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Functional visualization of the separate but interacting calcium stores sensitive to NAADP and cyclic ADP-ribose

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SUMMARY

Cells possess multiple Ca^{2+} stores and their selective mobilization provides the spatial-temporal Ca^{2+} signals crucial in regulating diverse cellular functions. Except for the inositol trisphosphate (IP_3)-sensitive Ca^{2+} stores, the identities and the mechanisms of how these internal stores are mobilized are largely unknown. In this study, we describe two Ca^{2+} stores, one of which is regulated by cyclic ADP-ribose (cADPR) and the other by nicotinic acid adenine dinucleotide phosphate (NAADP). We took advantage of the large size of the sea urchin egg and stratified its organelles by centrifugation. Using photolysis to produce either uniform or localized increases of cADPR and NAADP from their respective caged analogs, the two separate stores could be visually identified by Ca^{2+} imaging and shown to be segregated to the opposite poles of the eggs. The cADPR-pole also contained the IP_3-sensitive Ca^{2+} stores, the egg nucleus and the endoplasmic reticulum (ER); the latter was visualized using Bodipy-thapsigargin. On the other hand, the mitochondria, as visualized by rhodamine 123, were segregated to the opposite pole together with the NAADP-sensitive calcium stores. Fertilization of the stratified eggs elicited a Ca^{2+} wave starting at the cADPR-pole and propagating toward the NAADP-pole. These results provide the first direct and visual evidence that the NAADP-sensitive Ca^{2+} stores are novel and distinct from the ER. During fertilization, communicating signals appear to be transmitted from the ER to NAADP-sensitive Ca^{2+} stores, leading to their activation.

Key words: Ca^{2+} store, Cyclic ADP-ribose, NAADP, Inositol trisphosphate, Sea urchin egg

INTRODUCTION

Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are Ca^{2+} releasing metabolites of NAD and NADP, respectively (Clapper et al., 1987; Lee and Aarhus, 1995; Lee et al., 1989). The cADPR-sensitive Ca^{2+} stores were first described in sea urchin eggs (Clapper et al., 1987; Lee and Aarhus, 1995; Lee et al., 1989) and have since been shown to be widely present in cells from protist and plant to human (reviewed in Lee, 1997; Lee, 2000). Both the pharmacology (Galione et al., 1991; Lee, 1993) and the characteristics of the cADPR-sensitive Ca^{2+} release channels reconstituted in lipid bilayers are similar to those of the ryanodine receptor (Lokuta et al., 1998; Perez et al., 1998; Sitsapesan and Williams, 1995; Sonnleitner et al., 1998). The cADPR-sensitive Ca^{2+} stores are thus likely to be the endoplasmic reticulum, and this is also supported by subcellular fractionation studies (Dargie et al., 1990; Lee, 1991; Lee and Aarhus, 1995). Nevertheless, the cADPR-sensitive stores have never been directly visualized.

The NAADP-sensitive Ca^{2+} stores have recently been characterized in sea urchin eggs (Lee and Aarhus, 1995). They have since been found in two other invertebrate eggs, ascidian and starfish (Albrieux et al., 1998; Santella et al., 2000), as well as in plant (Navazio et al., 2000) and mammalian cells, pancreatic acinar cells and brain microsomes (Bak et al., 1999; Cancela et al., 1999), suggesting that these stores are also generally present in cells. A unique property of the NAADP-sensitive Ca^{2+} release mechanism is its insensitivity to antagonists of all the intracellular Ca^{2+} release channels known, which include the inositol trisphosphate-receptor, the ryanodine receptor and the cADPR-sensitive Ca^{2+} channels (Chini et al., 1995; Graeff et al., 1995; Lee and Aarhus, 1995). Fractionation studies indicate that the NAADP-sensitive Ca^{2+} stores are also distinct from the mitochondria (Lee and Aarhus, 1995). In this study, we describe the direct visualization of the cADPR- and NAADP-sensitive Ca^{2+} stores in sea urchin eggs using Ca^{2+} imaging. The results show that the stores are separate but appear to be interacting during fertilization.

MATERIALS AND METHODS

Stratification of sea urchin eggs

Lytechinus pictus eggs were stratified according to a previous procedure (Brandriff and Hinegardner, 1975; Craig and Piatigorski, 1971; Lee and Epel, 1983). The exact conditions for stratification were determined for each batch of eggs. Centrifuging for too long or at too

high a speed split the eggs, resulting in loss of cell fragments containing the relevant Ca^{2+} stores. Insufficient centrifugation, on the other hand, resulted in inadequate segregation of organelles. Generally, the egg suspension (0.4 ml) was layered on top of 1 ml of 1 M sucrose and centrifuged in a Beckman microfuge for 8-15 minutes at 11,000-13,000 rpm (7,000-8,000 ***g***).

