Intracellular Ca^{2+} is able to control numerous cellular responses through complex spatiotemporal organization. Ca^{2+} waves mediated by inositol trisphosphate or ryanodine receptors propagate by Ca^{2+}-induced Ca^{2+} release and therefore do not have an absolute requirement for a gradient in either inositol trisphosphate or cyclic ADP-ribose, respectively. In contrast, we report that although Ca^{2+} increases induced by nicotinic acid adenine dinucleotide phosphate (NAADP) are amplified by Ca^{2+}-induced Ca^{2+} release locally, Ca^{2+} waves mediated by NAADP have an absolute requirement for an NAADP gradient. If NAADP is increased such that its concentration is spatially uniform in one region of an egg, the Ca^{2+} increase occurs simultaneously throughout this area, and only where there is diffusion out of this area to establish an NAADP gradient is there a Ca^{2+} wave. A local increase in NAADP results in a Ca^{2+} increase that spreads by NAADP diffusion. NAADP diffusion is restricted at low but not high concentrations of NAADP, indicating that NAADP diffusion is strongly influenced by binding to immobile and saturable sites, probably the NAADP receptor itself. Thus, the range of action of NAADP can be tuned by its concentration from that of a local messenger, like Ca^{2+}, to that of a global messenger, like IP_3 or cyclic ADP-ribose.

Intracellular Ca^{2+} functions as a messenger that controls many processes including muscle contraction, secretion, fertilization, and development (1–3). Ca^{2+} is sequestered into intracellular stores by pumps and is released by at least three distinct pathways mediated by intracellular messengers inositol 1,4,5-trisphosphate (IP_3) (1, 2), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP) (4–6). The resulting increase in intracellular Ca^{2+} can be organized into complex patterns over time to give oscillations and waves mediated by either IP_3 or ryanodine receptors, the two well characterized families of intracellular Ca^{2+} channels (10, 11), propagate by a similar underlying mechanism whereby Ca^{2+} diffuses between channels and is then regenerated by CICR (1, 2, 7–9). The sensitivity of the IP_3 receptor and the ryanodine receptor to positive feedback by Ca^{2+} is modulated by IP_3 (1, 10, 12) and cyclic ADP-ribose (5, 13), respectively. An increase in IP_3 turns the cytoplasm into an excitable medium such that any increase in Ca^{2+} of sufficient magnitude is propagated as a regenerative Ca^{2+} wave (7, 9, 14). IP_3-mediated Ca^{2+} waves can propagate even when the concentration of IP_3 is constant and spatially uniform (7, 9), demonstrating that they do not have an absolute requirement for IP_3 diffusion.

NAADP was originally shown to mobilize Ca^{2+} in the sea urchin egg (4) and subsequently has been shown to be active in both mammalian (15, 16) and plant tissues (17). The Ca^{2+} stores targeted by NAADP are physically and pharmacologically distinct from those targeted by IP_3 and cADPR (4, 18). Subcellular fractionation has shown that both IP_3 and cADPR release Ca^{2+} from density-gradient fractions enriched in endoplasmic reticulum, whereas NAADP releases Ca^{2+} from all density-gradient fractions (4). Depletion of Ca^{2+} stores sensitive to the Ca^{2+} pump inhibitor thapsigargin eliminates Ca^{2+} release mediated by IP_3 and cADPR but not NAADP (18). Unlike IP_3 (12, 19) and cADPR (13), Ca^{2+} release mediated by NAADP is neither potentiated nor inhibited by Ca^{2+} (15, 18, 20). Uniquely, a pretreatment with a concentration of NAADP that does not release any Ca^{2+} can completely eliminate the response to a subsequent supramaximal concentration of NAADP (21, 22). This functional desensitization probably corresponds to irreversible binding of NAADP, suggesting that it occurs at the level of the NAADP receptor (22).

In intact sea urchin eggs, the microinjection of NAADP has been shown to elicit a global regenerative Ca^{2+} wave (21, 23). We now report that the mechanism underlying NAADP-mediated Ca^{2+} waves is fundamentally different from that of Ca^{2+} waves mediated by IP_3 or ryanodine receptors. Specifically, global Ca^{2+} waves mediated by NAADP have an absolute requirement for an NAADP gradient. Nevertheless, functional coupling to either IP_3 receptors or ryanodine receptors locally amplifies NAADP-mediated Ca^{2+} increases. At low concentrations NAADP is spatially restricted within a cell and acts as a local messenger, like Ca^{2+}, whereas at high concentrations NAADP is more mobile and can act as a global messenger, like IP_3 and cADPR.

