Fertilization and Nicotinic Acid Adenine Dinucleotide Phosphate Induce pH Changes in Acidic Ca\(^{2+}\) Stores in Sea Urchin Eggs\(^*\)

Anthony J. Morgan and Antony Galione
From the Department of Pharmacology, University of Oxford, Oxford OX1 3QT, United Kingdom

The second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) releases Ca\(^{2+}\) from the acidic Ca\(^{2+}\) stores of many organisms, including those of the sea urchin egg. We investigated whether the pH within the lumen of these acidic organelles changes in response to stimuli. Fertilization activates the egg by Ca\(^{2+}\) release dependent upon NAADP, and accordingly, we report that fertilization also alters organellar pH in a spatio-temporally complex manner. Upon sperm fusion, vesicles deep in the egg center slowly acidify, whereas cortical vesicles undergo a rapid alkalization. The cortical vesicle alkalization is independent of exocytosis and cytosolic pH but coincides with the NAADP-dependent fertilization Ca\(^{2+}\) wave. Microinjection of NAADP mimicked the fertilization cortical response, suggesting that it occurred within NAADP-sensitive acidic Ca\(^{2+}\) stores. Our data show that NAADP and physiological stimuli alter the pH within intracellular organelles and suggest that NAADP signals through pH as well as Ca\(^{2+}\).

The sea urchin egg is an invaluable cell model for studying Ca\(^{2+}\) signaling since the discovery of new second messengers and new Ca\(^{2+}\) stores in this system has subsequently proven to have a major impact on mammalian biology (1). The mechanism of activation of the egg upon fertilization encompasses the rapid increases in the cytosolic pH and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) important for fertilization envelope formation, DNA and protein synthesis, and embryological development (2). The pattern of [Ca\(^{2+}\)]\(_i\) changes within the egg are highly organized spatio-temporally and reflect the precisely timed and placed recruitment of different families of Ca\(^{2+}\) channels resident upon either the plasma membrane or intracellular organelles (2, 3). After the initial transient “cortical flash” caused by Ca\(^{2+}\) influx across the plasma membrane (2–5), Ca\(^{2+}\) release from intracellular stores is manifest as a Ca\(^{2+}\) wave that propagates across the entire egg initiating from the point of sperm entry (2). These phasic elevations in [Ca\(^{2+}\)]\(_i\) are driven by a complex interplay between the second messengers inositol 1,4,5-trisphosphate, cyclic adenosine diphosphoribose, and nicotinic acid adenine dinucleotide phosphate (NAADP)\(^2\) (1). Importantly, the relative contribution of each messenger to each phase of the Ca\(^{2+}\) signal is different, e.g. NAADP is the only messenger implicated in the cortical flash (4, 5), whereas cyclic adenosine diphosphoribose probably plays a later role in prolonging the main Ca\(^{2+}\) spike (6).

NAADP not only evokes a cortical flash but, as first revealed in sea urchin egg, also releases Ca\(^{2+}\) from acidic Ca\(^{2+}\) stores that are lysosome-like organelles (possibly yolk platelets in eggs), whereas inositol 1,4,5-trisphosphate and cyclic adenosine diphosphoribose release Ca\(^{2+}\) from the neutral endoplasmic reticulum (1, 7). Recently in sea urchin egg homogenate, we revealed that the luminal pH (pH\(_l\)) of these acidic stores is dynamic and increases upon NAADP-induced Ca\(^{2+}\) release, which is a direct effect of NAADP (and not cytosolic Ca\(^{2+}\)) via changes in luminal proton buffering/transport (8). We have now confirmed that the pH\(_l\) increases observed in homogenate are physiologically relevant in the intact egg upon fertilization. Unexpectedly, these changes are confined to the NAADP-sensitive stores in the egg cortex during a novel, early fertilization event, which may have ramifications for the pH\(_l\) of NAADP-sensitive stores in mammalian cells.

EXPERIMENTAL PROCEDURES

Reagents—NAADP was enzymatically synthesized (9) or purchased from Sigma-Aldrich. Mastoparan (synthetic, Vespuca lewissii) was also obtained from Sigma-Aldrich. Acridine Orange, LysoSensor Yellow/Blue DND-160, LysoTracker Red DND-99, Rhod Dextran (10 kDa, high affinity form), Fluo-4 Dextran (10 kDa), and Alexa Fluor 647 Dextran (10 kDa) were from Invitrogen.