Microinjection

Freshly stratified eggs were used in each experiment within 15-20 minutes of stratification to avoid complications due to redistribution of organelles in the eggs. Stratified eggs were washed once with artificial sea water, attached to coverslips coated with protamine sulfate and loaded by microinjection. The injection medium contained either 350 μM caged cADPR, 18-34 μM caged NAADP or 90 μM caged IP_3, and 10 mM fluo-3, 0.5 M KCl, 0.1 mM EGTA, 20 mM Hepes, pH 6.7. The injection volume was 1-2% of the egg volume. The site of injection is usually at the center of the egg, which was elongated by stratification. The injected fluid droplet dispersed within seconds as visualized in brightfield. The diffusion of the injected substances throughout the eggs was uniform and rapid as verified by observing the distribution of fluo-3 that was included in the injection medium. Fluo-3 has a size and charge similar to the caged compounds and can serve as a good tracer. That the injected caged compounds themselves indeed diffuse uniformly throughout the eggs is shown in unstratified eggs. A uniform pattern of Ca^{2+} elevation was seen in these eggs after whole-field illumination with UV-light, photolyzing the injected caged compounds (Aarhus et al., 1996; Aarhus et al., 1995; Lee et al., 1997).

Caged compounds and fluorescent probes

Caged cADPR and caged NAADP were synthesized as described previously (Aarhus et al., 1995; Lee et al., 1997). Caged IP_3 was purchased from CalBiochem (La Jolla, CA, USA) and further purified by anion exchange chromatography (Cellufine A800, Millpore, Bedford, MA, USA) to remove contaminating IP_3. A gradient of water and 1 M triethylamine bicarbonate (pH 8.8) was used for elution as previously described (Lee et al., 1997). Bodipy-thapsigargin and rhodamine 123, specific markers for the ER (Simpson and Russell, 1997) and mitochondria (Johnson et al., 1980), respectively, were from Molecular Probes (Eugene, OR, USA).

Photolysis

Calcium imaging and whole-field photolysis were obtained with an $InCa^{++}$ system (Intracellular Imaging Inc., Cincinnati, OH, USA) equipped with a 300-watt Xenon arc lamp. The light output was directed to the eggs via the epifluorescence attachment of a Nikon inverted microscope equipped with a dichroic mirror that reflected both 365 nm and 485 nm light. The exposure time was 0.5-2.5 seconds, which did not produce any Ca^{2+} changes in control eggs not loaded with the caged compounds. Fluorescence data were not collected during the photolysis exposure. Local photolysis used a nitrogen laser (VSL-377ND-S) obtained from LSI (Franklin, MA, USA). The laser provided 4 nanosecond pulses at 337.1 nm with a pulse energy of 120 μJ. Peak power was 30 kW and the average power was 2.4 mW at 20 Hz. Ten laser pulses were directed to a portion of the egg via a fused silica fibre (0.2 mm diameter) obtained from Ocean Optics (Dunedin, FL, USA). The fiber was heat-pulled to produce a tip of 10-20 μm.

Isolation of egg microsomes and ATPase measurements

Eggs were gently homogenized in a glass homogenizer fitted with a Teflon pestle. The homogenates were centrifuged for about 30 seconds in a microfuge to remove egg cortices containing the cortical granules as well as intact eggs (Clapper and Lee, 1985; Clapper et al., 1987; Lee, 1991). Egg microsomes were isolated by Percoll density centrifugation. 2 ml of 15% *L. pictus* egg homogenates were placed on top of 10 ml of 25% Percoll and centrifuged for 40 minutes at 25,000 rpm (41,000 ***g***) in a Beckman Ti50 rotor (10°C) as described previously (Lee and Aarhus, 1995). The supernatants were discarded and eight fractions were collected from each gradient by puncturing the bottom of the centrifuge tubes. ATPase activity was determined by diluting the microsomes twentyfold into an assay medium containing 250 mM N-methylglucamine, 250 mM potassium gluconate, 0.5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]dATP, 20 mM Hepes, pH 7.2, adjusted with acetic acid. After a 3 hour incubation at 20-25°C, the reaction was stopped with 0.1 M phosphoric acid containing 25 g/l activated charcoal. After centrifugation, the released $^{32}P_i$ in the supernatant was counted.

RESULTS

It is generally accepted that the stimulus-sensitive Ca^{2+} stores in cells are in the ER. Indeed, our fractionation results in the sea urchin egg show that both the cADPR- and IP_3-sensitive Ca^{2+} stores copurified in Percoll gradients with ER markers, such as glucose 6-phosphatase and Ca^{2+}-ATPase (Clapper et al., 1987; Lee, 1991; Lee and Aarhus, 1995). In contrast, the NAADP-sensitive Ca^{2+} stores have previously been proposed to be new and distinct from the ER (Lee and Aarhus, 1995). The main evidence is that their distribution in Percoll gradients is different from the other two Ca^{2+} stores and is also broader than the ER (Lee and Aarhus, 1995). It is possible, however, that fragmentation of Ca^{2+} stores could occur during cell homogenization, contributing to the apparently distinct fractionation patterns. To resolve this issue, it is necessary to demonstrate that the NAADP-sensitive stores can be distinguished, in intact cells, from the ER and the other two Ca^{2+} stores.