**EXPERIMENTAL PROCEDURES**

**Eggs**—Sea urchin eggs of Lytechinus pictus were obtained by in-tracoelomic 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), dejellied by passing through a 90-μm nylon mesh, and then washed twice.

**Microinjection**—Eggs were transferred to polylsine-coated glass coverslips for microinjection and microscopy. Fluo-3 or Oregon Green 488 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) Dextran (Molecular Probes) was pressure-microinjected (Picospritzer, World Precision Instruments) to 50 or 10 μM, respectively, as estimated by comparison with a standard curve. The standard curve was prepared by imaging pipettes containing the dye at various known concentrations with free Ca^{2+} set at 100 nM by a 10 mM Ca^{2+} EGTA buffer. To ensure that solutions containing heparin or 8-amino-cADPR differed from the control solution by only the presence of the inhibitor(s), dried down aliquots of chelex-treated inhibitor solutions were solubilized in the
Ca2+ Imaging — The Ca2+-sensitive dye was imaged by laser-scanning confocal microscopy (TCS NT, Leica) using the 488-nm line of an argon ion laser for excitation, and the emission was long-pass filtered (515 nm) and detected with a photomultiplier tube. Images were processed with the software NIS Image to create a self-ratio by dividing each image by an image acquired before stimulation to correct for any differences in path length and spatial differences in dye distribution.

Photorelease of Caged Compounds — Caged NAADP (2′,3′-P-1,2-nitrophenoxyethyl NAADP, Molecular Probes) was purified further by high pressure liquid chromatography to remove small amounts of contaminating free NAADP (24), which can potently desensitize the NAADP response at concentrations less than those required for activating Ca2+ release (21, 22). Caged NAADP and caged Ca2+ (2-nitrophényl EGTA, Molecular Probes) were photolyzed with ultraviolet light (351- and 364-nm lines) from an argon ion laser (Enterprise Model 651, Coherent) that was directed into the scanning head by a quartz fiber optic cable. A shutter was placed in the light-path of the ultraviolet laser to control the position and duration of the photorelease. This resulted in a band of UV across the image with the position and width of the band being controllable. Although the absolute amount of compound photolyzed was unknown, the relative differences are proportional to the amount of UV delivered to the egg. The amount of UV was controlled by varying the intensity of the UV laser and neutral density filters. Intensity is expressed as either μW emitted from the objective lens or integrated UV illumination calculated as the product of power, neutral density attenuation, area illuminated, and number of frames.

Statistical Analysis — Where appropriate, data were subjected to analysis of variance with means separated by Fisher’s least significant difference with probability set to 0.05.

RESULTS AND DISCUSSION

NAADP-mediated Ca2+ Waves Spread by NAADP Diffusion — A regenerative Ca2+ wave shows a constant amplitude and velocity (7–9, 25), whereas a diffusive Ca2+ wave shows a decrease in amplitude and velocity with time (26). Photorelease of NAADP in a band at the edge of an egg resulted in an immediate increase in Ca2+ in this region, which then spread across the cell with a decrease in amplitude and velocity (Fig. 1a, 25 μW UV). The spread of the Ca2+ increase appears diffusive, but this may have been due to insufficient NAADP being released to ignite a regenerative Ca2+ wave. To compare the amount of NAADP released among experiments, a UV dose was calculated. The normalized amplitude of the Ca2+ increase was proportional to the UV dose (Fig. 1c), demonstrating that UV dose is a valid measure of the amount of NAADP released. When NAADP was photoreleased to a higher concentration, both the velocity and the amplitude of the response increased (Fig. 1a, 50 μW UV) relative to the response to a lower concentration of NAADP (Fig. 1a, 25 μW UV). Nevertheless, regardless of the amount of NAADP released, the velocity of the Ca2+ spread decreased with time (Fig. 1b), as predicted for a process governed by diffusion. Moreover, the relationship between the position of the Ca2+ increase versus time can be fit (r2 = 0.99, 25 μW; r2 = 0.94, 50 μW) to an equation that describes diffusion in three dimensions (Fig. 1b), where distance ∝ (6Dt)1/2 and D is the diffusion coefficient (26), providing quantitative evidence for a diffusive mechanism.

