Pyridine Nucleotide Metabolites Stimulate Calcium Release from Sea Urchin Egg Microsomes Desensitized to Inositol Trisphosphate*

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Inositol trisphosphate (IP₃) was previously shown to release Ca²⁺ from a nonmitochondrial store in sea urchin eggs. In this study, egg homogenates and purified microsomes were monitored with either fura 2 or Ca²⁺-sensitive microelectrodes to determine whether other stimuli would induce Ca²⁺ release. Pyridine nucleotides (whose concentrations are known to change at fertilization) were found to release nearly as much Ca²⁺ as did IP₃. Average releases/ml of homogenate were: 0.6 μM IP₃, 10.9 nmol of Ca²⁺; 50 μM NADP, 7.3 nmol of Ca²⁺; and 100 μM NAD, 6.5 nmol of Ca²⁺ (n = 6). Specificity was demonstrated by screening a series of other phosphorylated metabolites, and none was found to reproducibly release Ca²⁺. Calcium release induced by IP₃ or NADP was immediate, whereas a lag of 1–4 min occurred with NAD. This lag before NAD-induced Ca²⁺ release led to the discovery that a soluble egg factor (Mᵢ > 100,000) converts NAD into a highly active metabolite that releases Ca²⁺ without a lag. The NAD metabolite (E-NAD) was purified to homogeneity by high pressure liquid chromatography and produced half-maximal Ca²⁺ release at about 40 nm. Injection of E-NAD into intact eggs produced both an increase in intracellular Ca²⁺ (as assayed with indo-1) and the subsequent alkalinization of internal pH by 0.5–0.6 pH unit (reviews by Refs. 1–3). A direct consequence of the Ca²⁺ transient is the induction of a massive exocytotic reaction leading to the formation of the fertilization envelope. The Ca²⁺ involved is released from internal stores (1–3), and several lines of evidence indicate that inositol trisphosphate (IP₃) is the most likely mediator of the Ca²⁺ release. Immediately after sperm binding, phosphatidylinositol metabolite in the egg is greatly stimulated (4, 5), with the phosphatidylinositol content reaching a minimum within 30 s postfertilization (4, 5). Concurrently, the phosphorylated intermediates, phosphatidyl 4,5-bisphosphate and phosphatidyl 3,4-bisphosphate, produced by the hydrolysis products, diacylglycerol and IP₃, were found to increase (4–6). Evidence suggests that a GTP-binding protein is responsible for activating the lipase activity that leads to the production of IP₃. Microinjection of IP₃ into a sea urchin egg triggers a transient increase in intracellular Ca²⁺ as monitored by fluorescent Ca²⁺ indicators (8) and also induces the cortical exocytotic reaction (7–9).

We have developed a cell-free system using egg homogenates and purified microsomes. This system shows ATP-dependent Ca²⁺ sequestration and can release Ca²⁺ in the presence of IP₃ (10). In the current study, we show that a novel metabolite of NAD is generated by a high molecular weight (≥100,000) enzyme present in the egg extract and can induce Ca²⁺ release in the cell-free system. The metabolite was purified to apparent homogeneity by HPLC and was found to be active at a concentration range similar to that of IP₃. Microinjection of the purified metabolite into an egg can induce transient Ca²⁺ release and initiate the cortical exocytotic reaction. Evidence is presented that IP₃, the NAD metabolite, and a similar metabolite produced from NADP all act through independent mechanisms in releasing Ca²⁺, thus suggesting the existence of multiple Ca²⁺ stores in sea urchin eggs. This interpretation was supported by partial separation of the Ca²⁺ stores using Percoll density gradient centrifugation.

EXPERIMENTAL PROCEDURES

Gamete Handling and Media Used—Dejellied eggs of Lytechinus pictus, artificial seawater, and Ca²⁺-free artificial seawater (0CaSW) were prepared as previously described (10). EGTA-0CaSW is 0CaSW containing 1 mM EGTA. Intracellular medium (GluIM) was modified from that used previously (10) by the deletion of Na⁺ and for this study was composed of 250 mM potassium gluconate, 250 mM N-methylglucamine, 20 mM HEPES, and 1 mM MgCl₂. The pH was

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adjusted to 7.2 by the addition of acetic acid. For acetate intracellular medium (AcIM), the potassium gluconate was replaced with 250 mM potassium acetate.

**Egg Homogenate Preparation and Purification of Microsomes**—Unfractionated dejellied eggs were sequentially washed in EGTA-0.5 mM Tris, pH 7.2, 0.5% (v/v) of each of the following: EGTA (10 μM), benzamidine (2.5 mM), and phenylmethylsulfonyl fluoride (0.5 mM) were added, and the eggs were homogenized with a Dounce-type glass tissue homogenizer with a size "A" pestle. The homogenates were centrifuged for 45 s (13,000 × g, 4 °C) in a microcentrifuge (model 235A, Fisher). The supernatant was saved and will be referred to here as a crude homogenate.

