Nicotinic acid adenine dinucleotide phosphate (NAADP+) is a recently identified metabolite of NADP+ that is as potent as inositol trisphosphate (IP3) and cyclic ADP-ribose (cADPR) in mobilizing intracellular Ca2+ in sea urchin eggs and microsomes (Clapper, D. L., Walseth, T. F., Dargie, P. J., and Lee, H. C. (1987) J. Biol. Chem. 262, 9561-9568; Lee, H. C., and Aarhus, R. (1995) J. Biol. Chem. 270, 2152-2157). The mechanism of Ca2+ release activated by NAADP+ and the Ca2+-stores it acts on are different from those of IP3 and cADPR. In this study we show that photoactivating caged NAADP+ in intact sea urchin eggs elicits long term Ca2+ oscillations. On the other hand, uncaging threshold amounts of NAADP+ produces desensitization. In microsomes, this self-inactivation mechanism exhibits concentration and time dependence. Binding studies show that the NAADP+ receptor is distinct from that of cADPR, and at subthreshold concentrations, NAADP+ can fully inactivate subsequent binding to the receptor in a time-dependent manner. Thus, the NAADP+-sensitive Ca2+ release process has novel regulatory characteristics, which are distinguishable from Ca2+-release mediated by either IP3 or cADPR. This battery of release mechanisms may provide the necessary versatility for cells to respond to diverse signals that lead to Ca2+ mobilization.

Three independent Ca2+ release mechanisms are present in sea urchin eggs. In addition to IP3, both NAD+ and NAADP+ are very effective in mobilizing internal Ca2+ (1). The Ca2+ release induced by NAD+ shows a characteristic initial delay due to conversion to cADPR (1-3). Cellular systems responsive to cADPR are not limited to sea urchin egg, an invertebrate cell (1-3), but include various mammalian cells (reviewed in Ref. 4), amphibian neurons (5), and plant vacuoles (6). Accumulation of evidence indicates the action of cADPR is to dramatically increase the Ca2+ sensitivity of the Ca2+-induced Ca2+ release mechanism in cells (5, 7-9).

Unlike NAD+, the Ca2+ release induced by NAADP+ shows no delay and is kinetically faster than that induced by IP3 (1). Systematic analyses show that the Ca2+-releasing activity of NAADP+ comes from a contaminant present in commercial NADP+ (1, 10). This contaminant can be produced from NADP+ by alkaline treatment (1, 10), and structural characterization shows that it is NAADP+ (10, 11). The NAADP+-sensitive Ca2+ release is likely to be a new pathway for mobilizing internal Ca2+ since it is unaffected by pharmacological agents of all known Ca2+ release mechanisms. It is insensitive to 8-aminocADPR, a specific antagonist of the cADPR receptor (12), as well as heparin, an antagonist of the IP3 receptor (1, 10). Antagonists of the ryanodine receptor, high concentrations of Mg2+ (13), procaine, and ruthenium red (11), also have no effect on the release. Fractionation studies show that the NAADP+-sensitive stores distribute separately from those sensitive to IP3 and cADPR as well as from mitochondria (1, 10). Although the NAADP+-sensitive mechanism is functionally distinct from that mediated by cADPR, the same metabolic enzymes that produce cADPR, ADP-ribose cyclase and CD38, can also catalyze the synthesis of NAADP+ from NADP+ under appropriate conditions (14). In this study, we provide conclusive evidence using a caged analog that NAADP+ is active in live cells. We demonstrate the presence of a specific receptor for NAADP+ in egg microsomes, and we describe a novel self-regulating mechanism of NAADP+.

MATERIALS AND METHODS

Microinjection and Photolysis of Caged NAADP+.—The injection buffer for all experiments contained 18 μM caged NAADP+, 10 mM flou 3, 0.5 mM KCl, 0.1 mM EGTA, 10 mM HEPES, pH 6.7. The injection volumes were 0.5-1.9% of the egg volumes. The final concentrations of caged NAADP+ in the eggs were 90-342 nM. The procedure for microinjection is as described previously (15). The UV photolysis and fluorescence measurements were done using the InCa2+ imaging system (Intracellular Imaging Inc., Cincinnati, OH). Excitation was from a 300-watt xenon lamp equipped with filters for 340 nm and 485 nm light. During photolysis, the excitation light was alternated between the uncaging (340 nm) and the monitoring (485 nm) wavelengths. The duty cycle can be programmed. Details of the setup will be described elsewhere. Caged NAADP+ was synthesized by derivatizing NAADP+ with 2-nitrophenethyl diazoethane using a procedure similar to that described for the synthesis of caged cADPR (15). The method for purifying and characterizing the product, caged NAADP+, will be described elsewhere. Caged NAADP+ has been designated with a research number (MPR 71041) by Molecular Probes, Inc.

