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NAADP Mobilizes Ca^{2+} from Reserve Granules, Lysosome-Related Organelles, in Sea Urchin Eggs

Grant C. Churchill,[1,4] Yuhei Okada[1], Justyn M. Thomas[1], Armando A. Genazzani[2], Sandip Patel[3], and Antony Galione[1]

[1]Department of Pharmacology
University of Oxford
Mansfield Road
Oxford OX1 3QT
United Kingdom

[2]Department of Pharmacology
University of Cambridge
Tennis Court Road
Cambridge CB2 1QJ
United Kingdom

[3]Department of Physiology
University College London
Gower Street
London WC1E 6BT
United Kingdom

Summary

Nicotinic acid adenine dinucleotide phosphate (NAADP) mobilizes Ca^{2+} in many cells and species. Unlike other Ca^{2+}-mobilizing messengers, NAADP mobilizes Ca^{2+} from an unknown store that is not the endoplasmic reticulum, the store traditionally associated with messenger-mediated Ca^{2+} signaling. Here, we demonstrate the presence of a Ca^{2+} store in sea urchin eggs mobilized by NAADP that is dependent on a proton gradient maintained by an ATP-dependent vacuolar-type proton pump. Moreover, we provide pharmacological and biochemical evidence that this Ca^{2+} store is the reserve granule, the functional equivalent of a lysosome in the sea urchin egg. These findings represent an unsuspected mechanism for messenger-mediated Ca^{2+} release from lysosome-related organelles.

Introduction

NAADP was first shown to mobilize Ca^{2+} in sea urchin eggs (Lee and Aarhus, 1995) and has subsequently been demonstrated to mediate Ca^{2+} mobilization in other species from plants to animals including mammalian cells (Berg et al., 2000; Cancela et al., 1999; Patel et al., 2001). NAADP is involved in initiating and coordinating Ca^{2+} signaling (Cancela et al., 1999; Churchill and Galione, 2000), which contribute to the ability of multiple extracellular signals to all use Ca^{2+} yet maintain specificity. Intriguingly, NAADP releases Ca^{2+} from a store distinct from the endoplasmic reticulum (Aarhus et al., 1996; Churchill and Galione, 2001a; Genazzani and Galione, 1996; Lee, 2001; Lee and Aarhus, 1995, 2000), the Ca^{2+} store traditionally thought to be important in Ca^{2+} signaling (Berridge et al., 2000; Pozzan et al., 1994). Despite the emerging importance of NAADP (Lee, 2001; Patel et al., 2001), the identity of organelle from which NAADP mobilizes Ca^{2+} remains unknown. Moreover, identification of this organelle could initiate a paradigm shift in Ca^{2+} signaling by demonstrating a mechanism by which organelles other than those closely related to the endoplasmic reticulum can be sources of messenger-mobilized Ca^{2+}.

[4]Correspondence: grant.churchill@pharm.ox.ac.uk

Results and Discussion

Sea urchin eggs have gained prominence for investigating Ca^{2+} signaling mechanisms because multiple Ca^{2+} mobilizing messengers are active in this cell (Galione and Churchill, 2000; Galione et al., 2000; Lee, 2001). We used sea urchin eggs, Ca^{2+} imaging, and caged compounds to investigate the identity of the organelle targeted by NAADP. Incubating intact eggs with glycyl-phenylalanine 2-naphthylamide (GPN, 100 μM), a substrate of the lysosomal exopeptidase cathepsin C that selectively disrupts these organelles via osmotic lysis (Jadot et al., 1984), significantly reduced the amount of Ca^{2+} mobilized by the photorelease of NAADP (Figure 1A), from an average peak ratio (F/F_o) of 2.1 ± 0.1 ($n = 6$) to 1.2 ± 0.07 ($n = 19$; $p < 0.0001$). GPN also reduced the response to free NAADP that was microinjected (data not shown), indicating that GPN was not interfering with photolysis. GPN (200 μM) did not affect the binding of [^{32}P]NAADP (Aarhus et al., 1996; Patel et al., 2000) to whole sea urchin egg homogenate (108 ± 5.8 percent of the control; $p = 0.23$), consistent with its known and selective action (Jadot et al., 1984). Moreover, binding of [^{32}P]NAADP was the same in homogenates prepared with or without a 2 hr preincubation with 200 μM GPN (100 ± 9.7 percent of control, $n = 3$; $p = 0.99$), indicating that the NAADP response was not lowered due to selective proteolyisis of the NAADP receptor by the release of lysosomal proteases. In contrast to the response to NAADP, GPN (100 μM) did not significantly reduce the amount of Ca^{2+} released by photorelease of either $InsP_3$ (peak ratio of 1.8 ± 0.09, $n = 6$, versus 1.9 ± 0.1, $n = 6$; $p = 0.38$; Figure 1A) or cADPR (peak ratio of 2.23 ± 0.1, $n = 17$, versus 2.08 ± 0.2, $n = 8$; $p = 0.48$; Figure 1A). These data indicate that GPN-mediated disruption of lysosomes was selectively eliminating the response to NAADP.