For some experiments, microsomes were purified by Percoll density gradient centrifugation using a procedure similar to that previously reported (10). Percoll was diluted into 1.5× concentrated AcIM to produce final concentrations of 25% Percoll and normal strength AcIM. ATP, phosphocreatine, creatine phosphokinase, benzamidine, and EGTA were added to the same concentration as that present in the crude homogenate. Then 3 ml of 25% crude homogenate was layered onto 7 ml of 25% Percoll and centrifuged for 40 min (25,000 × g, 10 °C). The upper band contained IP~3~sensitive microsomes, and it was used for Ca~2+~ uptake and release assays. This fraction will be referred to as purified microsomes. To prepare microsome-free egg supernatant, the top 2.5 ml of each Percoll gradient was removed and centrifuged an additional 20 min at 100,000 × g. This is referred to as a crude egg supernatant.

Throughout this study, the concentrations of crude homogenates, purified microsomes, or supernatant are expressed as a percentage of the original volume of packed eggs. The crude homogenate was prepared from packed eggs that were diluted 4-fold and is a 25% crude homogenate. Since the supernatant from the Percoll gradients is undiluted, it is a 25% supernatant. The volume of purified microsomes recovered from each Percoll gradient was typically 0.75 ml, and since 3 ml of a 25% homogenate was applied, the recovered microsomes would be suspended in the same volume as the original packed egg volume, representing a 100% recovery. For each experiment, typical concentrations used in this study (based on egg volume) and respective protein concentrations after dilution (mg/ml ± S.D.) are: 2.5% crude homogenate (2.84 ± 0.48 mg/ml protein, n = 5), 5% purified microsomes (0.30 ± 0.06 mg/ml, n = 4), and 25% egg supernatant (12.0 ± 3.5 mg/ml, n = 5).

**Monitoring Ca~2+~ Fluxes in Homogenates and Microsomes**—Ca~2+~ release and resequstration by egg microsomes were monitored by measuring changes in the Ca~2+~ concentration of the medium. Both crude homogenates and purified microsomes were diluted into GluIM containing 1 mM ATP, 2.5 mM benzamidine, and 10 μM EGTA. The method of Ca~2+~ release measurements (GluIM) was different from that used for the homogenization and Percoll separation (AcIM), because microsomes suspended in GluIM responded better to Ca~2+~ release agents (by releasing about twice as much Ca~2+~ as when suspended in AcIM), whereas the lower density AcIM was better for the Percoll procedure, since it allowed twice as much homogenate to be separated on each Percoll gradient. For most experiments, Ca~2+~ levels in 1-ml aliquots were monitored with the fluorescent Ca~2+~ indicator, fura 2 (0.5 μM) (11). To minimize the fluorescence interference of reduced pyridine nucleotides, fluorescence was measured at 400-nm excitation (2.5-nm slit) and 540-nm emission (10-nm slit). For each experiment, the relationship between fura 2 response and changes in Ca~2+~ concentration was determined by adding known amounts of Ca~2+~ to an aliquot of the same homogenate. As will be indicated later, certain key results were verified with Ca~2+~-sensitive minielectrodes (prepared and used as previously reported, Ref. 10).

**Membrane Filtrations and Incubations to Produce Enzyme-activated NAD**—To remove small molecular weight components present in the egg supernatant, aliquots of supernatant were washed on 100,000 M cutoff filters (XM-100 Ambers, Amicon Corp., Lexington, MA). Typically 6 ml of 25% supernatant was filtered, and the retained volume was 0.1-0.2 ml. The number of washes was repeated 3-4 times and the retentate recovered in 2.5 ml.

To produce enzyme-activated NAD (E-NAD), 1-2.5 ml NAD was added to washed retentate and incubated for 5-7 h at 17 °C. Ca~2+~ release was monitored by adding aliquots of the incubation mixture to 2.5% homogenates, and the incubation was stopped (by placing the mixture on ice) when maximum Ca~2+~ release activity had developed. The incubation mixture was filtered on a 10,000 M cutoff filter membrane (Amicon PM-10) to separate the E-NAD from the washed supernatant, and high levels of Ca~2+~ release activity were recovered in the filtrate.

**Alkal Treatment of NAD and NADP**—This procedure is based on a method by Lowrey and Passonneau (12) which was reported to alter NAD and NADP but not their activity. Solution of crude NAD and NADP were prepared in 80 mM K2CO3 and 20 mM KHCO3, the pH was adjusted to 10.5 with KOH if needed, heated at 60 °C for 6-8 min, placed on ice to stop the reaction, and the pH was readjusted to 8 to prevent further reaction. The resultant products with Ca~2+~ releasing activity are called alkali-activated NAD (A-NAD) and alkali-activated NADP (A-NADP).

