge the stores and inactivate the release mechanism, can effectively inhibit the Ca\(^{2+}\) oscillation. It is possible that the oscillation requires the functional interaction
between the InsP$_3$- and the NAADP-sensitive stores. Inactivating the NAADP-mechanism by the pretreatment could disrupt the critical interaction and thus block the oscillation, even if the InsP$_3$-sensitive stores are fully functional. Indeed, it has previously been proposed that the interaction between the NAADP- and the cADPR/InsP$_3$-sensitive Ca$^{2+}$ stores may be responsible for mediating the Ca$^{2+}$ oscillation seen in sea urchin eggs (32, 42). Irrespective of the exact mechanism, the inhibition by pretreatment with NAADP does suggest the involvement of NAADP, together with InsP$_3$, in mediating the post-fertilization Ca$^{2+}$ oscillation. Moreover, the known pH regulation of the NAADP production could allow the natural alkalization of the cytoplasmic pH that occurs after fertilization to influence the pattern of post-fertilization Ca$^{2+}$ oscillation (43).

The results presented in this study are consistent with a model where the three independent Ca$^{2+}$ stores are arranged in a hierarchical manner. It is proposed that the cADPR-sensitive stores are localized next to the plasma membrane. Their mobilization by cADPR would rapidly raise the cortical Ca$^{2+}$ concentration, resulting in activation of a fast decrease in oocyte current and membrane insertion. Since the action of cADPR can be blocked by ryanodine (24), it is suggested that a RyR-like release channel is involved. Such a channel has indeed been immunolocalized to the cortical region of the oocytes (25). The next level of the hierarchical organization is suggested to be the NAADP-sensitive stores. The farther distance of these stores from the plasma membrane accounts for the NAADP-induced current decrease being slower than that of cADPR. The attenuation of the Ca$^{2+}$ release from these stores by local buffering could also contribute to the slow change and could account for the ineffectiveness of NAADP to activate the capacitance increase. The cortical region where the cADPR- and NAADP-sensitive stores are localized may represent a diffusion barrier for either large molecules, such as Fura-2 dextran, or for Ca$^{2+}$ ions. The latter could be due to rapid resequestration of Ca$^{2+}$ and/or buffering by Ca$^{2+}$ binding proteins. Thus the cortical Ca$^{2+}$ changes can be detected readily by electrical measurements but not by the cytoplasmic Fura-2 dextran. In contrast, the Ca$^{2+}$ release induced by InsP$_3$ can be detected by the cytoplasmic probe, indicating that the stores are distributed in the cytoplasm. It is proposed that the InsP$_3$-sensitive stores interact specifically with the NAADP-sensitive stores to generate the observed Ca$^{2+}$ oscillation. The exact nature of the interaction remains to be determined. One possibility is that the Ca$^{2+}$ released from one type of store is sequestered by the other, resulting in overloading and activating spontaneous release. A similar proposal has been advanced to account for the effect of NAADP on Ca$^{2+}$ oscillations seen in sea urchin eggs (32, 42).

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