The approach we used to demonstrate that was to take advantage of the fact that sea urchin eggs are large cells and that their organelles can be stratified into well-defined layers by centrifugation (Brandriff and Hinegardner, 1975; Craig and Piatigorski, 1971; Lee and Epel, 1983). Fig. 1 shows that *Lytechinus pictus* eggs were stretched to elongated shapes following centrifugation on top of a sucrose cushion. The nucleus (indicated by arrowheads) together with the oil cap (dark patch, Fig. 1B) are moved to one pole (labeled N). These stratified eggs were injected with fluo-3 together with caged NAADP (Lee et al., 1997) (Fig. 1A), caged cADPR (Aarhus et al., 1995) (Fig. 1B) or caged IP_3 (Fig. 1C) and exposed to whole-field illumination of UV-light to photolyze the intracellular caged compounds. Although the photolyzing illumination was uniform, the resulting Ca^{2+} release was highly localized. The NAADP-induced release was mainly in the pole distal to the nucleus, while cADPR-induced release was localized to the nuclear pole (N). These localized patterns of Ca^{2+} mobilization are in striking contrast to the uniform elevation of intracellular Ca^{2+} following whole-field photolysis we reported previously in normal eggs not stratified by centrifugation (Aarhus et al., 1996; Aarhus et al., 1995; Lee et al., 1997). Uniform photolysis of caged IP_3 (Fig. 1C) produced a pattern of Ca^{2+} mobilization similar to that of caged cADPR and was distinct from that of the caged NAADP.

Similar localized patterns of release were seen in eight eggs injected with caged cADPR, nine eggs injected with caged NAADP and eleven eggs injected with caged IP_3. Fig. 2 shows the averaged fluo-3 fluorescence profiles of the eggs injected with the three caged compounds. The maximal fluorescence in

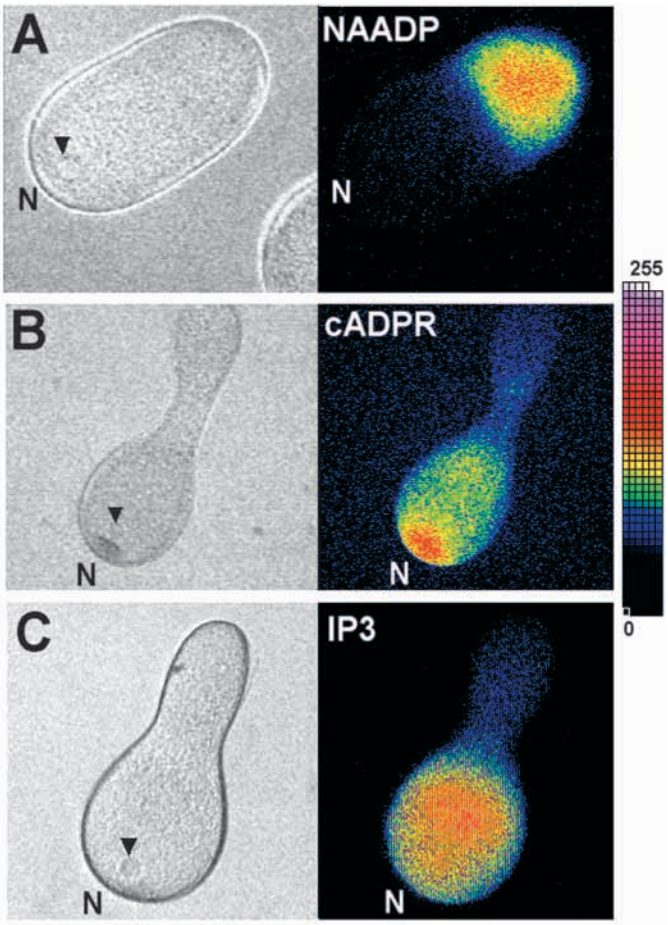

Fig. 1. Segregation of Ca^{2+} stores in stratified sea urchin eggs visualized by Ca^{2+} imaging. (A) A sea urchin egg was stratified by centrifugation and then loaded by microinjection with caged NAADP and fluo-3. The fluorescence image on the right was captured after photolysis with uniform whole-field illumination and shown in pseudocolor. An arrowhead indicates the location of the nucleus of the egg. N denotes the nuclear pole. (B) Similar to A except that the stratified egg was loaded with caged cADPR instead of caged NAADP. (C) Similar to A except that the stratified egg was loaded with caged IP_3. The calibration on the right shows the color palette corresponding to the 255 gray scale of the fluorescence images.