**Purification of Activated NAD and NADP by HPLC**—In order to quantitate the calcium release activity of E-NAD, A-NAD, and A-NADP during purification steps, a unit of Ca~2+~ release activity was defined as the amount sufficient to produce half-maximal Ca~2+~ release from 1 ml of a 2.5% crude egg homogenate. Therefore, each fraction-containing activity was serially diluted, and 2-10 μl was added to 1 ml aliquots of crude homogenate to determine the dilution and volume required to produce half-maximal Ca~2+~ release. From the volume (μl) added to the homogenate, the serial dilution required, and the initial volume of the fraction, the total units of activity in the initial fraction was calculated.

All three activators, E-NAD, A-NAD, and A-NADP, were prepared as previously indicated and purified by two sequential separations on 0.46 × 15-cm AG MP-1 anion exchange columns eluted with 1-150 mM trifluoroacetic acid gradients (13). For the first separation, each column was maximally loaded and produced incomplete separation of overlapping peaks. For each activator, the Ca~2+~ releasing activity applied was: E-NAD (1,230 units), A-NAD (1,980 units), and A-NADP (32,000 units). One-ml fractions were collected, vacuum evaporated, resuspended in 0.05-0.1 ml of distilled water, and assayed for Ca~2+~ releasing activity with 2.5% crude homogenates. For each activator, fractions containing activity from the first separation were pooled and rechromatographed, and this time each produced activity associated with a single clearly separated UV peak. Following the two AG MP-1 separations, the percent recoveries of activity and yields (based on initial NAD or NADP) were: E-NAD (110% recovery, 1.8% yield), A-NAD (20% recovery, 0.19% yield), A-NADP (68% recovery, 1.5% yield).

For each Ca~2+~-releasing factor, 80 units of activity previously purified by the two AG MP-1 separations were analyzed by reverse phase HPLC (14) to determine whether Ca~2+~-releasing activity co-purified with the absorbance at 284 nm. One-ml fractions were collected, dried, and analyzed for Ca~2+~-releasing activity as before.

**Ascorbing Intracellular Calcium in Intact Eggs**—To sequential iontophoretic injections were used to first load intact eggs with the fluorescent Ca~2+~ indicator, indo-1 (11), and then to introduce purified E-NAD or A-NADP. The current parameters used for these injections were 30 pulses/min, 1 s/pulse, and 2-3 nA. Reagent concentrations in the micropipettes were: E-NAD (1.7 units/μl), A-NADP (1.9 units/μl), indo-1 (10 mM), and NAD (5 mM). Resultant fluorescence changes were monitored at 365-nm excitation and 485-nm emission with a fluorescence microscope equipped with a silicon-intensified target camera (RCA TC1030/H) whose output was digitized. The details of the instrumental setup will be published elsewhere. Autofluorescence from an adjacent uninjected egg was also measured and subtracted from the fluorescence signal of the injected egg.

**Materials**—Fura 2 and indo-1 were obtained from either Molecular Probes or Behring Diagnostics. NAD and TMB-8 were from Behring Diagnostics. IP~3~ was prepared as previously described (10). NADP (sodium salt, Sigma grade), ATP (dipotassium), phosphocreatine (disodium), creating phosphokinase (type I), and all other reagents were obtained from Sigma.

**RESULTS**

**Calcium Release from Crude Homogenates**—In a previous study, we used *L. pictus* egg homogenates and microsomes as a cell-free system to study Ca~2+~ uptake and release and found that IP~3~ induced Ca~2+~ release from a nonmitochondrial store (10). In the present study, the same cell-free system is used, but the Ca~2+~ movement was monitored with a different fluorescent Ca~2+~ indicator, fura 2. The fluorescence properties of fura 2 allow the measurement of Ca~2+~ with little interference from the fluorescence of reduced pyridine nucleotides. At the
excitation wavelength used, fura 2 fluorescence decreases in response to increasing Ca\(^{2+}\). Fig. 1a shows IP\(_3\)-induced Ca\(^{2+}\) release from the egg homogenate as indicated by the observed decrease in fura 2 fluorescence.

To determine whether other cell metabolites might stimulate Ca\(^{2+}\) release, a series of phosphorylated molecules were screened. Since the concentrations of pyridine nucleotides are known to change after fertilization of sea urchin eggs (15), NAD and NADP were assayed first. Both were found to release Ca\(^{2+}\) at physiological concentrations (Fig. 1, a, b, and c). With six different homogenates, activators were added at concentrations that produced maximal Ca\(^{2+}\) release, and 1-ml aliquots of 2.5% crude homogenate produced Ca\(^{2+}\) releases (± S.D.) of: 10.9 ± 3.6 nmol of Ca\(^{2+}\) with 0.6 μM IP\(_3\), 6.5 ± 1.3 nmol of Ca\(^{2+}\) with 100 μM NAD, and 7.3 ± 2.4 nmol of Ca\(^{2+}\) with 50 μM NADP. The Ca\(^{2+}\) efflux began immediately upon addition of IP\(_3\) and NADP, whereas a delay of 1–2 min occurred before Ca\(^{2+}\) was released by NAD (Fig. 1c). Evidence will be presented later that the delay before NAD-induced Ca\(^{2+}\) efflux is due to the conversion of NAD to a more active form.