Gamete Preparation—Sea urchin eggs from Lytechinus pictus were harvested by intracoelomic injection of 0.5 M KCl, collected in artificial sea water (ASW; 435 mM NaCl, 40 mM MgCl\(_2\), 15 mM MgSO\(_4\), 11 mM CaCl\(_2\), 10 mM KCl, 2.5 mM NaHCO\(_3\), 20 mM Tris base, pH 8.0) and de-jellied by passage through 100-μm nylon mesh (Millipore). Sperm, on the other hand, were collected “dry” and maintained at 4 °C until use.

Confocal Laser Scanning Microscopy—Eggs adhering to polylsine-coated glass coverslips were placed on the stage of a Zeiss LSM 510 Meta confocal microscope equipped with 10× air and
40× and 63× oil immersion objectives (NA 0.3, 1.3, and 1.4, respectively) and maintained at room temperature in ASW. Simultaneous monitoring of several fluorescent dyes was carried out using the Multitrack mode of the microscope, which rapidly alternates channel collection and thereby greatly reduces bleed-through. For UV excitation, the 364-nm line of a Coherent Enterprise laser was used. The green channel was excited using the 488-nm line of an argon laser (emission, 505–530 nm), whereas the red channel used a 543-nm HeNe laser (emission, >560 nm). In the experiments using an Alexa Fluor 647 Dextran, this third fluorophore was excited with a 633-nm HeNe laser and emission collected at 645–719 nm using the Meta head.

Microinjection—Micropipettes were pulled from capillary glass with an internal filament and backfilled. The pipettes were then mounted in the electrode holder of an Injectman pressure injection system (Eppendorf) used at typical pressures of 200 hPa for 2 s, which produces ~1% injection volumes. Injectate compounds were usually prepared in 0.5 M KCl at 100 times the final required concentration, and insoluble debris was removed by centrifugation through a Centricon filter (100,000 molecular weight cutoff). Rhod Dextran was first prepared as 2 mM aliquots in 100 mM Tris-Cl, pH 8.0, to assist solubility and then diluted 1:1 with 1 mM KCl. The injectate concentration of Alexa Fluor 647 Dextran was 50–200 μM.

pH₇ Measurements—We measured pH₇ with two different approaches. First, eggs were loaded with 2–10 μM Lysosensor Yellow/Blue DND-160 for 15 min with qualitatively similar results at all dye concentrations. Lysosensor Yellow/Blue was imaged ratiometrically on the confocal microscope with excitation at 364 nm and dual emission recorded at 385–470 nm (channel 1) and >505 nm (channel 2). The results are expressed as the ratio of channel 1/channel 2, which is directly proportional to pH₇. In the majority of experiments, however, acridine orange was used because of its superior signal-to-noise and its documented use to monitor pH₇ in both sea urchin egg homogenate (8) and intact eggs (10). However, we modified the acridine orange protocol to measure pH₇ ratiometrically to circumvent movement and cell volume artifacts. Therefore, intact eggs were simultaneously loaded with 10 μM acridine orange and 1 μM Lysotracker Red DND-99 for 15–20 min at room temperature, at which time the fluorescence reaches an equilibrium; the dyes were present throughout the rest of the experiment. Acridine orange responds rapidly and profoundly to changes in pH₇, whereas Lysotracker Red responds only poorly (in agreement with Invitrogen) and remains essentially unchanged throughout the experiment until the addition of NH₄Cl. The results are expressed as the ratios of the acridine orange/Lysotracker Red signals such that an increase in the ratio reflects an increase in pH₇. At higher magnification (see Fig. 6), Lysotracker Red exhibited some photobleaching that resulted in an artifactual rise in the basal ratio in some eggs. This fall in fluorescence was corrected by using a fifth order polynomial curve fit.

In experiments investigating the effect of sodium removal, the eggs were dye-loaded in normal ASW prior to washes in Na⁺-free ASW (also containing the pH₇ dyes) immediately before recording. Na⁺-free ASW was prepared by replacing NaCl with 435 mM N-methyl-D-glucamine and replacing NaHCO₃ with 2.5 mM KHCO₃.

Measurement of [Ca²⁺]ᵢ—The eggs were microinjected with the Ca²⁺-sensitive dye fluo-4 dextran (10 kDa, injectate concentration 1 mM, plus 250 mM KCl, Tris pH 8). After a 15-min recovery period, a second micropipette containing 50–200 μM Alexa Fluor 647 Dextran with or without 100 μM NAADP was used for injection.

