Caged Nicotinic Acid Adenine Dinucleotide Phosphate

SYNTHESIS AND USE*

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a metabolite of NADP with Ca\(^{2+}\) mobilizing activity. The Ca\(^{2+}\) release mechanism activated by NAADP as well as the Ca\(^{2+}\) stores that it acts on are different from those activated by either cyclic ADP-ribose or inositol 1,4,5-trisphosphate (IP\(_3\)) (Lee, H. C., and Aarhus, R. (1995) J. Biol. Chem. 270, 2152–2157). In order to demonstrate unambiguously that NAADP can mobilize Ca\(^{2+}\) stores in live cells, a caged analog was synthesized by reacting NAADP with 1-(2-nitrophenyl)diazoethane. Anion exchange high pressure liquid chromatography (HPLC) was used to purify one particular caged form from the mixture of products. Photolysis analyses following specific enzymatic cleavage indicate that the caging group is on the 2'-phosphate. This is confirmed by \(^{23}P\) NMR spectroscopy, showing that the 2'-phosphate of the caged compound exhibits an altered chemical shift of –2.6 ppm as compared with 2.3 ppm determined for the 2'-phosphate of NAADP. Caged NAADP had no Ca\(^{2+}\) releasing activity at a concentration as high as 1 \(\mu M\) when tested on sea urchin egg microsomes. After photolysis, it released Ca\(^{2+}\), was effective in nanomolar range, and was indistinguishable from authentic NAADP. The regeneration of NAADP after photolysis was also confirmed by HPLC analyses. The analog is particularly susceptible to UV and can be efficiently photolyzed using a spectrofluorimeter. To demonstrate its utility in live cells, caged NAADP was microinjected into sea urchin eggs. Photolysis effectively regenerated NAADP and activated Ca\(^{2+}\) oscillations in the eggs. Removal of external Ca\(^{2+}\) did not prevent the Ca\(^{2+}\) oscillations but only delayed the second Ca\(^{2+}\) peak by about 45 s, indicating that the oscillations are due to release from internal stores and not caused by Ca\(^{2+}\) influx. A mechanism based on sensitization of the Ca\(^{2+}\) release by Ca\(^{2+}\) loading is proposed to account for the Ca\(^{2+}\) oscillation observed.

In addition to inositol trisphosphate, two other independent mechanisms for mobilizing internal Ca\(^{2+}\) stores have been identified in sea urchin eggs. Cyclic ADP-ribose (cADPR)\(^1\) and nicotinic acid adenine dinucleotide phosphate (NAADP) are Ca\(^{2+}\)-mobilizing metabolites derived, respectively, from NAD (1–3) and NADP (4). Two modes of action of cADPR have been documented (reviewed in Ref. 5). In one case it can function as a modulator of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism and, synergistically with calmodulin, increase the sensitivity of the release mechanism to divalent cations by several orders of magnitude (6–9). Alternatively, it can also function as a Ca\(^{2+}\) messenger. Nitric oxide, through elevating intracellular cGMP levels, can activate the synthesizing enzyme of cADPR, resulting in an increase in cellular cADPR and mobilization of Ca\(^{2+}\) stores (10, 11). Since the Ca\(^{2+}\) releasing activity of cADPR was first described in sea urchin eggs, a variety of mammalian, amphibian, and plant cells have been shown to be responsive to cADPR, indicating its general relevance (1) (reviewed in Ref. 12).