To verify that GPN was indeed disrupting lysosomes, intact sea urchin eggs were labeled with LysoTracker Red, a fluorescent weak base that accumulates in acidic compartments. Confocal microscopy revealed a granular pattern of fluorescence with discrete vesicles of ~1–2 μm diameter throughout the cytoplasm (Figure 1B, $n = 14$). Upon addition of 200 μM GPN to the extracellular medium, the bright vesicles were eliminated, as shown in both the image and the scan line, in which fluorescent intensity is plotted against distance (Figure 1B, $n = 9$). Ca^{2+} imaging of intact eggs revealed that 200 μM GPN evoked local Ca^{2+} release events (Figure 1D; 10/17 eggs), consistent with both the high luminal Ca^{2+} concentration in lysosomes (Christensen et al., 2002; Dell'-

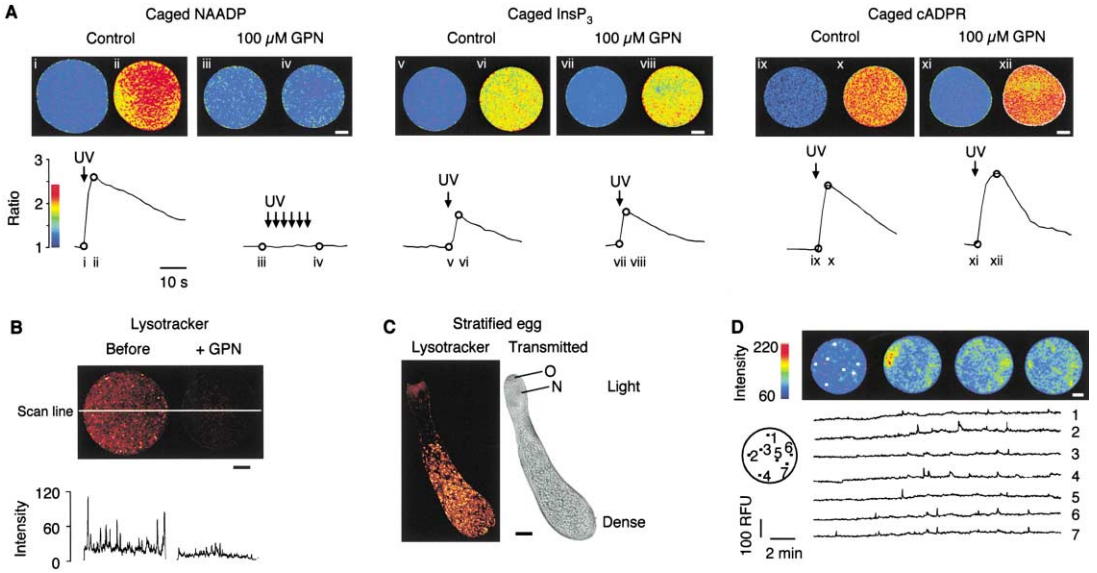

Figure 1. Effect of Glycylphenyl Alanine 2-Naphthylamide (GPN) on Lysosomes and Ca^{2+} Signaling in Intact Sea Urchin Eggs

(A) GPN eliminates NAADP-induced Ca^{2+} release but not $InsP_3$- or cADPR-induced Ca^{2+} release. Images are self-ratios with color representing the ratio according to the calibration scale. Traces are of the average ratio over time, with the open circles indicating the time points used for the images. Eggs contained Oregon Green 488 BAPTA Dextran (10 μM) and either caged $InsP_3$ (5 μM), caged cADPR (5 μM), or caged NAADP (0.5 μM) as labeled. Compounds were photoreleased at various intervals as indicated by the arrows. Eggs were exposed to GPN for at least 80 min before photolysis.