Simultaneous Measurement of pH₇ and [Ca²⁺]ᵢ—The eggs were first microinjected with Ca²⁺-sensitive Rhod Dextran (Kᵦ 0.7 μM, 1 mM injectate) and then loaded with acridine orange. However, because Rhod Dextran is a much dimmer dye under basal conditions than Lysotracker Red, acridine orange was used at the lower concentration of 1 μM to reduce bleed-through into the necessarily more sensitive red channel. Qualitatively similar results were seen at both 1 and 10 μM acridine orange when eggs were fertilized, but the fluorescence change was smaller with 1 μM.

Simultaneous Measurement of pH₇ and Exocytosis—Exocytosis was assessed with two different fluorescent techniques. First, by monitoring the disappearance of cortical granules; acridine orange monomers aggregate in acidic vesicles as a function of pH₇, and consequently their fluorescence undergoes a red shift as a function of aggregation (10, 11). When excited with the red laser, a selective staining of a narrow (1–2 μm) peripheral ring is observed that corresponds to the acidic cortical granules docked at the plasma membrane (12). The eggs were therefore labeled as before with 10 μM acridine orange for 15–20 min, but this time they were simultaneously viewed in the green and red channels using the Multitrack mode of the microscope (emission/excitation, 488 nm/505–530 nm (green); 543 nm/>560 nm (red)). Note that in the previous ratiometric experiments, the Lysotracker Red signal swamped the dimmer acridine orange red fluorescence.

We used another approach to validate the loss of red acridine orange fluorescence; the extracellular space was also fluorescently labeled with a long wavelength, cell-impermeant dye (10 μM Alexa Fluor 647 Dextran), whose signal was simultaneously collected with the red (cortical granule) and green (pH₇) channels as indicated above. At high magnification (63× objective, 2× digital zoom), the exocytosis of individual cortical granules observed with the red acridine orange fluorescence was confirmed when the space vacated by the granule filled with extracellular dye (data not shown), as originally developed by Terasaki (13). The data were analyzed by using Student’s t test, paired where appropriate, or by creating a 2×2 contingency table analyzed with Fisher’s exact test. The significance was set at p < 0.05.

RESULTS

Fertilization-induced pH₇ Changes—Sperm activate eggs and evoke Ca²⁺ release from acidic stores via NAADP (5). Measuring pH₇ changes within these acidic Ca²⁺ stores revealed that fertilization altered the pH₇ after a short lag (Fig. 1). The acridine orange signal changed substantially more than did Lysotracker Red (which is less pH₇-sensitive, see “Experimental Procedures”), consistent with an alteration in pH₇ rather than a morphological artifact (Fig. 1, B and C). Fig. 1D
Ca\textsuperscript{2+} and pH\textsubscript{L} at Fertilization

![Diagram](image)

**FIGURE 1.** Fertilization-induced changes in pH\textsubscript{L} in intact sea urchin eggs. The eggs were co-loaded 10 \textmu M acridine orange (AO) and 1 \textmu M Lysotracker Red (LTR) and fertilized with 0.1% (v/v) ejaculate where indicated. All of the images shown were captured using a 10× objective. A, pseudocolor images of the ratio of acridine orange/Lysotracker Red fluorescence where cool colors represent low pH\textsubscript{L} and warmer colors represent higher pH\textsubscript{L}. The time in seconds is shown in the upper left corner of each image. B, raw fluorescence of acridine orange (solid traces) or Lysotracker Red (broken traces) taken from the egg periphery in accordance with the inset schematic of a single egg. Sperm added when indicated followed by 10 mM NH\textsubscript{4}Cl (C) as for B except that the raw data is taken from the egg center. D, peripheral (red) and central (green) ratiometric (acridine orange/Lysotracker Red) pH\textsubscript{L} changes derived from the traces plotted in B and C. The broken portion of the red trace corresponds to the artifactual ratio because of the loss of fluorescence from the peripheral ROI as the fertilized egg apparently changes shape. E, the ratiometric pH\textsubscript{L} response proceeds as a wave around the egg cortex. The traces correspond to their color-matched ROIs in the inset for the same egg in B and C. F, bar graph showing the change in ratio in the egg periphery (Peri) and center (means ± S.E. of 77 eggs).