NAADP is not a cyclic molecule; instead, it is formed by replacing the nicotinamide group of NADP with nicotinic acid (4). The Ca\(^{2+}\) release mechanism activated by NAADP has many characteristics of an independent signaling pathway. In sea urchin eggs, NAADP is by far the most effective Ca\(^{2+}\) release agonist and is active at nanomolar concentrations (1, 4). Heparin, an antagonist of the IP\(_3\)-receptor, has no effect on the NAADP mechanism (1, 4, 13), and cell fractionation studies show that the NAADP-sensitive Ca\(^{2+}\) stores can be separated from those responsive to cADPR and IP\(_3\) (4). The NAADP-dependent Ca\(^{2+}\) release is not inhibited by high concentrations of Mg\(^{2+}\) (14), 8-amino-cADPR, an antagonist of the cADPR-receptor (4, 15), and does not require calmodulin (4). These properties distinguish it from the cADPR-dependent pathway. NAADP is likely to be operating through a distinct receptor. Specific binding of \(^{23}P\)-NAADP to sea urchin egg microsomes has been demonstrated, and cADPR has no effect on the binding (16). One novel property of the NAADP mechanism is that, at subthreshold concentrations, NAADP can completely inactivate the release system such that subsequent challenge with a maximal concentration of NAADP is ineffective (16, 17). Ligand binding studies show that the self-inactivation occurs at the level of the receptor (16). This novel property is not seen in either cADPR- or IP\(_3\)-dependent Ca\(^{2+}\) release and is likely to be the first description of such a process in receptor-mediated function. Although the structures and functions of cADPR and NAADP are totally distinct, the two Ca\(^{2+}\) agonists can, in fact, be synthesized by the same enzymes (18). Both ADP-ribosyl cyclase and CD38, a lymphocyte antigen which is also a bifunctional enzyme involved in the synthesis of cADPR (reviewed in Ref. 19), can catalyze the exchange of the nicotinamide in NAADP with nicotinic acid (18). The base exchange reaction dominates at acidic pH, while at neutral and alkaline pH, both

\(^\text{1}\) The abbreviations used are: cADPR, cyclic ADP-ribose; NAAD, nicotinic acid adenine dinucleotide; NAADP, nicotinic acid adenine dinucleotide phosphate; IP\(_3\), inositol 1,4,5-trisphosphate; NPE, 1-(2-nitrophenyl)diazoethane; HPLC, high pressure liquid chromatography; ASW, artificial seawater; 0CaSW, Ca\(^{2+}\)-free seawater.

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enzymes preferentially cyclize NADP to produce cyclic ADP-ribose phosphate (18). In this study, we describe the synthesis of caged NAADP and the usefulness of the analog in investigating the Ca$^{2+}$ release mechanism of NAADP in microorganisms as well as in live cells.

**EXPERIMENTAL PROCEDURES**

**Synthesis**—NAADP was synthesized by incubating NADP (1 mM) at pH 5.0 with the Aplysia ADP-ribose cyclase (25 mg/ml) in the presence of 30 mM nicotinic acid for several hours at 20–23°C and purified by HPLC using an AG MP-1 column as described previously (18). The procedure for microinjection by pressure was as described previously (21). Frozen egg homogenates (25%) were thawed at 17°C about 1% of the egg. Eggs were attached to the bottom of a protamine homogenate was continuously stirred. The volume of homogenate included zero-filling the free induction decays to 65536 data points and included spectral width of 48543 Hz; acquisition time of 0.338 s; number of transients ranging from 20 to 50”, depending on the experiment (PW$_{30}$ was about 50 μs); relaxation delay of 3–4 s, depending on the experiment; and number of transients ranging from 400 to 30,000, depending on the experiment. All 31P NMR spectra were proton-decoupled, with the decoupler gated on only during the acquisition period of the free induction decay. Data processing included zero-filling the free induction decay to 65536 data points and using an exponential weighting apodization function with a line broadening of 2.5 Hz to improve the signal-to-noise ratio in the transformed spectrum.