(B) GPN eliminates acidic organelles labeled with LysoTracker Red. Images show the same egg before and 5 min after 200 μM GPN. The plots below the images are taken from the scan line shown on the image.

(C) Distribution of acidic organelles labeled with LysoTracker Red in a sea urchin egg whose organelles were stratified by centrifugation. The positions of the oil droplet (O) and pronucleus (N) are indicated in the transmitted light image.

(D) GPN elicits localized Ca^{2+} release. Traces are from 5 μm square regions of interest. Eggs were injected with Oregon Green BAPTA dextran (10 μM) and then exposed to 100 or 200 μM GPN. Scale bars, 20 μm. Data are from individual experiments representative of the response observed in 6/6 (A, caged NAADP, control), 19/19 (A, caged NAADP, GPN), 6/6 (A, caged $InsP_3$, control), 6/6 (A, caged $InsP_3$, GPN), 14/14 (B), 6/9 (C), and 9/12 (D) experiments.

Angelica et al., 2000; Pozzan et al., 1994; Yagodin et al., 1999) and GPN-mediated bursting of lysosomes (Haller et al., 1996; Jadot et al., 1984). Additionally, in eggs centrifuged to stratify their organelles (Lee, 2001), the acidic organelles were depleted in the pole containing the nucleus and enriched in the opposite pole (Figure 1C). Thus, the localization of the acidic organelles mirrored the functional localization of the NAADP-sensitive Ca^{2+} stores, reported previously (Lee and Aarhus, 2000), and contrasted with the functional localization of the $InsP_3$- and cADPR-sensitive Ca^{2+} stores (Lee and Aarhus, 2000). Combined, these results support the contention that NAADP mobilizes Ca^{2+} from lysosomes.

We further investigated whether lysosomes were an NAADP-sensitive Ca^{2+} store by isolating and characterizing subcellular fractions prepared from sea urchin eggs (Clapper and Lee, 1985). Fractionation of crude homogenates by density gradient centrifugation yielded a subcellular fraction that responded to NAADP alone (Figures 2A and 2B) (Lee and Aarhus, 1995). Occasional sensitivity of this fraction to $InsP_3$ and cADPR was eliminated by further purification on a 50% Percoll gradient, a method used for isolating reserve (yolk) granules (McNeil et al., 2000), which, in the sea urchin egg, are the functional equivalent of lysosomes (Armant et al., 1986; Jadot et al., 1984). Of the subcellular fractions obtained, only one, the particulate fraction, was insensitive to both $InsP_3$ and cADPR but sensitive to NAADP (Figure 2B). We used marker enzymes (Clapper and Lee, 1985; Lee and Aarhus, 1995) to determine the distribution of organelles in the fractions (Figure 2C). The particulate fraction showed enrichment of the lysosomal markers acid phosphatase, glucosaminidase, and β-galactosidase (Figure 2C). Radiolabeled NAADP and cADPR both showed the highest binding in the microsome fraction (mainly endoplasmic reticulum; Clapper and Lee, 1985), but, importantly, the particulate fraction showed enrichment of NAADP relative to cADPR binding (Figure 2D). Our results are consistent with those published previously on homogenate fractionation (Lee and Aarhus, 1995) and provide further evidence that the physically separate NAADP-sensitive Ca^{2+} store is lysosomal.