shows the calculated ratio, and unexpectedly, this was not a spatially homogeneous response; around the periphery (cortex) of the egg, pH\textsubscript{L} promptly increased in a narrow band, whereas in the center of the egg pH\textsubscript{L} decreased, albeit with slower kinetics (Fig. 1, D and F). The transient and spatially restricted nature of the pH\textsubscript{L} increase was highly reminiscent of the [Ca\textsuperscript{2+}]i cortical flash (4, 5), for which reason we have termed this novel phenomenon a “pH\textsubscript{L}ASH” (pronounced “flash”). This peripheral vesicular alkalinization did not reflect an apparent unresponsiveness of the egg center because NH\textsubscript{4}Cl evoked a profound alkalinization across the entire cell (Fig. 1, A, C, and F). The results were confirmed using the chemically and spectrally dissimilar ratiometric dye, LysoSensor Yellow/Blue DND-160, albeit with an inferior signal-to-noise (see supplemental Fig. S1). Fertilization therefore induces changes in organellar pH that are highly organized in time and space.

In cells fortuitously inseminated in the right orientation, this pH\textsubscript{L}ASH proceeded as an alkalinization “wave” around the cell cortex (Fig. 1E). It is worth noting that in many eggs, the exocytotic lifting of the fertilization envelope results in such a dramatic change in cell shape/focus that a parallel fall in the fluorescence of both dyes occurs that is unrelated to changes in pH\textsubscript{L} and is depicted as a broken line in the ratio plot (Fig. 1D). (Note that this also accounts for the artifactual differences in the ratometric NH\textsubscript{4}Cl response in the periphery versus the center; Fig. 1F.)

When measuring pH\textsubscript{L} alone, cell-to-cell asynchrony (Fig. 1A) made it difficult to gauge how this pH\textsubscript{L}ASH related temporally with the fertilization Ca\textsuperscript{2+} response, i.e., would the pH\textsubscript{L}ASH coincide with the Ca\textsuperscript{2+} cortical flash (Ca\textsuperscript{2+} influx) or the main Ca\textsuperscript{2+} wave (intracellular Ca\textsuperscript{2+} release)? Therefore, we simultaneously measured pH\textsubscript{L} and [Ca\textsuperscript{2+}]i using acridine orange and the Ca\textsuperscript{2+} dye Rhod Dextran. In these dual labeling experiments, the first detectable insemination response was the Ca\textsuperscript{2+} cortical flash (Fig. 2A, left panels), followed after a delay by the main Ca\textsuperscript{2+} wave (Fig. 2).

Temporally, the pH\textsubscript{L}ASH overlapped with the main Ca\textsuperscript{2+} wave (Fig. 2, A and B), with the pH\textsubscript{L} upstroke occurring ostensibly with or just after the sharp [Ca\textsuperscript{2+}]i upstroke; the bar graph in Fig. 2C shows that the lag between sperm addition and the cortical flash is <30 s, whereas the main Ca\textsuperscript{2+} wave and pH\textsubscript{L}ASH both peak at ~65 s. In other words, despite their spatial overlap, the Ca\textsuperscript{2+} cortical flash and pH\textsubscript{L}ASH are temporally divorced; the latter coincides more with the main wave. This timing is consistent with the pH\textsubscript{L}ASH being associated with the intracellular Ca\textsuperscript{2+} release phase.

Spatially, the Ca\textsuperscript{2+} wave and pH\textsubscript{L}ASH wave initiated from the same pole in 7/10 cells (Fisher’s exact test, p < 0.02) and propagated through the egg periphery to the antipode (Fig. 2B). Despite a heterogeneous pH\textsubscript{L} response, the main Ca\textsuperscript{2+} wave
amplitude was the same in the periphery and center (data not shown). Because the Ca\textsuperscript{2+} wave initiates at the point of sperm entry (2), we conclude that the pH\textsubscript{L} wave also starts at the point of sperm fusion.

**pH\textsubscript{L} Events and Exocytosis**—However, it was possible that the pH\textsubscript{L} change is secondary to Ca\textsuperscript{2+}-stimulated exocytosis (and/or endocytosis), e.g., upon vesicle fusion with the plasma membrane, the alkaline extracellular medium exchanges with the acidic lumen of cortical granules, the primary egg exocytotic vesicles (12, 14). Therefore, we simultaneously recorded pH\textsubscript{L} changes and exocytosis to see whether there was any spatial or temporal overlap. Exocytosis was monitored as the disappearance of cortical granule labeling (12).