The screening of other phosphorylated metabolites showed that no Ca\(^{2+}\) release was induced by 1 mM 3′:5′-cyclic adenosine monophosphate, 1 mM 3′:5′-cyclic guanosine monophosphate, or 5 mM adenosine 5′-monophosphate, adenosine 5′-diphosphate, guanosine 5′-diphosphate, phosphocreatine, D-glucose 6-phosphate, phosphoenolpyruvate, or 6-phosphogluconate. However, GTP (2–4 mM) initiated Ca\(^{2+}\) release from four of six homogenates assayed (2–6 nmol of calcium released from 1-ml aliquots of 2.5% crude homogenates). Due to the high concentration of GTP required and the lack of reproducibility of its Ca\(^{2+}\)-releasing action, no further experiments with GTP are reported here.

**Desensitization Experiments Implicate Three Calcium Release Mechanisms**—To determine whether IP\(_3\), NAD, and NADP all release Ca\(^{2+}\) via the same mechanism, homogenates were desensitized to IP\(_3\) before addition of NAD and NADP. A previous study had shown that homogenates resequester Ca\(^{2+}\) after stimulation by IP\(_3\) but are desensitized to subsequent IP\(_3\) addition (10). Fig. 2 shows that the first addition of IP\(_3\) to the egg homogenate produced a large Ca\(^{2+}\) release followed by resequestration. Subsequent additions induced progressively less Ca\(^{2+}\) release indicating desensitization. The desensitized homogenate, however, could still respond to NAD and release Ca\(^{2+}\) upon its addition. Similar to IP\(_3\), NAD also produced desensitization in the homogenate. Finally, NADP-stimulated Ca\(^{2+}\) release after desensitization to both IP\(_3\) and NAD. The released Ca\(^{2+}\) was again resequestered by the homogenate. If the order of reagent addition is changed, each reagent still releases Ca\(^{2+}\), e.g. IP\(_3\) stimulated Ca\(^{2+}\) release from homogenates desensitized to NAD and NADP (data not shown).

These results implicate the presence of 3 different Ca\(^{2+}\) release mechanisms in *L. pictus* eggs. When the experiments depicted in Figs. 1 and 2 were repeated with a Ca\(^{2+}\)-sensitive minielectrode, the same results were obtained, therefore, verifying that the observed changes represent real Ca\(^{2+}\) fluxes and not artifacts of the fluorescent Ca\(^{2+}\) assay method. Also, the intracellular Ca\(^{2+}\) blocking agent 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate (3 mM) produced ≥90% inhibition of Ca\(^{2+}\) release by all three reagents.

**Calcium Release from Purified Microsomes**—We had previously shown that Percoll density gradient centrifugation could be used to purify microsomes that released Ca\(^{2+}\) upon stimulation by IP\(_3\) (10). Using the same protocol but with a lower density medium (AcIM) produced a similar banding pattern on Percoll gradients and allowed a larger volume of crude homogenate to be purified on each gradient.

The upper band (which contained IP\(_3\)-responsive vesicles) was removed and assayed for response to NAD and NADP. Fig. 3A shows that the purified microsomes responded to both NADP and IP\(_3\) but not to NAD. However, if the supernatant from the top of the Percoll gradients was centrifuged at 100,000 × g and added to purified microsomes, responsiveness to NAD was restored (Fig. 3B). A similar time delay between
NAD addition and Ca²⁺ release was observed with both purified microsomes (Fig. 3B) and crude homogenates (Fig. 1). The delay was found to be proportional to the concentration of supernatant added so that Ca²⁺ release started about twice as fast when the supernatant concentration was doubled (data not shown). After the release, the Ca²⁺ was again resorbed, and yet the microsomes did not respond to the second addition of NAD. Similar to that observed in the crude homogenate, the desensitized microsomes can still respond to NADP and IP₃ as shown in Fig. 3B. Comparing Fig. 3, A and B, it can be seen that both the amount and the kinetics of Ca²⁺ release induced by IP₃ and NADP were not affected by the presence of the high speed supernatant.

**NAD Is Converted into an Active Form**—The characteristics of NAD action are consistent with a soluble enzyme using NAD as a substrate to produce a Ca²⁺ release activator. To test this hypothesis, NAD (2.5 mM) was incubated with 25% supernatant before being added to a crude homogenate. Fig. 4a shows that such preincubated NAD (N+S in the Fig. 4a) induced Ca²⁺ release without a lag and at a lower concentration than would induce Ca²⁺ release without preincubation (Fig. 4b).

Although the preincubated NAD showed no lag in Ca²⁺-releasing activity when added to purified microsomes, supernatant addition was still required for maximal Ca²⁺ release. Fig. 4c shows that the magnitude of Ca²⁺ release was proportional to the concentration of supernatant added. In the presence of increasing amounts of high speed supernatant, the same amount of preincubated NAD produced increasing amounts of Ca²⁺ release. This result implies that an additional factor is necessary for purified microsomes to respond to the Ca²⁺-releasing factor.