We next established a functional correlation between the intact egg and fractionated homogenate by investigating the effect of GPN on the subcellular fractions (Figure 2A). GPN released Ca^{2+} from all subcellular fractions (Figure 2E), consistent with the broad density distribution of lysosomes, the functional distribution of the NAADP-sensitive Ca^{2+} store (Lee and Aarhus, 1995), and the ability of GPN to release Ca^{2+} in intact eggs (Figure 1D). Only in the microsome fraction was the GPN-mobilized Ca^{2+} resequestered, likely into the endoplasmic reticulum Ca^{2+} store, providing further evidence that these stores were almost absent in the yolk and particulate fractions. Following GPN-mediated Ca^{2+} release,

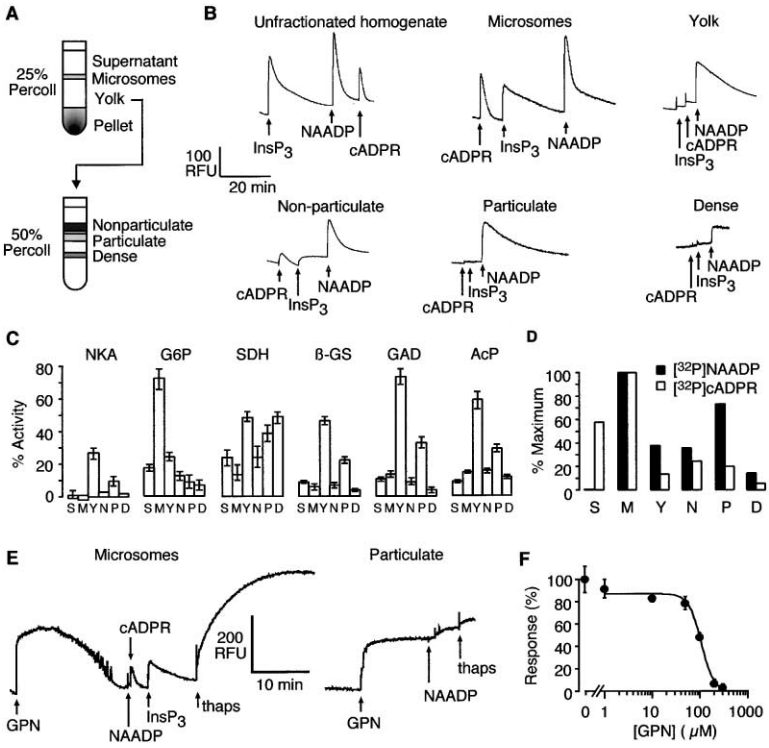

Figure 2. Purification and Characterization of a Subcellular Fraction Enriched in Ca^{2+} Stores Exclusively Sensitive to NAADP

(A) Schematic showing Percoll density gradient centrifugation and the banding patterns obtained with sea urchin homogenates.

(B) Sensitivity of subcellular fractions to Ca^{2+} releasing agents. The final concentrations of $InsP_3$, cADPR, and NAADP were 250 nM.

(C) Distribution of organelle marker enzymes in the subcellular fractions. The marker enzymes are Na^+/K^+ ATPase (NKA), glucose 6-phosphatase (G6P), succinate dehydrogenase (SDH), β-galactosidase (β-GS), glucosaminidase (GAD), and acid phosphatase (AcP). Fractions are labeled as S (supernatant), M (microsomes), Y (yolk), N (nonparticulate), P (particulate), and D (dense). Data are presented as mean ± SEM and normalized to the activity in the unfractionated homogenate ($n = 3–7$).

(D) Distribution of ^{32}P-labeled cADPR and NAADP binding in the subcellular fractions.

(E) Effect of the lysosome-disrupting agent glycylphenylalanine 2-naphthylamide (GPN) on the Ca^{2+} stores in the subcellular fractions.

(F) Effect of GPN concentration on the response to NAADP in sea urchin egg homogenate. The curve was obtained by fitting the data to the Hill equation. The final concentrations of $InsP_3$, cADPR, and NAADP were 250 nM, GPN 200 μM, and thapsigargin (thaps) 7.5 μM. Data are from individual experiments representative of the response observed in 5–21 (B), 3–7 (C), 3 (D), and 4–9 (E) experiments.

NAADP failed to release Ca^{2+} in both microsomes and the lysosome-enriched particulate fraction (Figure 2E, $n = 6$). The inhibition was dose-dependent as demonstrated with crude homogenate (Figure 2F). These data provide further evidence that the particulate fraction is enriched in lysosomes and that lysosomes represent a Ca^{2+} store exclusively sensitive to NAADP.