**RESULTS**

**Synthesis and Purification**—We have previously synthesized caged cADPR that is particularly photolabile. Effective photolysis can be accomplished with standard spectrofluorometers or epifluorescence setups, and no specialized equipment is required (21). NAADP represented a special challenge since it has three phosphate groups as well as a carbonyl group which are all reactive toward the NPE reagent (20). Since it is known that the NPE reagent reacts mainly with protonated groups, the caging reaction was performed at either pH 4.5 or 1.3 so that different ionizable groups could be sampled. The caged product obtained at the higher pH value was found to be difficult to photolyze. Even after prolonged exposure to a handheld UV lamp, no detectable NAADP was produced as judged by TLC analysis. Photolysis—Activation of caged cADPR in egg homogenates was achieved in a Hitachi spectrofluorometer (S-2000) by alternating the exciting wavelength every 2 s between 350 nm for photolysis and 485 nm for monitoring fluo-3 fluorescence.

In some experiments, photolysis with UV light (10$^{15}$ quanta/s) for 1 min was done in a Bayard–Alpert electrometer (Southern New England Ultraviolet Co.) at 0–4°C.

The UV photolysis and fluorescence measurement of individual eggs were done using the InCa$^{2+}$ imaging system (Intracellular Imaging Inc., Cincinnati, OH). Excitation was provided by a 300-Watt xenon lamp equipped with filters for 340- and 485-nm light. During photolysis, the xenon light was alternated between the uncaging (340 nm) and the monitoring (485 nm) wavelengths, both of which were reflected by a BICCF Sp dichroic filter toward the objective. The fluo-3 fluorescence was selected by a long pass filter with a 500-nm cutoff and monitored by a CCD camera. Fluo-3 fluorescence was measured every 4 s. Photolysis was performed for 3.5 s between measurements. As the xenon lamp aged, the intensity of UV light decreased. In some experiments, to compensate for the diminished UV intensity, the 340-nm filter was removed during photolysis.

**HPLC Analyses**—HPLC separation was done with columns packed with AG MP-1 resin (Bio-Rad) and eluted with a nonlinear gradient of trifluoroacetic acid similar to that described previously (15). The final purification of the caged NAADP was achieved using a 0.5 × 5-cm Mono Q column (Pharmacia Biotech Inc.). The product was eluted using a gradient of water (solvent A) and 1 M triethylamine bicarbonate (solvent B, pH 8.8): 0–12 min, 0% B; linearly increased to 20% B from 12 to 16 min, linearly increased to 30% B from 16 to 36 min, linearly increased to 100% from 36 to 37 min and held at 100% B for 3 min before returning to 0% B. Caged NAADP, NAADP, and photolyzed caged NAADP, all at 28 μM, were incubated with 2 units/ml alkaline phosphatase (Sigma), or 3.5 units/ml nucleotide pyrophosphatase (Sigma), or both enzymes together, in the presence of 10 mM MgCl$_2$ and 80 mM triethylamine bicarbonate, pH 8.8, for 20 min at 37°C. The total volume of the reaction mixture was 10 μl. The phosphate released by the enzymes was measured by adding 0.1 ml of the Malachite Green reagent (22) and 10 μl of 34% sodium citrate. Absorbance at 660 nm was measured and compared with sodium phosphate standards.

**Enzymatic Cleavage and Phosphate Measurements**—Caged NAADP, NAADP, and photolyzed caged NAADP, all at 28 μM, were incubated with 2 units/ml alkaline phosphatase (Sigma), or 3.5 units/ml nucleotide pyrophosphatase (Sigma), or both enzymes together, in the presence of 10 mM MgCl$_2$ and 80 mM triethylamine bicarbonate, pH 8.8, for 20 min at 37°C. The total volume of the reaction mixture was 10 μl. The phosphate released by the enzymes was measured by adding 0.1 ml of the Malachite Green reagent (22) and 10 μl of 34% sodium citrate. Absorbance at 660 nm was measured and compared with sodium phosphate standards.