The Ca2+ Release Assays—Ca2+ release measurement and homogenate preparation (1.25% w/v) from Strongylocentrotus purpuratus eggs were as described previously (15). All experiments were done at 17 °C using 3 μM flou 3 as the Ca2+ indicator.

Specific Binding of [32P]NAADP+ to Microsomes—Microsomes were purified from egg homogenates by Percoll density gradient centrifugation as described previously (1, 10). The binding reactions (final volume of 0.1 ml) were initiated by adding microsomes (400-800 μg) to tubes containing [32P]NAADP+ and competitors. After incubating at 4 °C for 20-30 min, the microsomes were filtered on GF/C filters and washed 2 times with 3 ml of ice-cold 10% polyethylene glycol in a buffer containing 250 mM N-methylglucamine, 250 mM potassium glutonate, 20 mM HEPES, 1 mM MgCl2 pH adjusted to 7.2 with acetic acid. [32P]NAADP+ was synthesized by phosphorylating [32P]NAD+ with NAD+ kinase to [32P]NADP+, which was then converted to [32P]NAADP+ using ADP-ribose cyclase as described previously (14). NAD+ kinase was isolated from sea urchin egg extracts (16) and further purified by a calmodulin affinity column. The purification procedure...
separates the NAD$^+$ kinase from a contaminating ATPase, which interferes with the phosphorylation of NAD$^+$. 

RESULTS AND DISCUSSION

To demonstrate conclusively that NAADP$^+$ is effective in live cells, caged NAADP$^+$ was synthesized by derivatizing NAADP$^+$ with 2-nitrophenethyl diazoethane using a procedure similar to that described for the synthesis of cADPR (15). Caged NAADP$^+$ had no Ca$^{2+}$-releasing activity in egg homogenates and produced no Ca$^{2+}$ change when injected into intact eggs. Fig. 1A shows that photolyzing with a brief exposure to UV light (340 nm) for 30 s produced a rapid elevation of intracellular Ca$^{2+}$ as indicated by a 30-fold increase in fluo 3 fluorescence. Thereafter, multiple Ca$^{2+}$ oscillations occurred over a period of more than 15 min. Similar Ca$^{2+}$ oscillations were seen in 14 other eggs. Although the exact patterns of oscillations differ between eggs, all 15 exhibited at least two oscillations, and all of them underwent cortical reactions when examined afterward (e.g., the black and white image in Fig. 1B). The Ca$^{2+}$ changes cannot be due to nonspecific UV exposure since control eggs loaded with a higher concentration of caged ATP and exposed for much longer periods to UV light produced no change in Ca$^{2+}$ (15). The changes also cannot be due to injection artifacts since they were induced by photolysis subsequent to injection. The use of caged NAADP$^+$, therefore, provides more convincing evidence than the microinjection protocols used previously in showing that NAADP$^+$ is effective in live cells (10, 17).

NAADP$^+$ is also a potent self-desensitizer of the Ca$^{2+}$ release mechanism as shown in Fig. 1C. By reducing the period of UV uncaging to 10.5 s, a threshold concentration of NAADP$^+$ was generated, which induced a much smaller Ca$^{2+}$ response. The egg, nevertheless, became totally desensitized because after as long as 11 min later, photolysis for 45.5 s produced no further change in Ca$^{2+}$. In 6 out of 8 eggs, an initial photolysis period of 10–20 s produced a fluo 3 increase of $2.8 \pm 0.5$ (±S.E.)-fold, and the eggs were strongly desensitized since a subsequent photolysis of 45–231 s elicited a fluo 3 increase of only $0.7 \pm 0.3$-fold.