Although the Ca^{2+} store that NAADP mobilizes is insensitive to thapsigargin (Churchill and Galione, 2001a; Genazzani and Galione, 1996), a selective inhibitor of sarco(endo)plasmic reticulum Ca^{2+} ATPase Ca^{2+} pump (Thastrup et al., 1990), the mechanism of Ca^{2+} uptake into this store has remained undefined. A previous attempt to characterize Ca^{2+} uptake by this store produced equivocal results (Genazzani and Galione, 1996), likely due to the confounding effects of multiple Ca^{2+} stores. In contrast, the lysosome-enriched particulate fraction, which is sensitive to NAADP but not $InsP_3$, cADPR, or thapsigargin (Figures 2B and 2E), provided us with the opportunity to characterize the mechanism of Ca^{2+} uptake without the complication of Ca^{2+} uptake into other stores. Ca^{2+} uptake into the lysosome-enriched particulate fraction was inhibited by apyrase, indicating a requirement for ATP (Figure 3A), but uptake was not affected by a pre-addition of a high concentration of thapsigargin (Figure 3A). Moreover, Ca^{2+} uptake was prevented by the protonophore FCCP (carbonyl cyanide p- (trifluoromethoxy) phenylhydrazone), the K^+/H^+ exchanger nigericin, and the weak base NH_3 (Figure 3A), indicating a requirement for a proton gradient. Bafilomycin A1, an inhibitor of vacuolar H^+ pumps powered by ATP (V-H^+-ATPase) (Docampo and Moreno, 1999), also prevented Ca^{2+} uptake. Vanadate, which inhibits Ca^{2+} pumps with a phosphorylated intermediate (P-type), including the countertransporting Ca^{2+}/H^+ ATPase (Rooney and Gross, 1992), did not block Ca^{2+} uptake (Figure 3A), consistent with the insensitivity of NAADP-mediated Ca^{2+} mobilization to thapsigargin (Genazzani and Galione, 1996). It was possible that the lysosome-enriched particulate fraction contained two independent Ca^{2+} stores: one dependent on a proton gradient for Ca^{2+} uptake and another sensitive to NAADP, but non-leaky and replete with Ca^{2+}. This possibility was excluded by the lack of response to NAADP when Ca^{2+} uptake was prevented (Figure 3A, no ATP) and the lack of Ca^{2+} uptake after co-addition of NAADP and bafilomycin (Figure 3). Together, these data indicate that the lysosome-related organelle sensitive to NAADP contains a V-H^+-ATPase and the resulting proton gradient drives Ca^{2+} accumulation likely by an H^+/Ca^{2+} exchanger (Figure 4). This mechanism of Ca^{2+} uptake is consistent with lysosomes maintaining acidic lumina and using the resulting proton gradient to drive Ca^{2+} uptake (Christensen et al., 2002; Dell'Angelica et al., 2000; Docampo and Moreno, 1999).

We next investigated the mechanism of Ca^{2+} reuptake into the NAADP-sensitive Ca^{2+} store in intact eggs. We used a protocol that consists of using a local photorelease of NAADP to release Ca^{2+} from and desensitize a local region of an egg followed by a global photo-