**31P NMR Analyses**—Spectra were collected at an observation frequency of 161.19 MHz using a Varian UNITY plus 400 FT-NMR spectrometer which was equipped with a computer switchable Nalorac 4N400–4 × 5 mm-nucleus probe (V, 1H, 13C, 31P). All samples were dissolved in a D$_2$O solution of 32% in a 25% D$_2$O solution at pH 7.5. Partially deuterated spectra were acquired at an ambient room temperature of 22–23°C. A phosphoric acid (H$_3$PO$_4$) solution in methanol in a 2-mm coaxial tube was used as external 31P NMR standard for zero chemical shift calibration for all spectra. Negative chemical shifts are uplifted of (lower frequency than) H$_3$PO$_4$. The acquisition conditions used for all the spectra include spectral width of 48543 Hz; acquisition time of 0.338 s; number of scans (1024); and binary data points, 32768; pulsed flip angles ranging from 20° to 50°, depending on the experiment (PW$_{30}$ was about 50 μs); relaxation delay of 3–4 s, depending on the experiment; and number of transients ranging from 400 to 30,000, depending on the experiment. All 31P NMR spectra were proton-decoupled, with the decoupler gated on only during the acquisition period of the free induction decay. Data processing included zero-filling the free induction decay to 65536 data points and using an exponential weighting apodization function with a line broadening of 2.5 Hz to improve the signal-to-noise ratio in the transformed spectrum.
by TLC analysis. In sea urchin egg homogenate, the caged material was inactive as a Ca\(^{2+}\) mobilizer, but prolonged photolysis only slowly and weakly generated free NAADP (data not shown). The product was not characterized further, but the poor photolysis properties are characteristic of carboxylates caged as NPE esters (23).

We next reduced the pH of the reaction to 1.3, a condition at which all four groups would be reactive. We did not have prior knowledge of which phosphate group would be the most appropriate for caging and which specific groups should be protected from being caged. Therefore, the strategy was not to focus on a particular group, but instead, to separate the mixture of products by chromatography and analyze each fraction using sea urchin egg homogenates as a bioassay for Ca\(^{2+}\) release (1). The product was expected not to release Ca\(^{2+}\) on its own but to be easily photolyzed using a spectrofluorimeter and to regenerate the Ca\(^{2+}\) release activity of NAADP.

TLC analyses of the caged products produced at pH 1.3 showed two main spots with \(R_p\) values of 0.87 and 0.46, respectively. The more polar products \(R_p \approx 0.46\) were separated using a Sephadex column (see “Experimental Procedures”). Fig. 1 (top) shows a HPLC chromatograph of the caged products analyzed on an AG MP-1 column. It was found to contain several components. All three peaks were collected and tested for Ca\(^{2+}\) release activity before and after photolysis. Only the smallest peak, indicated with an asterisk, satisfied our criteria. The product was purified one more time on an AG MP-1 column followed by twice on a Mono-Q column. Fig. 1 (bottom) shows the purified product eluted as a single peak on the second Mono-Q column. Starting from 5 mg of the product mixture, about 50 \(\mu\)g of purified caged NAADP was obtained.

**Structural Determination**—To determine which of the phosphates is caged, we used nucleotide pyrophosphatase to cleave the pyrophosphate linkage of the molecule and alkaline phosphatase to release the phosphate groups as inorganic phosphate. Alkaline phosphatase should also cleave the 2'-phosphate group if it is not caged. The Pi released was measured using the Malachite Green method (22). Fig. 2 shows that treatment with alkaline phosphatase released about 1 mol of Pi per mol of NAADP (open bars), which corresponds to the 2'-phosphate of the molecule. Nucleotide pyrophosphatase cleaves the pyrophosphate bond and allows the phosphate groups to be released by alkaline phosphatase. Indeed, treatment with both enzymes \(MIX\) produced about 3 mol of Pi/mol of NAADP, as expected. In contrast to NAADP, treatment of caged NAADP \(black\ bars\) with alkaline phosphatase produced no Pi, indicating that the 2'-phosphate is caged. Treatment with the combined enzymes released the two phosphates forming the pyrophosphate linkage. The pattern of Pi released from the photolyzed caged NAADP \(gray\ bars\) following the enzyme treatments is the same as that of NAADP. These results are consistent with the caged group being attached to the 2'-phosphate of NAADP.