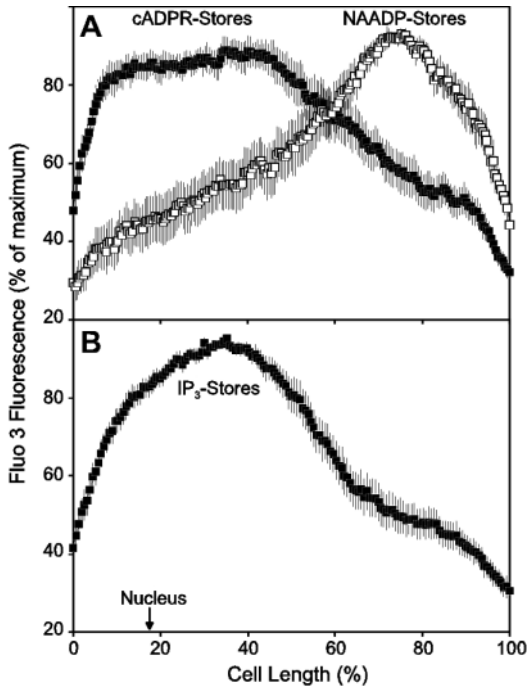

Fig. 2. Fluo-3 fluorescence profiles of Ca^{2+} mobilization. (A) Fluorescence profiles along the long axes of nine eggs injected with caged NAADP (open squares) and eight eggs injected with caged cADPR (filled squares) were measured after uniform photolysis, respectively averaged and normalized. (B) The averaged fluo-3 fluorescence profiles of eleven eggs loaded with caged IP_3 and uniformly photolyzed by whole field illumination. The averaged distance of the nucleus from the nuclear pole in these 28 eggs is indicated. Values are means ± s.e.m.

the eggs following photolysis of caged cADPR was seen at a distance 29±5% (± s.e.m., *n*=8) of the cell length starting at the nuclear pole (Fig. 2A). On the other hand, the maximal Ca^{2+} release in the caged NAADP-injected eggs determined in the same manner occurred at 74±9% (± s.e.m., *n*=9) of the cell length (Fig. 2A). The nuclei of these eggs were located at 16±2% (± s.e.m., *n*=28) of the cell length. The profile of Ca^{2+} mobilization induced by caged IP_3 peaked at 36±4% of the cell length (± s.e.m., *n*=11) (Fig. 2B), very similar to that induced by caged cADPR. Since it is generally accepted that the diffusion of Ca^{2+} in cells is limited, the localized release patterns observed suggest that the three Ca^{2+} stores were segregated to opposite poles of the stratified eggs.

This remarkable segregation of the three Ca^{2+} stores was further demonstrated using local photolysis. The output of a nitrogen laser was delivered to a stratified egg via a fused silica fiber with a tip diameter of about 10 μm (Parpura and Haydon, 1999) (Fig. 3). The egg was previously injected with caged NAADP and fluo-3. Local photolysis at the nuclear pole (labeled N in Fig. 3A) produced only a small and localized Ca^{2+} release that dissipated quickly. The fiber was then placed at the distal pole of the same egg (Fig. 3B) and the same numbers of photolyzing laser pulses were delivered. The resulting Ca^{2+} release spread throughout the distal half of the egg. The magnitude of the release was higher and the duration was also longer. Eight of the eleven eggs measured in this manner responded similarly. Fig. 3C shows that the averaged fluo-3 fluorescence increase (F/Fo) induced by local photolysis at the distal poles of these eggs was 6.2±1.1 (± s.e.m.) -fold as compared with an increase of only 2.5±0.6 (± s.e.m.) -fold when the photolysis was directed at the nuclear poles of the same eggs. The time courses of the fluorescence increase at the two poles (Fig. 3C) indicate that the duration of the Ca^{2+} release (time from the start of photolysis to the return of the fluo-3 fluorescence to half of the maximum) at the distal pole was also about 2.2-fold longer.

The small and brief Ca^{2+} release in the nuclear end (blue squares, Fig. 3C) suggests that some NAADP-sensitive stores remained there. This is consistent with the results shown in Fig. 2A. After whole-field illumination, the averaged Ca^{2+}-release profile of the eggs injected with caged NAADP showed fluorescence in the nuclear pole that was about 2-3 times lower than the peak in the distal end. Fractionation rarely separates organelles completely, even when done in vitro by density