For a non-regenerative Ca2+ wave the diffusion coefficient of the propagating molecule can be calculated directly from changes in the position of the wave front over time. Diffusion coefficients were obtained by fitting the diffusion equation to plots of distance versus time, as shown in Fig. 1b. The resulting diffusion coefficients varied with UV dose from approximately 50 to 150 μm2/s (Fig. 1d). The estimate of the diffusion coefficient is based on diffusion in three dimensions. If it is based on diffusion in two dimensions or one dimension, the values are scaled larger by a factor of 1.5 or 3, respectively (26), but the relative magnitude of the concentration dependence remains the same. The concentration dependence of the diffusion coefficient of NAADP is probably not due to metabolism of NAADP because its time constant for degradation in sea urchin homogenate is 118 min (27), which amounts to less than 1% degradation in 1 min. Rather, the concentration dependence suggests...
that NAADP diffusion is restricted by immobile buffers in the cytoplasm, as is the case for Ca$^{2+}$ (28). The values of the calculated diffusion coefficient overlap with that for Ca$^{2+}$ (approximately 100 $\mu$m$^2$/s (28) when free Ca$^{2+}$ is 3 $\mu$m (the maximum reached during these experiments)). However, a local increase in Ca$^{2+}$ of greater amplitude than the NAADP response remained highly localized (Fig. 1d), demonstrating that the diffusion coefficient is for NAADP and not Ca$^{2+}$. The EC$_{50}$ of the UV dose is similar for both the amplitude of the Ca$^{2+}$ increase (Fig. 1c) and the value of the diffusion coefficient (Fig. 1d), suggesting that the NAADP receptor itself is restricting NAADP diffusion. In conclusion, these experiments show that a local release of NAADP does not trigger a global regenerative Ca$^{2+}$ wave propagated by CICR but rather results in a Ca$^{2+}$ wave that spreads by NAADP diffusion.

The dependence of spatial Ca$^{2+}$ signaling on NAADP diffusion is strikingly similar to that reported recently for an agonist of the IP$_3$ receptor, adenophostin (29). For adenophostin, the size of the Ca$^{2+}$ signaling domain is dependent on adenophostin diffusion, which in turn is dependent on the concentration of adenophostin (29). The diffusion of adenophostin is both restricted and concentration-dependent because of the high affinity ($\approx 1$ nM) of adenophostin for the IP$_3$ receptor (30). The effect of binding to its receptor on messenger diffusion should be much greater for NAADP than adenophostin because NAADP binding is irreversible (22). From a spatial perspective, we predict that the relative spread of an NAADP-mediated Ca$^{2+}$ increase will be highly dependent on NAADP concentration. At low NAADP concentrations the receptors will be excess over free NAADP and bind most of the NAADP before it can diffuse, resulting in a localized spatial response. At high NAADP concentrations, however, the receptors will saturate and NAADP will be free to diffuse resulting in a more global spatial response.

Spatial Control of Ca$^{2+}$ Signaling by NAADP Concentration—If NAADP-initiated Ca$^{2+}$ waves are mediated by diffusion of NAADP, and NAADP diffusion is limited by endogenous and immobile buffers, then releasing NAADP slowly in part of an egg should limit the Ca$^{2+}$ increase to that area of the egg. Only upon saturating the binding sites for NAADP would NAADP be able to diffuse and mediate the spread of the Ca$^{2+}$ increase into other regions of the egg. To test this, we released NAADP with three periods of UV illumination in the same egg. The first photorelease of NAADP resulted in a Ca$^{2+}$ increase that was mostly restricted to within the area of UV illumination (Fig. 2). Although the second photorelease of NAADP resulted in a larger Ca$^{2+}$ increase, the Ca$^{2+}$ increase was still mostly restricted to the area of UV illumination (Fig. 2). The third photorelease of NAADP was more prolonged, and although this resulted in only a slightly larger Ca$^{2+}$ increase, this Ca$^{2+}$ increase spread across the entire egg (Fig. 2). During the third photorelease the increase in Ca$^{2+}$ was simultaneous in the three leftmost regions of interest in the area of irradiation and then transitioned into a regenerative-like spread through the rest of the egg with near constant velocity (3.6 $\mu$m/s) and amplitude (Fig. 2, rightmost plot). Propagation of the Ca$^{2+}$ increase was primarily through the cortex, with the spread through the center of the egg arising from the collision of the two cortical Ca$^{2+}$ waves. Even though the Ca$^{2+}$ wave is propagated by NAADP diffusion, the wave does not decrease in velocity or amplitude because NAADP is continuously added to the other half of the egg. This result shows that a diffusive Ca$^{2+}$ wave can resemble a regenerative Ca$^{2+}$ wave when the concentration of NAADP is saturating as it diffuses throughout the egg.