The structural assignment is further confirmed by \(^{31}\)P NMR analyses. Fig. 3 compares the \(^{31}\)P NMR spectra of two standards, NAADP and nicotinic acid adenine dinucleotide (NAAD), with that of the purified caged NAADP. The chemical shift was...
The structure of caged NAADP is shown in Fig. 4. The chemical shift of the 2'-phosphate of NAADP is as described in Lee and Aarhus (4). The caging group is shown attached to the 2'-phosphate.

The proposed structure of caged NAADP. The structure of NAADP is as described in Lee and Aarhus (4). The caging group is shown attached to the 2'-phosphate.

The structure of caged NAADP is shown in Fig. 4.

Photolysis—Fig. 5B shows that addition of caged NAADP from 90 to 900 nM to egg homogenates produced no Ca\(^{2+}\) release. The small jumps of fluo-3 fluorescence at high concentrations of caged NAADP were due to addition artifacts (e.g., small Ca\(^{2+}\) contamination in the samples), since they were present even when the release mechanism was totally inactivated (tracing labeled 8' in Fig. 5A). A novel property of the NAADP-sensitive Ca\(^{2+}\) release is that the release mechanism can be totally inactivated by pretreatment with a subthreshold concentration of NAADP as low as 1 nM (16, 17). The multistep procedure described above for the purification of caged NAADP was designed to ensure that the contaminating NAADP is below the self-inactivating levels. Fig. 5 also shows the results of testing the inactivating effect of caged NAADP. Egg homogenates were pretreated with 1 nM NAADP and subsequently challenged with a maximal concentration (40 nM) (Fig. 5A). After 2 min of pretreatment, the response to 40 nM NAADP was substantially reduced (tracing labeled 2') as compared to without pretreatment (tracing labeled 0').

After 8 min of pretreatment (tracing labeled 8'), and was totally eliminated after 8 min of pretreatment (tracing labeled 8'). Pretreatment of the homogenates with 90 nM caged NAADP for 2 min produced very little inactivation (Fig. 5B). At 540 nM caged NAADP, the extent of inactivation following the pretreatment was similar to that effected by 1 nM of NAADP (comparing Fig. 5A, 2', with Fig. 5B, 540 nM). This extent of inactivation by the caged compound can be accounted for if the sample is contaminated with about 0.1–0.2% NAADP.

This appears to be the case since freshly purified samples of the caged compound at 200 nM exhibited essentially no inactivation as shown in Fig. 6. Even at 900 nM of caged NAADP, the inactivation due to pretreatment was only about 30–40%. Results in Fig. 2 indicate NAADP is very sensitive to alkaline phosphatase while the caged compound is not. Treatment of the 900 nM sample with alkaline phosphatase (+APase), indeed, essentially removed all the inactivation. Results described in Figs. 5 and 6 thus show that caged NAADP is biologically inactive. It does not release Ca\(^{2+}\) nor does it induce inactivation.

Fig. 7 shows that photolysis of caged NAADP regenerates NAADP. The samples, before and after photolysis, were analyzed by HPLC. The retention time of the photolyzed product was essentially the same as that of NAADP, which was shifted as compared with caged NAADP. The photolyzed product was effec-

[Caged NAADP]

Fig. 3. \(^{31}\)P NMR spectra of NAADP, NAAD, and caged NAADP. The numbers shown denote chemical shift values in parts/million. External phosphoric acid was used as calibration, and its chemical shift was set at zero. The two tracings above the spectrum of caged NAADP represent graphical integration of the two peaks.
tive in releasing Ca\(^{2+}\) as shown in Fig. 8 and, in fact, its concentration dependence was indistinguishable from that of NAADP. Also shown in Fig. 8 is that caged NAADP, before photolysis, had no Ca\(^{2+}\) releasing activity at concentrations as high as 1 \(\mu\)M. Further evidence that the photolyzed product is NAADP is provided by its desensitization of the NAADP-dependent Ca\(^{2+}\) release in egg homogenates. The results are shown in the inset of Fig. 8. At 28 nM, the photolyzed product induced rapid Ca\(^{2+}\) release. The egg homogenate became totally desensitized such that subsequent addition of 80 nM NAADP, after the Ca\(^{2+}\) was resequestered, did not produce any release. Homogenates desensitized to prior exposure to 80 nM NAADP also did not respond to the photolyzed product. This cross-desensitization indicates the photolyzed product is indeed, NAADP.