Measuring the fluorescence at the periphery, fertilization induced a prompt fall in the red fluorescence, which corresponded to the loss of cortical granules via exocytosis (Fig. 3, A and B). By altering the focus, we verified that this was a true loss of staining rather than egg movement in the focal plane (data not shown). The initiation of the pH\textsubscript{L}AS and the loss of cortical granules were, on average, simultaneous (Fig. 3A) with a lag of only 5 \pm 3 s, which was not significant (n = 34, p > 0.05). However, this obscured the fact that the pH\textsubscript{L}AS readily evoked cortical granule loss (76 \pm 3% of sperm control, n = 37), it did so without any detectable pH\textsubscript{L}ASH (\texttext{-4 \pm 6% sperm, p < 0.001; Fig. 3D}). Moreover, the addition of sperm after mastoparan elicited no response compared with parallel fertilization controls (\text{-8 \pm 1%, p < 0.001}). This is consistent with a physical barrier (i.e., the fertilization envelope) being induced by the venom.

The fact that exocytosis and pH\textsubscript{L}AS can be divorced under a variety of circumstances strongly supports an independent mechanism for the latter. Because it is well known that endocytosis takes place minutes after the exocytotic burst (12, 17), we conclude that neither of these events is responsible for the pH\textsubscript{L}AS.

**Unitary pH\textsubscript{L} Events**—Not only did the higher resolution images confirm that the pH\textsubscript{L}AS occurred well away from the fusion with the plasma membrane, but pH\textsubscript{L}AS was revealed as a summation of smaller events (which we term “pH\textsubscript{L}ARES, pronounced “flares”). When focused at the egg equator, pH\textsubscript{L}ARES were seen to propagate as a wave along a 15-\textmu m-wide cortical band (Fig. 4A). When plotted as a function of distance from the plasma membrane, both the rate of rise and amplitude of these events were well maintained up to \textsim 15 \textmu m.

**Ca\textsuperscript{2+} and pH\textsubscript{L} at Fertilization**
**Ca\(^2+\) and pH\(_L\) at Fertilization**

**FIGURE 3. Comparison of pH\(_L\) and exocytosis during stimulation by sperm or mastoparan.** Sea urchin eggs labeled with 10 \(\mu\)M acridine orange were viewed under green (pH\(_L\)) and red (cortical granule, CG) fluorescence. A, sequence of images of both channels showing the raw green acridine orange fluorescence (upper left), and pH\(_L\)ASH (a pseudo-colored self-ratio) and red cortical granule exocytosis, at times indicated in seconds (sperm added 58 s). The traces in A depict the mean peripheral fluorescence changes of a single egg, B, correlation plot of the initiation times of pH\(_L\)ASH versus exocytosis with the line of best fit. C, profile plot along the equatorial line drawn on the cell in the upper left of A. Maximum fluorescence changes (mean of two or three consecutive images) were calculated as the difference between basal and fertilized eggs and normalized to their maximum values (pH\(_L\) green; cortical granules, red). D, effect of 200 \(\mu\)M mastoparan (and subsequent sperm and 10 mM NH\(_4\)Cl) upon pH\(_L\) (green trace) and cortical granule exocytosis (red trace). The results are representative of 34–55 eggs.

**FIGURE 4. Sperm-induced pH\(_L\)ASH unitary events monitored at two focal planes.** Shown is a series of high magnification images focused at either the equator of the egg (A) or at the bottom of the egg viewed side by side (B) monitoring pH\(_L\) changes with 10 \(\mu\)M acridine orange. Time(s) of each image is indicated in white. Sperm were added at 56 s (A) or 60 s (B). The traces are fluorescence records underlying the color-matched squares indicated in the first panel of the series. The images for A and B are depicted at the same magnification. The results are representative of four eggs/condition.

from the cortical granules (Fig. 4A, red to yellow ROIs), but both parameters fell off dramatically at deeper loci.

Discrete pH\(_L\)ARES were more clearly observed when focused along the bottom of the egg in the plane of the egg cortex. Viewed en face (Fig. 4B), individual pH\(_L\)ARES with a \(~2\ \mu\)m diameter were more clearly resolved (Fig. 4B, white arrows) presumably because of reduced scatter and turbidity at the egg-substratum interface. Unlike the equatorial slice, the bottom of the egg shows pH\(_L\)ARES firing across the entire field of view that were invariant with distance from the cell edge (Fig. 4B, cf. red and black ROIs). Together, the data indicate that pH\(_L\) changes can be resolved as small events that are spatially divorced from exocytosis.