The soluble components of the NAD system were fractionated by membrane filtration. Utilizing a series of membrane filters of defined pore sizes, it was determined that the Ca²⁺-releasing factor itself is a small molecular weight metabolite of NAD, while the converting enzyme and the large molecular weight components were removed by filtration on the 10,000 molecular weight cutoff membrane, and the resultant Ca²⁺-releasing activity was recovered in the eluent. This enzyme-activated NAD (E-NAD) was further purified by anion exchange HPLC using a procedure known to provide good separation of nucleotides (13). Fig. 5A shows that the initial preparation contained several peaks that absorb at 254 nm, with Ca²⁺-releasing activity correlating with a small UV peak that eluted at 24 min.

**Purification of Enzyme-activated NAD**—The behavior of these factors on membrane filters was used to prepare an extract highly enriched for the NAD-derived Ca²⁺-releasing factor. The procedure is described under “Experimental Procedures,” and in summary consists of first washing the supernatant on the 100,000 molecular weight cutoff filter to both remove small molecular weight cytoplasmic components and concentrate the enzyme, and then incubating with NAD until maximum Ca²⁺-releasing activity was developed. Finally the large molecular weight components were removed by filtration on the 10,000 molecular weight cutoff membrane, and the resultant Ca²⁺-releasing activity was recovered in the eluent.

This enzyme-activated NAD (E-NAD) was further purified by anion exchange HPLC using a procedure known to provide good separation of nucleotides (13). Fig. 5A shows that the initial preparation contained several peaks that absorb at 254 nm, with Ca²⁺-releasing activity correlating with a small UV peak that eluted at 24 min.
Upscaling the procedure to preparative range using a sample 8.5x larger than that shown in Fig. 5A resulted in incomplete separation of UV-absorbing peaks. All the fractions containing Ca\(^{2+}\)-releasing activity were, therefore, combined and rechromatographed on a second AG MP-1 column. The second chromatogram showed clearly separated UV peaks with the Ca\(^{2+}\) release activity correlating with a single peak that was eluted at 17.8 min (data not shown). The slight difference in elution times for E-NAD between Fig. 5A (24 min) and the second preparative run (17.8 min) is due to either different AG MP-1 columns being used or else the column in Fig. 5A was overloaded.

Next, an aliquot of E-NAD purified by the two sequential separations was further analyzed by reverse phase HPLC (14). A single UV peak with a retention time of 9.4 min copurified with the Ca\(^{2+}\)-releasing activity (Fig. 5B). Since anion exchange and reverse phase HPLC separate according to different principles, these results, therefore, provide strong evidence that E-NAD is purified to homogeneity and contains a moiety that absorbs at 254 nm.

In a first attempt to characterize E-NAD, UV spectra were compared for E-NAD, NAD, adenosine 5'-diphosphoribose (ADP-ribose), adenosine 5'-monophosphate (AMP), and β-nicotinamide mononucleotide (NMN). Therefore, the spectra for E-NAD, NAD, ADP-ribose, and AMP were nearly identical with each showing a peak at 257-260 nm and a minimum at 227-233 nm. NMN, on the other hand, showed a significantly different spectrum with a small peak at 262 nm and a minimum at 245 nm. These results indicate that the adenine group is retained in E-NAD but provide no information concerning other possible modifications of the NAD structure.

The absorbance at 258 nm was then used to estimate the active concentration of E-NAD. Assuming the extinction coefficient at 258 nm is the same for both NAD and E-NAD, it could be determined that 80 units/ml of E-NAD would correspond to 2.8 μM NAD. One unit/ml was then calculated to be 35 nM; and from the definition of a unit of activity, 35 nM is the approximate concentration that produces one-half maximal Ca\(^{2+}\) release. Since the extinction coefficient of E-NAD is unlikely to be too much higher than NAD, this estimate, although quite rough, does put the active concentration of E-NAD in the range of that of IP3.

Ca\(^{2+}\) release by E-NAD and IP3 was then compared, with each being used at concentrations that produce greater than 90% maximal Ca\(^{2+}\) release. In two experiments with 2.5% crude homogenates, IP3 (600 nM) released 14 and 8.6 nmol of Ca\(^{2+}\)/ml of homogenate, and E-NAD (5 units/ml) released 11.5 and 6.3 nmol of Ca\(^{2+}\)/ml. Therefore, E-NAD released an average of 78% as much Ca\(^{2+}\) as did IP3. Desensitization experiments showed that E-NAD desensitized the component of the Ca\(^{2+}\) release activity which was sensitive to NAD but did not desensitize microsomes to either IP3 or NAD.