Ca\(^{2+}\) Release Activity—Fig. 9 shows that NAADP can be regenerated from caged NAADP using a spectrofluorimeter. The excitation wavelength was alternated between 350 nm for photolysis and 485 nm for monitoring the fluorescence of the Ca\(^{2+}\) indicator, fluo-3. Addition of caged NAADP to the egg homogenates with the alternating UV excitation turned on resulted in Ca\(^{2+}\) release after a brief delay (Fig. 9A). The delay was more prominent at the lower concentrations. Comparison of the Ca\(^{2+}\) release activity with that induced by NAADP itself shows that about 1% of the caged NAADP added was photolyzed. This low efficiency is due to the relative weak UV excitation light of the spectrofluorimeter. Fig. 9B compares the concentration-response of NAADP and caged NAADP with or without UV photolysis. Because NAADP is effective in releasing Ca\(^{2+}\) at nanomolar concentrations, the low efficiency of photolysis by the spectrofluorimeter does not hamper its use in

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**FIG. 5.** Assay of the contamination level of NAADP in the caged NAADP preparation. The self-inactivation property of NAADP was used as an assay to estimate its contamination in the caged NAADP preparation. Trace a shows that treatment of sea urchin egg homogenates with 1 nM NAADP for 8 min (A, 8') effectively inactivates the Ca\(^{2+}\) release system such that the response to 40 nM NAADP is essentially eliminated. The extent of inactivation induced by 540 nM caged NAADP (B) is similar to that elicited by 1 nM NAADP (A, trace 2'), indicating the level of contamination is about 0.1–0.2%. Ca\(^{2+}\) release was monitored by fluo-3.

**FIG. 6.** Elimination of the inactivating effect of caged NAADP by treatment with alkaline phosphatase to remove NAADP contamination. Caged NAADP was freshly purified using a Mono Q column and incubated with (+) or without (−) alkaline phosphatase (APase) for 1 h at 37°C and pH 8.5 as described under “Experimental Procedures.” Egg homogenates were incubated with the samples at the concentrations indicated for 2 min and subsequently challenged with 100 nM NAADP. Error bars represent the S.D. of triplicates.

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**FIG. 7.** An HPLC analysis of the photolyzed product. Caged NAADP samples were analyzed by anion exchange HPLC before and after 1 min of exposure to UV light in a photochemical reactor. The solid curves indicate absorbance values at 254 nm and dashed lines indicate the trifluoroacetic acid (TFA) gradient used for elution.