**pH\(_L\) and Cytosolic pH**—In a previous egg population study, the slow vesicular acidification upon fertilization was secondary to changes in cytosolic pH driven by the plasma membrane sodium-proton exchanger (NHE) (10). Therefore, we tested whether the NHE was also involved in the subplasmalemmal pH\(_L\)ASH by inhibiting the NHE with Na\(^+\)-free medium. However, this had no effect upon the pH\(_L\)ASH amplitude (Fig. 5, \(p > 0.1\)), pH\(_L\)ASH kinetics (time to peak in s: control, 67 ± 2; Na\(^+\)-free, 69 ± 2; \(p > 0.4\)), or fertilization envelope formation (data not shown). Nonetheless, it entirely blocked the acidification of granules deeper in the egg (Fig. 5, B and C, \(p < 0.001\)) in agreement with previous work (10). We conclude that the cortical pH\(_L\) response was not secondary to NHE-driven changes in cytosolic pH.

**NAADP-induced pH\(_L\)ash**—It was unexpected that fertilization could evoke vesicular alkalinization in such a restricted subcellular locus when acidic vesicles are found throughout the egg (7, 10). Given that fertilization is NAADP-dependent, and NAADP is the only Ca\(^2+\)-mobilizing second messenger that evokes an increase in pH\(_L\) (8), we microinjected eggs with NAADP to map the spatial distribution of NAADP-sensitive acidic Ca\(^2+\) stores that respond with a change in pH\(_L\).

In control eggs microinjected with a fluorescent injection marker alone, no substantial change of the pH\(_L\) (Fig. 6, A and C) or Ca\(^2+\) (Fig. 6, D and F) was observed in any part of the egg. By contrast, when NAADP was co-injected into the center of the egg (as judged by the fluorescent marker, left hand images), a rapid and profound alkalinization was evoked in the characteristic peripheral pattern of the pH\(_L\)ASH (Fig. 6, B and C). This was not due to a heterogeneous Ca\(^2+\) response because NAADP evoked a global increase (Fig. 6, E and F). In other words, the pH\(_L\)ASH response seen with sperm is entirely consistent with the intrinsic nature of a subpopulation of NAADP-sensitive stores rather than upstream signal compartmentation.

Closer examination revealed that the pH\(_L\) response to NAADP exhibited aspects that were both similar to as well as different from those induced by sperm. The spatial aspects of the pH\(_L\)ASH were clearly well preserved and sometimes pro-
The release of Ca\(^{2+}\) from internal stores has long been recognized as the primary drive for the fertilization-induced Ca\(^{2+}\) signal (2) as well as other mammalian processes (1), but the involvement of non-endoplasmic reticulum Ca\(^{2+}\) stores was underscored when NAADP was shown to evoke Ca\(^{2+}\) release from acidic, lysosome-related organelles in both sea urchin egg and mammalian cells (1, 7). In sea urchin egg, NAADP-induced Ca\(^{2+}\) release seems to contribute to all phases of the fertilization Ca\(^{2+}\) spike from the initial cortical flash through to the falling phase of the main Ca\(^{2+}\) transient (5). Recently we demonstrated that the pH\(_{1}\) of these acidic Ca\(^{2+}\) stores is increased by NAADP in sea urchin egg homogenate by a mechanism coupled to Ca\(^{2+}\) release via the NAADP receptor on acidic Ca\(^{2+}\) stores (8). Interestingly, this pH\(_{1}\) change is not secondary to an increase in [Ca\(^{2+}\)] at the cytosolic face, nor can it be mimicked by Ca\(^{2+}\) release from the endoplasmic reticulum but rather is a direct effect of NAADP upon the vesicles themselves (8). However, this is not at the level of the H\(^{-}\)-ATPase or the vesicle membrane potential but may be due to changes in luminal H\(^{+}\) buffering (8). In view of this, we have investigated NAADP-dependent pH\(_{1}\) events under physiological conditions, i.e. at fertilization.

The pH\(_{1}\) of acidic vesicles in unfertilized eggs has been reported to be approximately pH 5.5 for both cortical granules (14) and yolk platelets (18), and it has been proposed that the pH within these vesicles serves to regulate luminal enzyme activity (14, 19–21) and/or vesicular integrity (18). That the pH\(_{1}\) within egg acidic vesicles can slowly acidify has been suggested during oogenesis (11, 20, 21) and post-fertilization (10), but with the exception of the rapid alkalinization of cortical granules upon fusion and exocytosis (12, 14), rapid increases in the pH\(_{1}\) of acidic vesicles have not been reported.