Desensitization by Injection of Activated NAD into Intact Eggs—The egg was first loaded with the fluorescent Ca\(^{2+}\) indicator, indo-1; then the fluorescence was monitored as E-NAD was iotophoretically injected. Fig. 6 shows that each injection was followed by a transient decrease in fluorescence, thus demonstrating Ca\(^{2+}\) release followed by resesquestration. After the first injection, the egg was observed with phase contrast optics and had undergone a complete cortical reaction. If more E-NAD was injected 5.5 min after the first injection, additional Ca\(^{2+}\) was released. This shows that any desensitization produced by E-NAD is reversed by about 5 min and implies that the egg possesses a system for removing E-NAD.

Three more eggs were injected with indo-1 followed by E-NAD, and all showed free Ca\(^{2+}\) increases followed by cortical reactions. In three control experiments, the same protocol was used, only the pipettes were filled with 5 mM NAD instead of E-NAD. All three eggs showed much less increases in Ca\(^{2+}\) (6-7-fold smaller fluorescence decreases than with E-NAD) and no cortical reactions. Two of these control eggs were subsequently fertilized by adding sperm, and both showed large Ca\(^{2+}\) increases followed by cortical reactions, indicating they were not damaged by the microinjection.

NAD Analogs and Alkaline-activated NAD—Several commercially available analogs for NAD were screened for Ca\(^{2+}\)-releasing activity upon addition to 2.5% crude homogenates. Only NADP released Ca\(^{2+}\) at less than 100 μM; however, NADP cannot be E-NAD since NADP does not desensitize homogenates to E-NAD and vice versa. Molecules found to release Ca\(^{2+}\) at 100-500 μM include NADH, NADPH, adenosine 5'-diphosphoribose (ADP-ribose), and nicotinic acid adenine dinucleotide. All four showed no lag before Ca\(^{2+}\) release, and all four desensitized homogenates to E-NAD; therefore, they are most likely acting as analogs to E-NAD. Little or no Ca\(^{2+}\) release was induced by 200-500 μM nicotinamide, nicotinamide mononucleotide, or α-NAD. Also none of these desensitized homogenates to E-NAD.

A comparison of the structures of active NAD analogs indicates that activation of NAD may involve neutralization of the positive charge on the nicotinamide moiety. Lowry and Passonneau (12) reported an alkali treatment that alters NAD but not NADH, with a major initial reaction being a hydrolisis that opens the nicotinamide ring and neutralizes the positive charge originally associated with the ring (16).

Alkali treatment of NAD (using the procedure described under "Experimental Procedures") produced a substance with Ca\(^{2+}\)-releasing activity very similar to that of E-NAD. The alkali product produced Ca\(^{2+}\) release without a lag, desensitized homogenates only to NAD, and required the equivalent of 50 μM original NAD for maximal Ca\(^{2+}\) release. This alkali-activated NAD is called A-NAD.

A-NAD was then purified by anion exchange HPLC and analyzed by reverse phase HPLC using the same procedures as for E-NAD. Respective elution times for the UV peaks associated with Ca\(^{2+}\)-releasing activity for E-NAD and A-NAD were identical in both the anion exchange system (17.8 min) and the reverse phase system (9.4 min). The UV spec-
trium of the HPLC-purified A-NAD was also found to be the same as E-NAD and NAD.

The active concentration of A-NAD was determined from the absorbance at 258 nm, assuming the same extinction coefficient as NAD, and 48 nm was found to induce half-maximal Ca\(^{2+}\) release from 2.5% crude homogenates. This is close to the 35 nm active concentration determined for E-NAD. Furthermore, A-NAD also desensitized microsomes only to E-NAD and NAD but not to IP\(_3\) or NADP. All together, these results indicate that E-NAD and A-NAD are probably the same molecule.

**Alkaline-activated NADP**—Both the enzyme and the alkaline treatments as developed for NAD were also tested on NADP to determine if NADP could also be converted to a more active form. Incubation of NADP plus supernatant using the same conditions as used for the production of E-NAD did not produce a convincing increase in Ca\(^{2+}\)-releasing activity; however, alkaline treatment of NADP produced about a 30-fold enhancement. Before the alkaline treatment, the concentration of NADP was measured using a marker enzyme, cytochrome c oxidase, on the anion exchange HPLC as was used for E-NAD and A-NAD. The UV spectrum of A-NADP was the same as for NADP and NAD; therefore, the adenine group was retained. Again, assuming the extinction coefficient of A-NADP is the same as NAD, its half-maximal active concentration can be estimated to be 38 nm, very similar to that of E-NAD. The maximal amount of Ca\(^{2+}\) release in crude homogenates induced by A-NADP was on average 67% of that released by E-NAD. Furthermore, A-NADP also desensitized microsomes to both IP\(_3\) and E-NAD, as previously indicated. Arrows labeled IP\(_3\), E-NAD, and A-NADP indicate the respective additions of 600 nm IP\(_3\), 5 units of E-NAD, or 5 units of A-NADP. E-NAD and A-NADP were purified as indicated under "Experimental Procedures."