**FIG. 8.** A comparison of the dependence of Ca\(^{2+}\) release on the concentration of NAADP and photolyzed caged NAADP. Ca\(^{2+}\) release was measured in sea urchin egg homogenates using fluo-3. Photolysis was induced by UV exposure in a photochemical reactor for 1 min at 0–4°C. The concentration-response of the photolyzed caged NAADP (filled squares) was indistinguishable from that of NAADP (open squares), and caged NAADP (filled triangles) was inactive. The inset shows the cross-desensitization between NAADP and the photolyzed product, indicating the latter was, in fact, NAADP.
this setting. A common problem in measuring Ca\textsuperscript{2+} release from a suspension of permeabilized cells or cell-free assays, such as that shown in Fig. 9, is distinguishing Ca\textsuperscript{2+} release from Ca\textsuperscript{2+} contamination in the samples. The caged analog should be useful since it is not biologically active until photolysis, which can be accomplished conveniently by simply alternating the excitation wavelength in a spectrofluorimeter. The caged analog is also useful in single cell measurements. We have previously shown that photolyzing caged NAADP loaded into sea urchin eggs can induce Ca\textsuperscript{2+} oscillations (16). In some cases, these Ca\textsuperscript{2+} oscillations persist for more than 30 min. The possibility that Ca\textsuperscript{2+} influx may be involved in generating these oscillations was investigated by removing external Ca\textsuperscript{2+}. We focused on the first two Ca\textsuperscript{2+} oscillations that occur within 6–7 min after photolysis. Fig. 10 shows that removal of external Ca\textsuperscript{2+} does not prevent the Ca\textsuperscript{2+} change induced by photolysis nor does it inhibit the subsequent Ca\textsuperscript{2+} oscillation that occurs spontaneously. The main effect of removal of external Ca\textsuperscript{2+} is a delay of the occurrence of the second Ca\textsuperscript{2+} peak. This delay separates the second peak farther from the first peak and makes it appear more prominent in the case of 0CaSW. Table I summarizes the results from 16 eggs in 0CaSW and 17 eggs in ASW. Both the magnitude of the two Ca\textsuperscript{2+} peaks and the time of the first Ca\textsuperscript{2+} peak are independent of external Ca\textsuperscript{2+}. These results show that the internal stores are the main source of the Ca\textsuperscript{2+} changes induced by photolyzing caged NAADP as well as the subsequent Ca\textsuperscript{2+} oscillation. It should be noted that in another five eggs in 0CaSW, photolysis induced no change in internal Ca\textsuperscript{2+}. It is likely that these eggs had suffered damage during microinjection. The leakage of EGTA into the eggs could have buffered the Ca\textsuperscript{2+} changes. In ASW the second peak occurred at 115.7 ± 6.6 s after the start of photolysis. In 0CaSW, the second peak occurred at 161.5 ± 12.6 s, a 45-s delay. The exact mechanism of how removal external Ca\textsuperscript{2+} can delay the Ca\textsuperscript{2+} oscillation remains to be elucidated. One possibility is proposed in the "Discussion."

**DISCUSSION**

As described in the introduction, the Ca\textsuperscript{2+} release mechanism activated by NAADP has many properties of a signaling pathway. In this study, we describe two other properties of NAADP that strengthen its signaling role. First, it is highly effective in live cells, which can be unambiguously demonstrated using the caged analog. Indeed, loading of eggs with about 200 nM caged NAADP was more than sufficient (Table I), a concentration which is 10-fold lower than that required for caged cADPR (21). Removal of external Ca\textsuperscript{2+} did not inhibit the Ca\textsuperscript{2+} change induced by photolyzing caged NAADP in live eggs, indicating the source of Ca\textsuperscript{2+} is from internal stores.

Second, NAADP can be degraded effectively by phosphatases, such as nucleotide pyrophosphatase and alkaline phosphatase.
Caged NAADP

Calcium oscillations induced by photolyzing caged NAADP

Eggs were loaded with caged NAADP and 30–190 μM fluo-3 and incubated either in ASW or 0CaSW. Photolysis was achieved by exposure to UV for 17–25 s. Maximal fluorescence increase (F\textsubscript{max}) was normalized to the initial fluo-3 fluorescence before photolysis (F\textsubscript{i}). T\textsubscript{1} and T\textsubscript{2}, respectively, denote the time of the first and second Ca\textsuperscript{2+} peak from the start of photolysis. All values are mean ± S.E. The number of eggs used for measurements was 17 for ASW and 16 for 0CaSW.