cADPR but Not NAADP Creates an Excitable Medium That Mediates Regenerative Ca$^{2+}$ Waves—It has been demonstrated previously that when intracellular IP$_3$ is increased slowly by photorelease there is only a small increase in resting Ca$^{2+}$ until a global Ca$^{2+}$ wave is triggered, because the IP$_3$ turns the cytoplasm into an excitable medium, and the resulting Ca$^{2+}$ wave propagates by CICR (9, 14, 25). Because cADPR acts by sensitizing ryanodine receptors to CICR (13), it should elicit a regenerative rather than diffusive Ca$^{2+}$ wave similar to that induced by IP$_3$ (9, 14, 25). To provide further evidence that NAADP-mediated Ca$^{2+}$ waves are dependent on a non-regenerative diffusive mechanism, the responses of cADPR and NAADP were compared at their threshold concentrations for evoking Ca$^{2+}$ release. The threshold was achieved by slowly increasing the messenger concentration with a low UV irradiation intensity applied for a long duration.

As predicted, cADPR elicited only a small elevation in Ca$^{2+}$ (Fig. 3a, arrow) until there was a rapid increase that propagated as a fast (14.7 ± 1.5 $\mu$m/s, $n = 6$) regenerative Ca$^{2+}$ wave throughout the irradiated area of the egg (Fig. 3, a and c). The decrease in Ca$^{2+}$ wave amplitude in the center of the egg probably relates to spatial differences in sensitivity to Ca$^{2+}$-releasing messengers, as discussed below; the important feature is the constant velocity throughout the irradiated area. This response is similar to that reported for IP$_3$ when it was uncaged in half of a Xenopus oocyte because the resulting regenerative Ca$^{2+}$ wave was limited to the half of the egg being irradiated (25). In both the Xenopus oocyte (25) and the sea urchin egg (Fig. 3, a and c) the Ca$^{2+}$ increase spread into the non-irradiated area, but this “spillover” is more noticeable in the sea urchin egg due to its smaller size (100 $\mu$m versus 1 mm). In contrast to the response to cADPR, low threshold concentrations of NAADP elicited a slowly spreading (1.4 ± 0.7 $\mu$m/s) Ca$^{2+}$ increase that was graded with respect to both amplitude and distance traveled (Fig. 3, b and c). The direction of the Ca$^{2+}$ wave is a precise reflection of the NAADP gradient but not of the cADPR gradient. The relatively slow spread of the NAADP-mediated Ca$^{2+}$ increase is consistent with there being a gradient of NAADP resulting from restricted diffusion of NAADP. Indeed, continuous release of NAADP progressively overcame...
the images the pseudocolors correspond to the fluo-3 self-ratio (F_o/F). Fertilization-induced Ca^{2+} waves are amplified by IP_3-mediated CICR and IP_3-mediated Ca^{2+} release pathways, possibly by CICR amplification (16, 36). To determine whether NAADP-mediated Ca^{2+} waves are amplified by coupling to IP_3- and cADPR-sensitive Ca^{2+} release mechanisms, caged NAADP was photolyzed in eggs containing either heparin or 8-amino-cADPR to block IP_3- and cADPR-sensitive Ca^{2+} release mechanisms, respectively. In these experiments photorelease was restricted to approximately half of the egg so that the effects of the inhibitors could be determined simultaneously both in a region where NAADP was increasing uniformly and in a region where NAADP was forming a gradient by diffusion. As shown in Figs. 2 and 3, a continuous release of NAADP was required to push the Ca^{2+} increase across the entire egg (Fig. 4). Heparin (5 mg/ml) alone and 8-amino-cADPR (5 μM) alone decreased the amplitude of the Ca^{2+} increase, but neither inhibitor significantly affected any other Ca^{2+} release mechanisms, possibly by CICR amplification (16, 36). To determine whether NAADP-mediated Ca^{2+} waves are amplified by coupling to IP_3- and cADPR-sensitive Ca^{2+} release mechanisms, caged NAADP was photolyzed in eggs containing either heparin or 8-amino-cADPR (37) to block IP_3- and cADPR-sensitive Ca^{2+} release mechanisms, respectively. In these experiments photorelease was restricted to approximately half of the egg so that the effects of the inhibitors could be determined simultaneously both in a region where NAADP was increasing uniformly and in a region where NAADP was forming a gradient by diffusion. As shown in Figs. 2 and 3, a continuous release of NAADP was required to push the Ca^{2+} increase across the entire egg (Fig. 4). 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NAADP-induced Ca\(^{2+}\) Waves