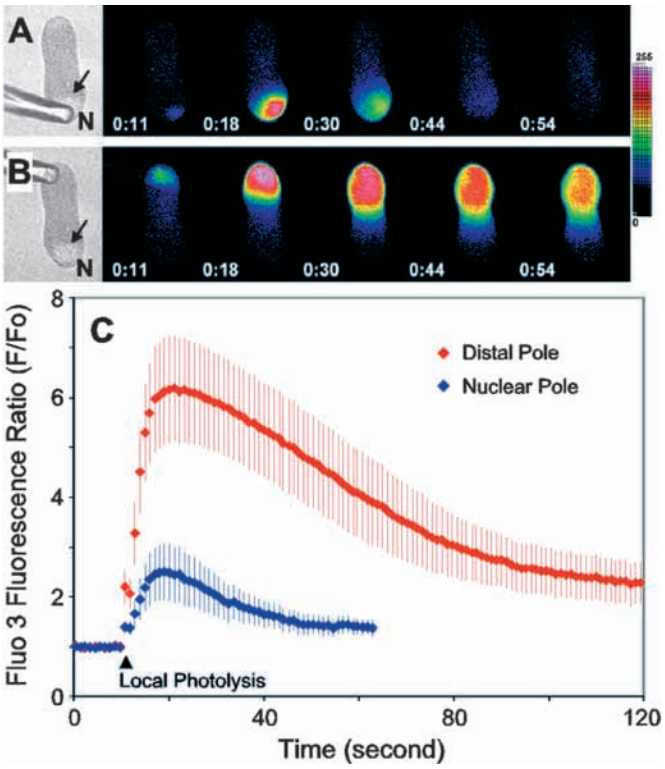

Fig. 3. Mobilization of the segregated NAADP-sensitive Ca^{2+} stores by local photolysis. The light output of a nitrogen laser was delivered through a fused silica fiber to effect local photolysis. (A) The photolyzing fiber is visible in the bright-field image, which was placed at the nuclear pole of the stratified egg. An arrow indicates the location of the nucleus. A sequence of fluorescence images was taken immediately after local photolysis was initiated at 10 seconds. The numbers on each image indicate minutes:seconds. (B) After local photolysis at the nuclear pole the same procedure was repeated at the distal pole of the same egg. The resulting Ca^{2+} increase was higher, spread wider and lasted longer, indicating the NAADP-sensitive Ca^{2+} stores were segregated to the distal pole. (C) The time course of the averaged fluo-3 fluorescence increase in eight stratified eggs at the nuclear pole (blue) was compared to that at the distal or NAADP pole (red). The fluorescence intensity measured after local photolysis (F) was normalized to that before photolysis (Fo). Values are means ± s.e.m.

centrifugation. It is thus reasonable that some NAADP-sensitive stores remained in the nuclear pole even after stratification.

The two types of Ca^{2+} stores have different sensitivity to thapsigargin, which effectively discharges the cADPR-sensitive stores by inhibiting the Ca^{2+}-ATPase, while the NAADP-sensitive stores are resistant (Genazzani and Galione, 1996). Bodipy-thapsigargin, a fluorescent analog, was used to visualize the stores containing the Ca^{2+}-ATPase (Simpson and Russell, 1997). Eggs were incubated with the fluorescent probes and subsequently stratified. Fig. 4A shows that the eggs exhibited prominent staining, mainly in regions close to nuclear pole (N), similar to the distribution of the cADPR- and IP_3-sensitive Ca^{2+} stores. The absence of fluorescence around the distal pole is consistent with the NAADP-stores possessing a thapsigargin-insensitive Ca^{2+}-ATPase. That the eggs do have this type of Ca^{2+}-ATPase is shown directly by measuring ATPase activity in the egg microsomes. The presence of 100 μM Ca^{2+} stimulated the activity about twofold (Fig. 4B). A portion of 80-90% of the Ca^{2+}-ATPase was completely blocked by 10 nM of thapsigargin while the remaining 10-20% of the Ca^{2+}-ATPase was not sensitive to as high as 10 μM

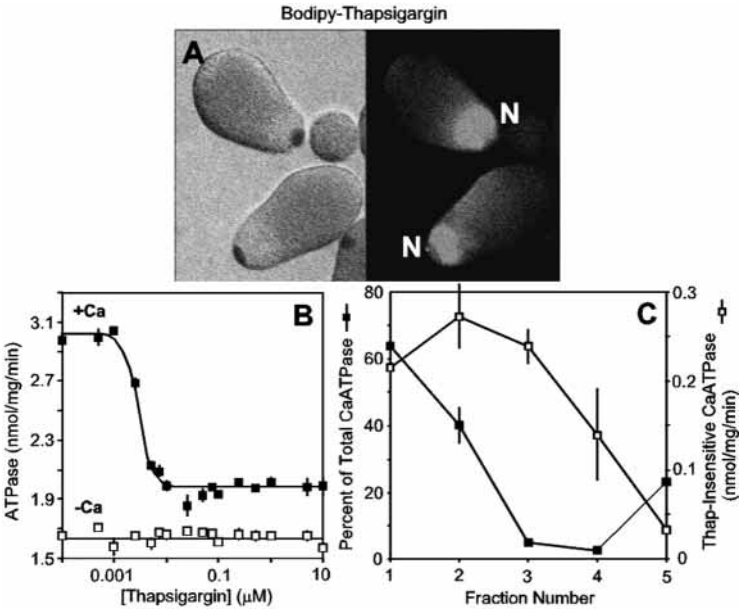

Fig. 4. (A) Distribution of thapsigargin-sensitive organelles. Eggs were incubated with 0.1-0.5 μM Bodipy-thapsigargin for 2 hours at 20-25°C and subsequently stratified by centrifugation as described in Materials and Methods. (B) ATPase activity in egg microsomes was measured as described in Materials and Methods, in the presence of 100 μM Ca^{2+} (+Ca^{2+}) or 1 mM EGTA (–Ca^{2+}), and at various concentrations of thapsigargin. (C) Egg homogenates were fractionated by Percoll density centrifugation and the total Ca^{2+}-ATPase activity in each fraction was determined as described in B. The thapsigargin-insensitive ATPase activity in each fraction was measured as the Ca^{2+}-ATPase activity in the presence of 1 μM thapsigargin (open squares). The same activity in each fraction is also plotted as a percentage of the total Ca^{2+}-ATPase activity in that fraction (filled squares). Values are means ± s.e.m.