| First Ca\textsuperscript{2+} peak            | Second Ca\textsuperscript{2+} peak | [Caged NAADP] \textsubscript{i} | T\textsubscript{1}–T\textsubscript{2} |
|-------------------------------------------|-----------------------------------|---------------------------------|-------------------------------------|
| F\textsubscript{max}/F\textsubscript{i}  | s s nM s                          | T\textsubscript{1}              | T\textsubscript{2}                  |
| 0CaSW 6.7 ± 0.8                          | 17.0 ± 1.0                        | 5.1 ± 0.7                       | 161.5 ± 12.6                       |
| ASW 7.3 ± 0.6                            | 16.8 ± 1.8                        | 4.6 ± 0.3                       | 115.7 ± 6.6                        |

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REFERENCES
1. Clapper, D. L., Walseth, T. F., Dargie, P. J., and Lee, H. C. (1987) J. Biol. Chem. 262, 9561–9568
2. Lee, H. C., Walseth, T. F., Bratt, G. T., Hayes, R. N., and Clapper, D. L. (1989) J. Biol. Chem. 264, 1608–1615
3. Lee, H. C., Aarhus, R., and Levitt, D. (1994) Nat. Struct. Biol. 1, 143–144
4. Lee, H. C., and Aarhus, R. (1995) J. Biol. Chem. 270, 2152–2157
5. Lee, H. C. (1996) Recent Prog. Hormone Res. 52, 357–391
6. Galione, A., Lee, H. C., and Buss, W. B. (1991) Science 253, 1143–1146
7. Lee, H. C. (1993) J. Biol. Chem. 268, 293–299
8. Lee, H. C., Aarhus, R., Graeff, R., Gurnack, M. E., and Walseth, T. F. (1994) Nature 370, 307–309
9. Lee, H. C., Aarhus, R., and Graeff, R. M. (1995) J. Biol. Chem. 270, 9060–9066
10. Galione, A., White, A., Willmot, N., Turner, M., Potter, B. V. L., and Watson, S. P. (1993) Nature 365, 456–459
11. Willmot, N., Sethi, J., Walseth, T. F., Lee, H. C., White, A. M., and Galione, A. (1996) J. Biol. Chem. 271, 3699–3705
12. Lee, H. C. (1995) in CRC Series on Pharmacology and Toxicology (Sorrentino, V., ed) pp. 31–50, CRC Press, Boca Raton, FL
13. Chini, E. N., Beers, K. W., and Dousa, T. P. (1995) J. Biol. Chem. 270, 3216–3223
14. Graeff, R., Polein, R. J., Aarhus, R., and Lee, H. C. (1995) Biochem. Biophys. Res. Commun. 206, 766–791
15. Walseth T. F., and Lee, H. C. (1993) Biochim. Biophys. Acta 1178, 235–242
16. Aarhus, R., Dickey, D. M., Graeff, R. M., Gee, K. R., Walseth, T. F., and Lee, H. C. (1996) J. Biol. Chem. 271, 8513–8516
17. Genazzani, A. A., Empson, R. M., and Gallyone, A. (1996) J. Biol. Chem. 271, 11959–11962
18. Aarhus, R., Graeff, R. M., Dickey, D. M., Walseth, T. F., and Lee, H. C. (1995) J. Biol. Chem. 270, 30327–30333
19. Lee, H. C., Graeff, R., and Walseth, T. F. (1995) Biochimie (Paris) 77, 345–355
20. Walker, J. W., Reid, G. P., McCray, J. A., and Trentham, D. R. (1988) J. Am. Chem. Soc. 110, 7170–7177
21. Aarhus, R., Gee, K., and Lee, H. C. (1995) J. Biol. Chem. 270, 7745–7749
22. Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A. (1979) Anal. Biochem. 100, 95–97
23. Wilcox, M., Viola, R. W., Johnson, K. W., Billington, A. P., Carpenter, B. K., McCray, J. A., Guzikowski, A. P., and Hess, G. P. (1990) J. Org. Chem. 55, 1585–1589
24. Galione, A., McDougall, A., Buss, W. B., Willmot, N., Gillot, I., and Whitaker, M. (1993) Science 261, 348–352
25. Dargie, P. J., Agre, M. C., and Lee, H. C. (1990) Cell Regul. 1, 279–290