**Fig. 5.** Diagram illustrating how NAADP propagates diffusive Ca\(^{2+}\) waves. Channels activated by NAADP are depicted as black rectangles, and both IP\(_3\) receptors and ryanodine receptors are depicted as white mushroom shapes. The released Ca\(^{2+}\) is shown as hemispheric clouds emanating from the channels. The clusters of ryanodine and IP\(_3\) receptors are recruited by CICR. The bottom graph shows the concentration-distance curve for NAADP that illustrates the effect of CICR amplification on wave front position. The horizontal dashed lines show the thresholds for detecting a Ca\(^{2+}\) increase arising from the NAADP gradient with or without amplification.

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regenerative response, or localized CICR. A regenerative Ca\(^{2+}\) wave was not triggered by an increase in Ca\(^{2+}\) alone (Fig. 1d), indicating that amplification is probably due to local CICR. A necessary conclusion from this mechanism is that resting levels of IP\(_3\) and cADPR must be sufficiently elevated in resting eggs so that their target channels open in response to an increase in Ca\(^{2+}\). The redundancy of these two amplification pathways is similar to that reported previously for the Ca\(^{2+}\) wave that accompanies fertilization, which can propagate via either IP\(_3\) or ryanodine receptors (39, 40). Functional coupling is not detected in sea urchin egg homogenates (4), suggesting that it is related to the architecture of the Ca\(^{2+}\) stores in the intact egg and is lost upon homogenization. The physical relationship between the NAADP-activated channels and the CICR channels might be analogous to that between the dihydropyridine receptors (Ca\(^{2+}\) channels in the plasma membrane) and ryanodine receptors in cardiac cells (11). This arrangement enables precise delivery of extracellular Ca\(^{2+}\) directly to the ryanodine receptors, thereby activating them and amplifying the Ca\(^{2+}\) increase (11).

**Model for the Control of Ca\(^{2+}\) Signaling by NAADP—**A model showing how NAADP mediates spatial Ca\(^{2+}\) signaling is presented in Fig. 5. A gradient in the concentration of NAADP defines the domain over which the wave propagates. The NAADP gradient could result from either local synthesis and diffusion of NAADP or locally different rates of NAADP synthesis to yield a phase wave. Functional coupling to either IP\(_3\) receptors or ryanodine receptors contributes to the amplitude of the Ca\(^{2+}\) increase, but the effect is spatially localized. Functional coupling has been reported between IP\(_3\) receptors and ryanodine receptors in both portal vein smooth muscle cells (41) and PC12 cells (42). Both Ca\(^{2+}\) and NAADP could activate Ca\(^{2+}\) release from the clusters of channels, but unlike Ca\(^{2+}\), NAADP can diffuse between the clusters to coordinate a diffusive Ca\(^{2+}\) wave. The lower part of Fig. 5 shows how inhibition of amplification both reduces the Ca\(^{2+}\) increase and retards the position of the wave front, despite the fact that the two waves are dependent on the same underlying NAADP gradient. This is because it is not the NAADP concentration but the resulting Ca\(^{2+}\) increase that is monitored.

The implication of the mechanism by which NAADP mediates spatial Ca\(^{2+}\) signaling is that NAADP can be tuned to be either a local or global messenger. In contrast, IP\(_3\) and cADPR are always global messengers (28, 43), and Ca\(^{2+}\) is always a local messenger (28). A potentially important implication of diffusive NAADP-mediated Ca\(^{2+}\) waves is that they encode information such as the intensity of the originating signal, distance from the originating signal, and the direction of the signal. Uniquely, due to its ability to self-desensitize, this information could serve as a memory of the NAADP gradient such that past responses would affect subsequent responses. Although we are only now beginning to demonstrate a physiological role for NAADP in Ca\(^{2+}\) signaling (16, 44), we propose that the unique properties of NAADP may make it important in forming Ca\(^{2+}\) gradients such as those that may dictate cellular polarity and developmental patterning (3).

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