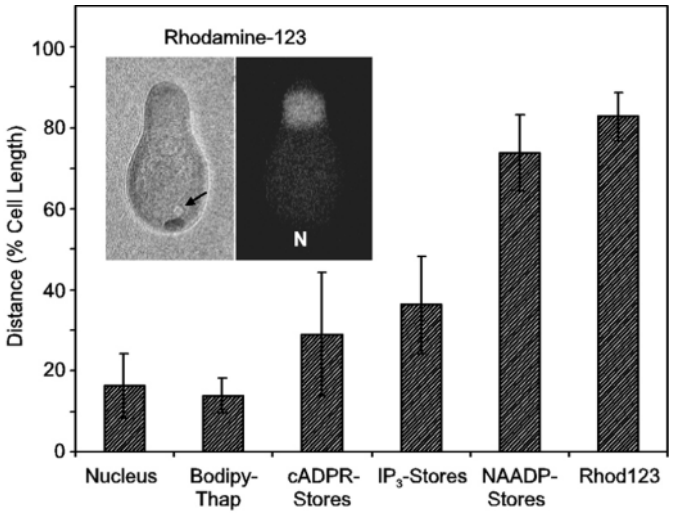

Fig. 5. The distribution of various organelles in the stratified eggs. The distances were measured from the nuclear pole to the peak of the fluorescence profile of each organelle along the long axis of the egg. The values shown are normalized to the total cell length and values are means ± s.d. Each organelle was determined in 8-28 stratified eggs. (Inset) The distribution of mitochondria in the eggs was visualized by incubating with 10-20 μM Rhodamine 123 for 0.5-1 hour and subsequent stratification.

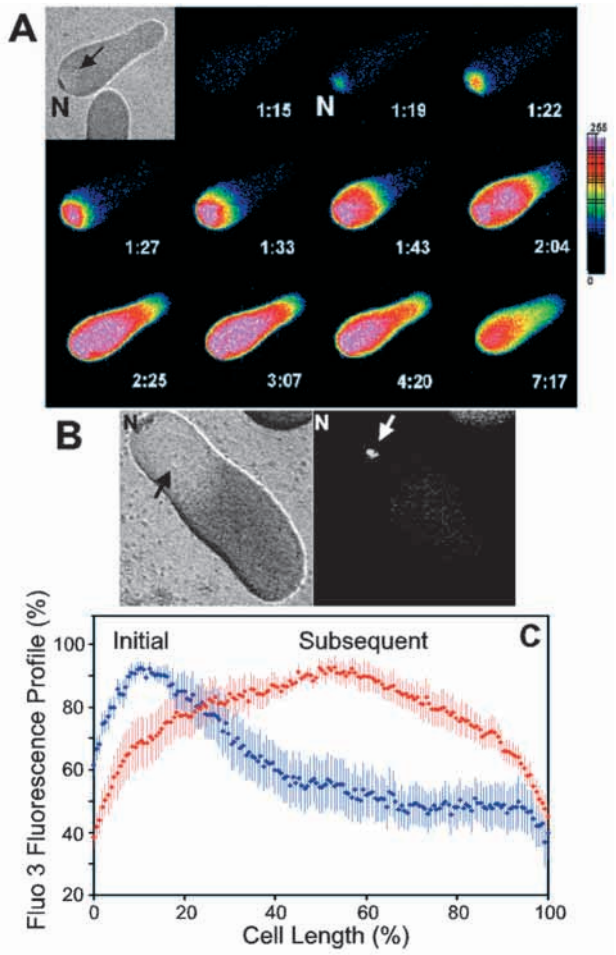

Fig. 6. (A) The Ca^{2+} wave associated with fertilization. The wave is always initiated close to the nuclear (N) pole. The arrow in the bright-field image indicates the location of the nucleus. The sequence of fluorescence images shows the propagation of the Ca^{2+} wave from the point of initiation at the nuclear pole to the rest of the cell. The numbers on each image indicate time (minutes:seconds) the image was taken. (B) The fertilization sperm was visualized by staining with the Hoechst 33342 (4 μM). Eggs were preincubated with the dye for 30 minutes and subsequently stratified. The arrow in the bright-field image indicates the location of the nucleus. The white arrow in the fluorescence image shows the location of the fertilizing sperm nucleus. The nuclear pole is denoted by N. (C) The average fluo-3 fluorescence profiles along the long axes of the seven fertilized eggs measured immediately after the Ca^{2+} wave was initiated and compared to those measured 2-3 minutes subsequently. Values are means ± s.e.m.