Corroborating our egg homogenate studies (8), the luminal pH of acidic organelles in intact eggs is coupled to both fertilization and NAADP-induced Ca\(^{2+}\) release with a prompt alkalinization. We would argue that our assays of pH\(_{1}\) are reliable because similar results were ratiometrically recorded with both acridine orange, which has been well characterized in this system (8, 10), as well as with LysoSensor Yellow/Blue DND-160. Importantly, pH\(_{1}\) changes were clearly independent of exocytosis/endocytosis and cytosolic pH changes. Moreover, acridine orange is unlikely to be responding to the oxidative burst at fertilization because this initiates much later, 2–3 min after insemination (19, 22–25), and direct addition of H\(_{2}\)O\(_{2}\) (10 \(\mu\)M to 1 mM) to eggs did not induce a pH\(_{1}\) ASH (data not shown). Taken together, our data strongly support an alkalinization of cortical vesicles.

**Spatial Characterization of the pH\(_{1}\) Response**—Fertilization evoked a spatially heterogeneous change in organelar pH, with a rapid, cortical alkalinization followed by a central, slow acidification. Only the latter was previously detected in asynonomous populations of eggs (10), hardly surprising when the transient pH\(_{1}\) ASH only extends 15–25 \(\mu\)m into the cortex (in an egg ~120 \(\mu\)m in diameter). Moreover, alkalinization propagated as a wave around the egg cortex at ~5 \(\mu\)m/s, a velocity similar to the main [Ca\(^{2+}\)] wave and the exocytotic lifting of the fertilization envelope (26, 27). At higher magnification, the alkalinization wave was manifest as the summation of smaller events.
**Ca\(^{2+}\) and pH\(_{L}\) at Fertilization**

![Diagram](Image)

**FIGURE 6. Effect of NAADP upon pH\(_{L}\) and \([Ca^{2+}]\) in intact eggs.** Acidine orange/Lysotracker Red was used to monitor pH\(_{L}\) changes (A–C) with injectate containing 200 \(\mu\)M Alexa Fluor 647 Dextran alone (A) or together with 100 \(\mu\)M NAADP (B). To measure \([Ca^{2+}]\) changes, unloaded eggs were injected with fluo-4 dextran prior to a second injection with or without NAADP (D–F). Images (A, B, D, and E) show the time in seconds in the upper left corners. The left-hand image of each trio shows the raw fluorescence of the location (and time) of Alexa Fluor 647 Dextran injection (bright center), along with the peripheral (Peri) (red) and central (green) ROIs used to derive the fluorescence traces. The central image shows a preinjection basal acridine orange/Lysotracker Red ratio (A and B) or F/F\(_0\) image (D and E), whereas the right-hand image depicts the ratio image at the peak of each response. Broken portions of the traces in B and E correspond to the artificial fall because of the change in cell shape. Summary of maximal peak responses to injection measuring pH\(_{L}\) (C) or \(Ca^{2+}\) (F). The data are the means ± S.E. of 12–16 eggs for pH\(_{L}\) and for 3 (Control) and 9 (NAADP) eggs for \(Ca^{2+}\).

**FIGURE 7. Working model of pH\(_{L}\) changes in cortical acidic \(Ca^{2+}\) stores at fertilization.** The upper images show pH\(_{L}\) and \([Ca^{2+}]\) changes corresponding to the lower schematics of eggs and cortical acidic \(Ca^{2+}\) stores. For the upper images, blue represents basal levels, whereas warmer colors reflect increases in either pH\(_{L}\) or \([Ca^{2+}]\). Similarly, in the egg schematic a rainbow scheme reflects the concentration of \(Ca^{2+}\). Cortical granules and acidic vesicles are red when acidic, and green when alkalinated during the pH\(_{L}\) ASH. The red arrows correspond to \(Ca^{2+}\) influx across the plasma membrane (the cortical flash) or release from acidic stores through NAADP receptors (NAADP R).

(pH\(_{L}\)ARES, 2–3 \(\mu\)m in diameter) that appear to represent alkalization at the individual vesicle level.

From which vesicles these pH\(_{L}\)ARES emanate is presently unclear. From electron micrographs the 1–3-\(\mu\)m diameter yolk platelets (20, 28, 29) and other candidate “acidic vesicles” (28) correlate well with the size of a pH\(_{L}\)ARES, particularly if one allows for diffusion of acidine orange lost from the vesicle upon alkalization. Deeper exocytotic cortical vesicle classes have been described (30) and the so-called “basal laminar” and “apical” vesicles are both in the right place to host the pH\(_{L}\)ARES. Defining the vesicle population will be crucial to understanding the ramifications of this novel phenomenon.