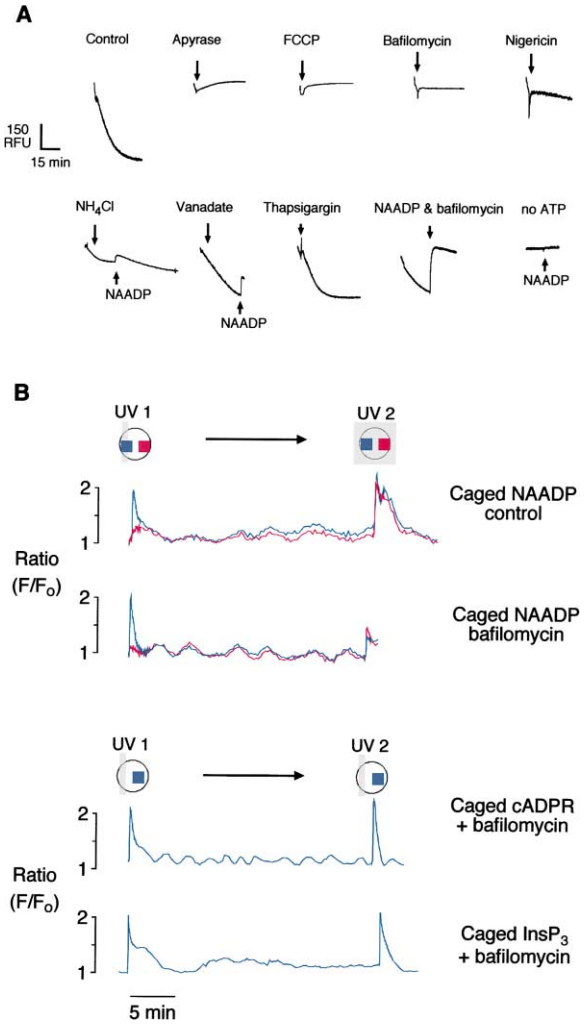

Figure 3. Mechanism of Ca^{2+} Uptake into the NAADP-Sensitive Store

(A) Characterization of Ca^{2+} uptake into a purified NAADP-sensitive lysosomal Ca^{2+} store. The final concentrations of the compounds are as follows: apyrase, 100 U/mL; FCCP, 5 μM; bafilomycin, 1 μM; nigericin, 1 μM; NH_4Cl, 20 mM; vanadate, 1 mM; and thapsigargin 8 μM. The reaction was initiated by adding an aliquot of particulate fraction to intracellular medium containing 1 mM ATP and an ATP regenerating system. Traces are from individual experiments representative of 4–11 experiments.

(B) Effect of the V-H^+-ATPase inhibitor bafilomycin on the recovery from messenger-specific depletion of Ca^{2+} stores. Messengers were photoreleased (arrows) first locally then globally as shown in the schematic diagram. Bafilomycin (1 μM) was present for at least 30 min before the first photorelease. Eggs contained Oregon Green 488 BAPTA Dextran (10 μM) and either caged $InsP_3$ (5 μM), caged cADPR (5 μM), or caged NAADP (0.5 μM) as labeled.

release of NAADP to evaluate recovery from local desensitization (Churchill and Galione, 2001b). Bafilomycin (1 μM) did not affect the response to the initial localized photorelease of NAADP (peak ratio of 1.84 ± 0.02, n = 8, versus 2.06 ± 0.12 for the control, n = 12; p = 0.79), but significantly reduced the response to the second global photorelease of NAADP (peak ratio of 1.26 ± 0.07 compared to 2.09 ± 0.08 for the control; $p < 0.0001$; Figure 3B). In the bafilomycin-treated egg, the responses to the global release of NAADP were equivalently lower in both regions (Figure 3B, red and blue) because of the redistribution of lysosomes during the period of recovery. That is, desensitized organelles moved into the naïve region and vice versa. Such lysosome redistribution was verified by monitoring the movement of lysotracker-labeled lysosomes with fluorescence recovery after photobleaching (data not shown). This result indicates that the lysosomal Ca^{2+} store is replete and non-leaky in resting cells, and that bafilomycin sensitivity is only revealed once the store is mobilized, making its inhibition use-dependent. In contrast, bafilomycin did not affect the initial response or recovery from the photorelease of either $InsP_3$ (peak ratios: UV 1 of 2.11 ± 0.09, UV 2 of 2.08 ± 0.26, n = 3, and control of 2.10 ± 0.05, n = 4; p = 0.99) or cADPR (peak ratios: UV 1 of 2.15 ± 0.12, UV 2 of 2.44 ± 0.29, n = 3, and control of 2.27 ± 0.1, n = 15; p = 0.69) even when the initial response was global (Figure 3B). These data are consistent with an NAADP-sensitive Ca^{2+} store that is non-leaky and dependent on a proton gradient for Ca^{2+} uptake.