thapsigargin (Fig. 4B). Fig. 4C shows the distribution of the thapsigargin-insensitive Ca^{2+}-ATPase in egg homogenates fractionated using Percoll density centrifugation. 40-60% of the Ca^{2+}-ATPase activity measured in the denser fractions (1 and 2) was insensitive to thapsigargin-inhibition. We have previously shown that the NAADP-sensitive Ca^{2+} stores, unlike the cADPR-sensitive stores, have a broad distribution in the Percoll gradient (Lee and Aarhus, 1995). In fractions 3 and 4, which contained the cADPR-sensitive stores (Lee and Aarhus, 1995), the majority of the Ca^{2+}-ATPase was inhibited by thapsigargin and only about 2-5% was insensitive.

Fig. 5 summarizes the distributions of various organelles in the stratified eggs. The nucleus, the ER containing the thapsigargin-sensitive Ca^{2+}-ATPase, the cADPR-sensitive and the IP_3-sensitive Ca^{2+} stores were all concentrated in about a third of the egg (36±4%) closest to the nuclear pole. In contrast, the NAADP-sensitive Ca^{2+} stores were mainly localized in the distal half (74±3% of the cell length distal to the nuclear pole). Included in Fig. 5 is another organelle marker, the mitochondria, which were visualized by Rhodamine-123 staining. The inset shows that they were also concentrated in the distal part of the stratified eggs.

Fig. 6A shows that fertilization of these stratified eggs produced a striking Ca^{2+} wave, starting at the nuclear pole (N, nucleus; arrow) (Eisen and Reynolds, 1985) and propagating throughout the entire egg. The Ca^{2+} concentration in the distal end that contained the NAADP-sensitive Ca^{2+} stores elevated also, suggesting that the NAADP-stores were activated during fertilization. Even though sperm were added uniformly as a suspension to the eggs, in all seven eggs observed, the Ca^{2+} waves were initiated near the nuclear pole, close to the cADPR-sensitive stores. This is further documented in Fig. 6C by averaging the fluo-3 fluorescence profiles along the long axis of the eggs shortly after fertilization. On average, the initial Ca^{2+} release was most prominent in regions at distances about 10% of the cell length from the nuclear pole. 1-2 minutes afterwards, the release pattern changed to be essentially uniform throughout the cells. These results suggest that a signal is sent from the initiation site in the nuclear half of the egg, which contains the majority of the cADPR-sensitive Ca^{2+} stores, and propagates to the distal half, resulting in the activation of the NAADP-sensitive Ca^{2+} stores.

The remarkable selectivity of the initiation of the Ca^{2+} wave in the nuclear half suggests that the fertilizing sperm may have a preference for the nuclear half. The fertilizing sperm can be visualized by the nuclear stain, Hoechst 33342. The nucleus

of the fertilizing sperm, once incorporated into the egg, accumulated the dye preincorporated into the eggs and appeared as a bright fluorescent spot (Lee et al., 1993; Twigg et al., 1988). In all seven monospermic eggs observed in this manner, the fertilizing sperm nuclei were located in the nuclear half. An example is shown in Fig. 6B. In three polyspermic eggs, one showed three sperm nuclei in the nuclear half and two showed one sperm nucleus in each half of the egg (data not shown). It thus appears that under the normal monospermic condition, the fertilizing sperm do have a remarkable preference for the nuclear half, where the cADPR-sensitive Ca^{2+} stores are present.

DISCUSSION

Direct and visual evidence is provided in this study to show that the cADPR- and the NAADP-sensitive Ca^{2+} stores are distinct and separable, firmly establishing the reality of these two novel Ca^{2+} stores. That centrifugation can segregate these stores indicates they are free cytoplasmic organelles not attached to the cortex of the egg. The cADPR-stores are likely to be the ER since they segregate near the nuclear pole similar to the thapsigargin-sensitive Ca^{2+}-ATPase, a marker for the ER. This is consistent with subcellular fractionation studies showing that the cADPR-stores copurify with the Ca^{2+} sequestration activity and a marker of the ER (Lee, 1991; Lee and Aarhus, 1995).

It is generally believed that the nuclear envelope is closely associated, or even continuous, with the ER. Indeed, recent work shows that not only the cADPR-sensitive Ca^{2+} release mechanism but also CD38, a homolog of ADP-ribosyl cyclase, are both present in the nucleus (Adebanjo et al., 1999; Gerasimenko et al., 1995; Khoo et al., 2000; Santella, 1996). That the nucleus is found to colocalize with the cADPR-sensitive stores in the stratified eggs further indicates the close association of the two organelles. This strategic localization may allow cADPR to play a direct and important role in regulating gene expression, which has, in fact, been demonstrated in plants. Thus, cADPR is shown to be the Ca^{2+} messenger mediating gene expression induced by the plant hormone, abscisic acid (Wu et al., 1997), as well as that induced by nitric oxide and viral infection (Durner et al., 1998).