The concentration of NADP required to produce half-maximal and maximal Ca\(^{2+}\) release was found to be about 75 and 400 \(\mu\)M, respectively. After alkaline treatment, these values were reduced to about 2.5 and 10 \(\mu\)M, respectively.

Alkaline-treated NADP (A-NADP) was then purified by anion exchange HPLC as was used for E-NAD and A-NAD. On the anion exchange column, activity eluted with a UV peak at 30 min as compared to the elution time of 17.8 min for E-NAD. These elution times clearly show that A-NADP is different from E-NAD. Also, the purified A-NADP induced Ca\(^{2+}\) release from microsomes desensitized to both IP\(_3\) and E-NAD as shown in Fig. 7.

The UV spectrum of A-NADP was the same as for NADP and NAD; therefore, the adenine group was retained. Again, assuming the extinction coefficient of A-NADP is the same as NAD, its half-maximal active concentration can be estimated to be 38 nm, very similar to that of E-NAD. The maximal amount of Ca\(^{2+}\) release in crude homogenates induced by A-NADP was on average 67% of that released by IP\(_3\) and 85% of that released by E-NAD (n = 2). Also, all three reagents showed similar kinetics of Ca\(^{2+}\) release, with release beginning immediately and being maximal by 2 min.

Purified A-NADP was injected into intact eggs, and free Ca\(^{2+}\) was monitored using the same protocol as for E-NAD. A-NADP produced both transient Ca\(^{2+}\) increases (similar to that shown in Fig. 6) and cortical reactions in all four eggs injected, therefore, showing that intact eggs respond to A-NADP.

**Two Calcium Stores Can Be Separated on Percoll Gradients**—When comparing purified microsomes to crude homogenates, the response to NADP always decreased more than the response to either IP\(_3\) or NAD (e.g. Figs. 3 and 7). One possible explanation would be if part of the NADP-responsive microsomes was lost when the upper band was removed from the Percoll gradients. To test this hypothesis, upper and lower bands (the only two bands present on those Percoll gradients) were recovered separately and assayed for response to IP\(_3\), E-NAD, and A-NADP.

Table I shows that the upper band contained over 90% of the responsiveness to IP\(_3\) and E-NAD. On the other hand, the A-NADP sensitivity distributed mainly in the lower band with only about 23% of the activity in the upper band. These results clearly show that the responsiveness to A-NADP resides in a different Ca\(^{2+}\) store.

**Table I**

| Activity       | Upper band | Lower band |
|----------------|------------|------------|
| Calcium release\(^a\) | 91 ± 4     | 9.0 ± 3.6  |
| IP\(_3\) \(^b\) | 93 ± 7     | 7.3 ± 6.7  |
| E-NAD \(^c\)  | 23 ± 17    | 77 ± 17    |
| A-NADP         | 0.47 ± 0.21| 99.5 ± 0.21|

\(^a\) Homogenates were separated on Percoll gradients as described under "Experimental Procedures." Results from three homogenate preparations are averaged, and percent activity (± SD) is reported to normalize differences in absolute values.

\(^b\) Ca\(^{2+}\) release was assayed as described under "Experimental Procedures." Concentrations of reagents used were: IP\(_3\) (600 nm), purified E-NAD (5 units/ml), and purified A-NADP (5 units/ml). Supernatant (2.5%) was added to each microsome fraction to allow responsiveness to E-NAD.

\(^c\) Cytochrome c oxidase was assayed following the procedure of Smith (29).

The discovery that IP\(_3\) can induce Ca\(^{2+}\) release directly from internal stores attracts a great deal of interest focusing on the role of IP\(_3\) as a second messenger for intracellular Ca\(^{2+}\) changes. In most of the systems studied so far, IP\(_3\) production is stimulated as a result of the interaction between external stimuli and cell surface receptors (reviews by Refs. 17 and 18). Similarly, interaction between sperm and sperm receptors on the surface of sea urchin eggs activates a GTP-binding protein which then leads to the production of IP\(_3\) and Ca\(^{2+}\).
release from internal stores (7). However, not all intracellular increases are mediated by surface receptor activation. For example, in the sea urchin egg, about 15 min after the first Ca\(^{2+}\) changes at fertilization, the intracellular Ca\(^{2+}\) increases transiently again (19). This occurs in the absence of external stimulus and is correlated with the pronuclear migration (19). Thereafter, multiple Ca\(^{2+}\) transients can be detected correlating with various mitotic events (19).

We are interested in knowing if all of these Ca\(^{2+}\) transients are triggered by IP\(_3\) and, if not, whether other activators can be identified. We had previously developed a cell-free system for studying the IP\(_3\)-sensitive Ca\(^{2+}\) release mechanism (10). This system is ideally suited to screening a large number of potential Ca\(^{2+}\) release activators. Pyridine nucleotides were tested first because it is known that shortly after fertilization, there is a large and rapid conversion of NAD to NADP which is followed by the reduction of NADP to NADPH (15). These changes in pyridine nucleotides have been postulated to have important consequences for the later activation of the nucleus (20).