In summary, we have identified the reserve granule in sea urchin eggs as a Ca^{2+} store mobilized exclusively by NAADP (Figure 4). Similar organelles function as a nutrient source in the developing embryo in other organisms (Williams, 1967). Such a function in sea urchin remains conjectural, however, because there is no detectable decrease in reserve granule protein, lipids, RNA, or carbohydrates during embryonic development up to the pluteus stage (Armant et al., 1986). Rather, sea urchin reserve granules are functionally analogous to lysosomes (Schuel et al., 1975). Traditionally, lysosomes have been viewed as terminal degradative compartments for macromolecules (Dell'Angelica et al., 2000), but recently lysosomes/reserve granules have been shown to play dynamic roles in processes such as plasma membrane repair by acting as secretory vesicles (McNeil et al., 2000; Reddy et al., 2001).

That reserve granules function as a Ca^{2+} store involved in messenger-mediated cellular signaling ascribes to lysosome-related organelles another non-traditional function. Although such a role for lysosomes in Ca^{2+} signaling has been proposed previously during burst activity in snail neurons (Sugaya and Onozuka, 1978) and in agonist-mediated Ca^{2+} signaling in MDCK cells (Haller et al., 1996), the molecular details are unknown. Our current results may provide a mechanistic explanation. Indeed, agents that collapse proton gradients impair both cholecystokinin-induced Ca^{2+} oscillations in pancreatic acinar cells (Gonzalez et al., 1997) and antigen-mediated signaling in T-lymphocytes (Guse et al., 1994); both of which are signaling events that employ NAADP (Berg et al., 2000; Cancela et al., 1999; Patel et al., 2001).

Experimental Procedures

Egg Collection, Homogenization, and Fractionation

Sea urchin eggs from *Lytechinus pictus* (Marinus, Long Beach, CA) or *Psammechinus miliaris* (Roscoff Marine Station, France) were obtained by intracoelomic injection of 0.5 M KCl, shed into artificial sea water (in mM, NaCl 435, $MgCl_2$ 40, $MgSO_4$ 15, $CaCl_2$ 11, KCl 10, $NaHCO_3$ 2.5, EDTA 1, and [pH 8]), dejellied by passing through 90

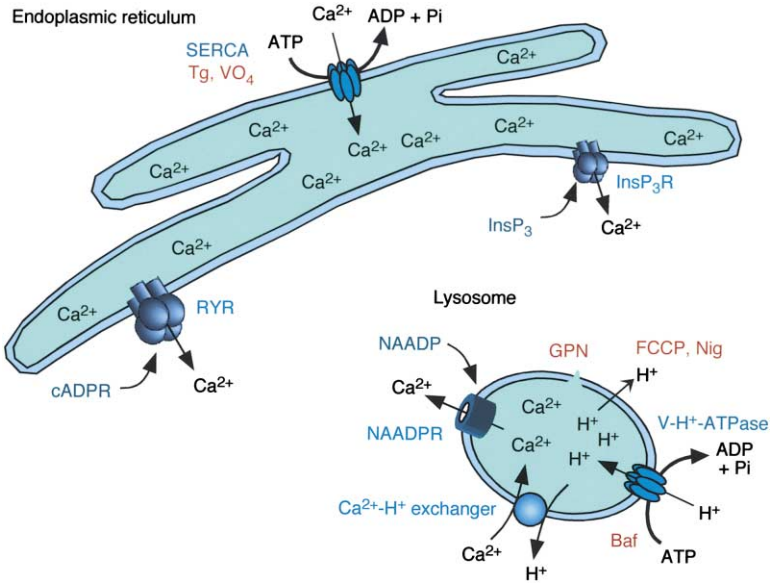

Figure 4. Summary Schematic of the Dynamic Ca^{2+} Regulation in Lysosomes (Reserve Granules) and Endoplasmic Reticulum in Sea Urchin Eggs

Components are distinguished by color as follows: channels and pumps are blue and inhibitors are red. Abbreviations: sarco(endo)plasmic reticular ATPase, serca; thapsigargin, Tg; vanadate, VO_4; ryanodine receptor, RYR; $InsP_3$ receptor, $InsP_3$ R; putative NAADP receptor, NAADPR; vacuolar-proton ATPase, V-H^+ ATPase; carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone, FCCP; nigericin, Nig; and bafilomycin, Baf.