The clear separation of the cADPR and IP_3 stores from the NAADP-stores indicates the latter are organelles distinct from the ER. In the stratified eggs, they appear to colocalize with mitochondria to the distal pole as shown by rhodamine staining, which suggests that they may be the same. Mitochondria have also been shown to contain an NADase capable of synthesizing cADPR (Ziegler et al., 1997). However, neither cADPR nor NAADP have been reported to be involved in mobilizing the mitochondrial Ca^{2+}. Evidence against the possibility that the NAADP-stores are mitochondria comes from previous fractionation studies, which shows that the distribution of the NAADP stores is distinct from cytochrome *c* oxidase, a mitochondrial marker (Lee and Aarhus, 1995). More importantly, mitochondria in unfertilized sea urchin eggs are essentially devoid of Ca^{2+}. Thus, treatment with mitochondrial uncouplers produces Ca^{2+} release only in fertilized but not unfertilized eggs (Eisen and Reynolds, 1985). This is in striking contrast to the dramatic Ca^{2+} release induced by NAADP in the unfertilized eggs. The NAADP stores are also unlikely to be the cortical granules since we have previously shown by electron microscopy that they remain attached to the egg cortex after stratification (Lee and Epel, 1983). The NAADP stores may thus be a new Ca^{2+} storing organelle that has not been previously described.

A unique feature of these novel stores is the presence of a thapsigargin-insensitive Ca^{2+}-ATPase (Genazzani and Galione, 1996), which is consistent with the NAADP stores segregating to a pole opposite to the one stained by Bodipy-thapsigargin. We have also directly verified that the eggs do possess a thapsigagin-insensitive Ca^{2+}-ATPase. An intracellular Ca^{2+}-ATPase called PMR1, showing similar insensitivity to thapsigargin, has been cloned from yeast (Sorin et al., 1997). A homologous protein has also been identified in rat, containing 919 amino acids and having the same apparent transmembrane organization and all of the conserved domains present in other P-type ATPases (Gunteski-Hamblin et al., 1992). The approach described in this study provides the first visualization of this new Ca^{2+} store and should be useful for characterizing it further.

A remarkable observation reported in this study is that the Ca^{2+} wave associated with fertilization always begins at the nuclear pole of the stratified eggs, which is consistent with that reported previously (Eisen and Reynolds, 1985). This long-standing puzzle can now be accounted for by the equally remarkable preference for sperm-egg fusion to occur at the nuclear pole of the stratified eggs. These results suggest an intriguing possibility that the sperm-egg fusion sites on the surface of the eggs may be physically connected to internal organelles, such as the cADPR-sensitive Ca^{2+} stores. Stratification of the eggs could thus result in the sperm-egg fusion sites concentrating in the nuclear halves, together with the stores. Alternatively, the egg surface may contain hot zones for sperm-egg fusion. Unfertilized sea urchin eggs are perfectly symmetrical with no visible marks for distinguishing one point on the egg surface from another. Stratification of the eggs provides the necessary asymmetry that allows these hot zones to be visualized.

The presence of multiple, separate and interacting Ca^{2+} stores provides cells with a versatile Ca^{2+} signaling mechanism. Diverse stimuli could be specifically linked to separate Ca^{2+} stores. Thus, activation of surface receptors by impermeant molecules is generally linked to the inositol trisphosphate pathway (Berridge, 1993), while nitric oxide, a permeant molecule, has been shown, in some cases, to signal via the cADPR-sensitive stores (Clementi et al., 1996; Reyes-Harde et al., 1999; Willmott et al., 1996). In plants, the cADPR- and not the inositol trisphosphate-sensitive Ca^{2+}-stores are involved in mediating the response to abscisic acid (Wu et al., 1997). Similar exclusive involvement of the cADPR-sensitive stores has been reported for insulin secretion by pancreatic β-cells (Takasawa et al., 1998; Takasawa et al., 1993). The distribution of different types of Ca^{2+} stores need not be uniform. Segregated distribution of the stores in cells allows them to serve localized signaling functions (Albrieux et al., 1998; Zhang et al., 1999). This is the case in *Ascidian* oocytes, in which the NAADP- and cADPR-sensitive stores appear to be restricted to the cortex and their mobilization effectively modulates membrane events, while the inositol trisphosphate-sensitive stores are distributed in the cytoplasm

mediating cytosolic Ca^{2+} oscillations (Albrieux et al., 1998). Likewise, in pancreatic acinar cells, the NAADP-induced Ca^{2+} signal has been proposed to function as a local trigger, which is then amplified by Ca^{2+}-induced Ca^{2+} release involving the cADPR- and inositol trisphosphate-sensitive stores to produce a global and propagative Ca^{2+} wave (Cancela et al., 1999). Clearly, our understanding of Ca^{2+} mobilization as a signaling mechanism would not be complete without elucidating the complexity of regulation and interaction among these diverse and novel Ca^{2+} stores.

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