The self-desensitization can be best demonstrated in egg homogenates. Addition of 4 nm NAADP$^+$ to microsomes elicited minimal Ca$^{2+}$ release, but the microsomes failed to respond to subsequent challenge by a saturating concentration of NAADP$^+$ administered 2 min later (Fig. 2b). A higher subsequent Ca$^{2+}$ response was seen if the concentration of NAADP$^+$ used in the pretreatment was reduced to 0.13 nm (Fig. 2a), but it was still less than control without pretreatment (inset of Fig.
Indeed, treating the microsomes for 2 min with as low as 0.5 nM NAADP\(^+\) was sufficient to reduce the subsequent Ca\(^{2+}\) response by half (inset of Fig. 2). The self-desensitization is also concentration- and time-dependent. Pretreatment of the homogenates with 1 nM NAADP\(^+\) for 1 min produced significant reduction of the subsequent response to 0.97 μM NAADP\(^+\) (Fig. 3A). After a 10-min pretreatment, the microsomes became totally refractory, but the response to cADPR remained normal as compared with its control without pretreatment. The desensitization was faster and more complete the higher the concentration of NAADP\(^+\) used in the pretreatment (Fig. 3B). For a 10-min pretreatment, 0.25 nM NAADP\(^+\) was sufficient to produce 50% inactivation.

It is likely that the inactivation occurs at the receptor level. To demonstrate specific binding, \(^{32}\)P]NAADP\(^+\) was synthesized from \(^{32}\)P]NAD\(^+\) in the presence of nicotinic acid using the Aplysia ADP-ribosyl cyclase as described previously (14). Fig. 4A shows the specificity of the binding of \(^{32}\)P]NAADP\(^+\) to egg microsomes purified by Percoll density centrifugation. The binding was inhibited by nanomolar concentrations of NAADP\(^+\) but was not affected by 10 μM NAD\(^+\), NAAD\(^+\), cADPR, or cyclic ADP-ribose 2'-phosphate (14). A small inhibition of the binding was seen with 10 μM NADP\(^+\). This is likely due to the contaminating NAADP\(^+\) in commercial NADP\(^+\) preparations (1, 10), since the inhibition can be removed by purification of the sample by high pressure liquid chromatography (data not shown). Under similar conditions as described in Fig. 4, the binding reached steady state in 2.5 min at 4°C. The saturation of the binding reaction was tested by varying the concentration of radiolabeled NAADP\(^+\), and maximal specific binding was attained with ligand concentrations higher than about 10 nM (data not shown).

The competitive binding studies shown in Fig. 4A were done with the competitors and \(^{32}\)P]NAADP\(^+\) added simultaneously. If the microsomes were pretreated with labeled NAADP\(^+\) (2 nM) for various periods before the addition of 100 nM unlabeled NAADP\(^+\), the displacement of \(^{32}\)P]NAADP\(^+\) showed a time-dependent decrease. After 5 min of pretreatment with the label (2 nM), NAADP\(^+\) no longer could displace the binding (Fig. 4B). This is consistent with the time-dependent inactivation of Ca\(^{2+}\) release shown in Fig. 3. It thus appears the binding of subthreshold concentrations of the radioactive label (1 nM) to the microsomes is sufficient to alter the binding site in such a manner that it is no longer accessible to NAADP\(^+\) added afterward.

The self-inactivation mechanism described in this study is
novel in that it is complete and can be induced by remarkably low concentrations of NAADP$^+$. It occurs in live cells and in microsomes. To our knowledge, that subactivating concentrations of ligand can induce desensitization of its receptor, possibly through alteration of the receptor conformation, has not been described previously. Equally novel is the effectiveness of NAADP$^+$ to release Ca$^{2+}$ in live eggs. Photolyzing caged NAADP$^+$ induces a 17.6 ± 2.9-fold (n = 15) increase in fluo 3 fluorescence (cf. Fig. 1), as compared with the 3–6-fold increase induced by either microinjecting cADPR or photolyzing caged cADPR (15). UV photolysis simultaneously releases NAADP$^+$ from all parts of the eggs, circumventing possible desensitization produced by high local concentrations, which is likely to occur during microinjection (10, 17). With this procedure, remarkable patterns of long term Ca$^{2+}$ oscillations are revealed. Because of its self-inactivating property, it is unlikely that NAADP$^+$ itself is responsible for the long term oscillations. The IP$_3$- and/or cADPR-sensitive mechanism may be involved. However, neither cADPR nor IP$_3$ alone has been reported to be able to generate these types of oscillations in eggs. This suggests that the global elevation of NAADP$^+$ may specifically set off complex interactions between cADPR- and IP$_3$-sensitive Ca$^{2+}$ stores, resulting in the generation of the long term changes. Indeed, these changes are reminiscent of the multiple Ca$^{2+}$ oscillations occurring after fertilization, which are correlated with various developmental events (18).

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