μm nylon mesh, and then washed twice by centrifugation. Homogenates of sea urchin eggs were prepared as described previously (Clapper and Lee, 1985). Briefly, eggs were disrupted in an intracellular-like medium consisting of 250 mM potassium gluconate, 250 mM N-methylglucamine, 20 mM HEPES, and 1 mM $MgCl_2$, [pH 7.2] supplemented with 1 mM ATP, 10 U/mL creatine kinase, and 10 mM phosphocreatine and protease inhibitors. The homogenate was fractionated by Percoll density gradient centrifugation first at 25% Percoll (Clapper and Lee, 1985; Lee and Aarhus, 1995) and then the yolk fraction was layered onto a solution of 50% Percoll to increase the purity of the reserve granules (McNeil et al., 2000). Homogenate (1.25 ml of 50% v/v eggs) was layered onto 9 ml of Percoll solution and centrifuged at 27,000 × g for 30 min at 15°C. Fractions were removed with a syringe by puncturing the vessel wall and stored at −80°C until use.

Marker Enzymes

The abundance of organelles in the subcellular fractions was determined with the marker enzymes Na^+/K^+-ATPase (plasma membrane), glucosaminidase (lysosomes), glucose-6-phosphatase (endoplasmic reticulum), succinate dehydrogenase (mitochondria), β-galactosidase (lysosomes), and acid phosphatase (lysosomes), as described previously (Clapper and Lee, 1985; Lee and Aarhus, 1995).

Radioligand Binding

^{32}P-labeled cADPR and NAADP, prepared enzymatically as described previously (Aarhus et al., 1996; Galione et al., 2000; Patel et al., 2000), were incubated with subcellular fractions in an intracellular-like medium for 10 min at room temperature. The reaction was terminated by rapid filtration (Brandel cell harvester) through GF/B filters (Whatmann) with two 2.4 ml washes with ice-cold buffer (intracellular-like for cADPR and 10 mM Tris, [pH 7.2] for NAADP). Specific binding was taken by subtracting nonspecific (bound in the presence of 10 μM unlabeled cADPR or 1 μM NAADP) from total bound.

Fluorimetry

For the egg homogenate and subcellular fractions, Ca^{2+} concentration was measured with fluo-3 (3 μM) at 17°C, using 500 μl of homogenate in a fluorimeter (Perkin-Elmer LS-50B) at 506 nm excitation and 526 nm emission.

Ca^{2+} Imaging

Intact eggs were imaged as described previously (Galione et al., 2000). Eggs were transferred to polylysine-coated glass coverslips, pressure microinjected with Oregon Green 488 BAPTA Dextran (Molecular Probes) and caged compounds and/or inhibitors. The Ca^{2+}-sensitive dye was imaged by laser-scanning confocal microscopy (TCS NT, Leica) and caged compounds were photolyzed with an ultraviolet laser. Caged $InsP_3$ (Calbiochem) and caged cADPR (Molecular Probes) were obtained commercially, whereas caged NAADP was synthesized essentially as described previously (Lee et al., 1997). Images were processed with the software NIH Image to create a self-ratio by dividing each image (F) by an image acquired before stimulation (F_o). To confirm the activity of the caged compounds, free compounds were coinjected with Texas Red Dextran (Molecular Probes) as a tracer dye used to monitor the injection. Texas Red Dextran was monitored with excitation at 568 nm and emission collected above 590 nm selected with a long-pass filter.

Imaging Lysosomes

Acidic organelles were labeled by incubation with 0.5 nM LysoTracker Red (Molecular Probes) for 20–40 min. Confocal microscopy (as described above) was used to detect LysoTracker Red, with the same settings as for Texas Red Dextran. Eggs were stratified by layering eggs in artificial seawater on a 1 M solution of sucrose and centrifuging at 10,000 × g for 10–13 min as reported previously (Lee, 2001).

Data analysis

Data are presented as mean ± standard error of the mean. Differences were detected with either Student's t test (two means) or analysis of variance (multiple means) followed by Fisher's Least Significant Difference test (StatView, Abacus Concepts).

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