Why the fertilization pH\(_{L}\)ASH is restricted to the egg cortex is not entirely clear but might reflect either localized messenger accumulation or a unique property of the acidic stores themselves. Our data are more consistent with the latter because global NAADP microinjection evoked a local pH\(_{L}\)ASH, and it is known that the cortical \(Ca^{2+}\) stores are the most sensitive to NAADP (31, 32). Nonetheless, we cannot formally exclude local messenger production as a contributory factor at fertilization.

**Temporal Characterization of the pH\(_{L}\)ASH**—Despite the spatial similarity of the pH\(_{L}\)ASH and \(Ca^{2+}\) cortical flash, there was no temporal overlap, and the pH\(_{L}\)ASH occurred with or just after the main \(Ca^{2+}\) wave, ∼35 s later. However, this timing makes sense because it coincides with NAADP-induced \(Ca^{2+}\) release from intracellular stores (5). Indeed, both the pH\(_{L}\)ASH and main \(Ca^{2+}\) response propagate as waves, which is additional evidence for their being interrelated phenomena. Note that the difference in the upstroke kinetics of the \(Ca^{2+}\) and pH\(_{L}\) changes in intact eggs is consistent with those observed in homogenate (8).

We were concerned that the pH\(_{L}\)ASH may be related to \(Ca^{2+}\)-stimulated exocytosis, which occurs...
6–8 s after the main [Ca\(^{2+}\)]\(_{i}\) rise in urchin eggs (13, 33) and which alkalizes cortical granules upon plasmalemmal fusion (12, 14). Our conclusion is that the pH\(_{i-HASH}\) is not due to exocytosis based on the following evidence: (a) Deeper pH\(_{i}\) events are too remote to be due to fusion with the plasma membrane, although it might be argued that these deeper pH\(_i\) ARES artifically represents the “tail” of exocytotic events emanating from out of the plane of focus, recording from thin optical slices (2–3% of the egg diameter) at the egg equator means that the curvature of the plasma membrane is minimal, and out-of-focus cortical granules will not overlap this in-focus region. In addition, the amplitude and upstroke kinetics of the pH\(_{i-HASH}\) are well maintained over 15–25 \(\mu\)m, which is inconsistent with the tail of out-of-focus events, which would be blunted; (b) Exocytosis and pH\(_i\) changes can be divorced, at the level of individual eggs, as well as by mastoparan, which stimulated cortical granule exocytosis but not pH\(_{i-HASH}\). Therefore, the approximate post-fertilization sequence of events is Ca\(^{2+}\) cortical flash \(\gg\) main Ca\(^{2+}\) wave \(\approx\) pH\(_{i-HASH}\) > exocytosis (Fig. 7).

Not only do we describe a novel fertilization pH\(_i\) event, but we also offer a mechanism whereby sperm elevate NAADP levels in the egg (5), which in turn raise pH\(_i\) via activation of the Ca\(^{2+}\)-mobilizing NAADP receptor on acidic vesicles (8) (Fig. 7). Moreover, we reveal that the unique spatial profile of the pH\(_{i-HASH}\) reflects the intrinsic nature of the cortical stores themselves rather than some upstream mechanism. We also conclude that vesicle alkalization is independent of changes in cytosolic Ca\(^{2+}\), vesicular membrane potential, H\(^+\)-ATPase activity (8), cytosolic pH, and exocytosis and appears to be at the level of NAADP-induced changes in H\(^+\) buffering (8).

The importance of the egg cortex for egg function is under-scored by many cell biological aspects (reviewed in Ref. 34). In addition to supporting layers of exocytotic vesicle populations (30), it also hosts the [Ca\(^{2+}\)]\(_{i}\), cortical flash (4, 5), a unique tubular endoplasmic reticulum morphology with an exclusive complement of luminal Ca\(^{2+}\)-binding proteins (35, 36), as well as a greater density of ryanodine receptors (37). To this and other properties, we add the fertilization-dependent alkalization of NAADP-sensitive acidic Ca\(^{2+}\) stores. The regulation of organelle pH by activation of the NAADP receptor on the vesicle membrane poses exciting questions relating not just to sea urchin biology but also to mammalian biology. Certainly, key enzymes in the lumen of egg vesicles are regulated by pH changes at fertilization (14, 38), but in the acidic organelles of mammalian cells, luminal pH influences processes as diverse as secretory vesicle fusion (39), autophagy (40), and proteolysis (41). Our results may have far-reaching implications for NAADP regulating various cellular pathways via pH and not just by Ca\(^{2+}\).

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