In this study, we showed that both NAD and NADP at physiological concentrations of 50-100 \(\mu\text{M}\) were able to induce Ca\(^{2+}\) release in the cell-free system. On the average, the amount of Ca\(^{2+}\) released is 60-70\% of that released by IP\(_3\). Unlike that of IP\(_3\), which releases Ca\(^{2+}\) immediately upon addition, the kinetics of Ca\(^{2+}\) release induced by NAD shows a lag period. This suggested that NAD needed to be converted to an active form before it could release Ca\(^{2+}\). This was found to be the case, since preincubation of NAD with a high speed supernatant of the egg homogenate produced an active form of NAD (E-NAD) which was purified to apparent homogeneity by HPLC. Microinjection of purified E-NAD into sea urchin eggs induced Ca\(^{2+}\) release and triggered cortical exocytosis, thus indicating it is active within a living cell.

E-NAD was found to be of small molecular weight, since it passed through a 10,000 molecular weight cutoff filter. The UV absorption spectrum of E-NAD was identical to that of NAD and ADP-ribose suggesting that the modification was likely to be on the nicotinamide and not on the adenine group. An alkaline treatment (known to attack the nicotinamide group) was tested on NAD and was found to be effective in generating the active form of NAD. The active substance (A-NAD) was shown to be identical to E-NAD in both the UV absorption characteristics and also retention times on anion exchange and reverse phase HPLC. This strongly indicates that both A-NAD and E-NAD are the same substance. A similar alkaline treatment was also able to activate NADP. The active substance (A-NADP) has an identical UV absorption spectrum as E-NAD and A-NAD but is retained longer on anion exchange HPLC. This is likely due to the additional phosphate group and indicates that A-NADP is structurally different from E-NAD and A-NAD.

The active concentrations of E-NAD, A-NAD, and A-NADP that produced half-maximal Ca\(^{2+}\) release were all estimated to be 30-50 \(\mu\text{M}\). This estimate was based on the assumption that they all have the same extinction coefficient as NAD. This assumption should be quite reasonable since the extinction coefficient of NAD is quite insensitive to modifications on the nicotinamide group. Thus, a survey of published extinction coefficients for NAD, NADP, NADH, NADPH, ADP-ribose, ADP, adenosine, and adenine shows that none of them differ by more than 25\%. The uncertainty in the estimated half-maximal concentrations is, therefore, also very likely not to exceed 25\%, and this puts them in the same active range as IP\(_3\). Screening of common derivatives of NAD showed that none of them can induce Ca\(^{2+}\) release at this concentration range. This suggests that E-NAD is likely to be a novel derivative of NAD specifically serving as a Ca\(^{2+}\) release activator.

The enzyme in the supernatant of the egg extract which was responsible for converting NAD to its active form was found to be of high molecular weight, since it was retained by a 100,000 molecular weight cutoff filter. Also found in the retentate is another factor which was required for E-NAD to release Ca\(^{2+}\) from egg microsomes. The presence of these factors in the high speed supernatant of the egg extract suggests both are cytoplasmic components, although the possibility that they were solubilized during homogenization cannot be excluded. In any case, it is likely that regulatory mechanisms exist in vivo for controlling either the enzyme activity or the availability of the enzyme itself. Thus, the NAD system, as characterized in this study, possesses at least three potential controlling sites: first, the availability of the substrate NAD; second, the regulation of the converting enzyme; and third, the availability of the high molecular weight factor. Regulation at one or more of these sites should provide a tight control of the Ca\(^{2+}\) release activity of E-NAD. Furthermore, the egg also possesses a very active removal system for E-NAD. This can be inferred from the microinjection experiment shown in Fig. 6. The egg took only about 5 min to recover from the first injection of E-NAD and released Ca\(^{2+}\) again in response to a second injection. In comparison, a similar microinjection experiment using IP\(_3\) showed a recovery period of about 15 min (8). Therefore, the NAD system appears to have all the necessary characteristics of a signaling system.

Recent studies have suggested that GTP (21) and arachidonic acid (22) can also induce Ca\(^{2+}\) release from internal stores independently of IP\(_3\). We have tested both in our system and found that GTP could induce Ca\(^{2+}\) release only at a much higher concentration (2-4 mM) than reported to be effective (3-5 \(\mu\text{M}\)) in cultured neuronal cells (21). The effect was also not very reproducible and was not studied further. Arachidonic acid at 20 \(\mu\text{M}\) (twice the effective concentration in pancreatic islets (22)) did not release Ca\(^{2+}\) in our system and neither did it potentiate nor inhibit Ca\(^{2+}\) release induced by IP\(_3\) or NAD. However, it (20 \(\mu\text{M}\)) did specifically block 90-98\% of the Ca\(^{2+}\) release induced by NADP and A-NADP with half-maximal inhibition occurring at 5 \(\mu\text{M}\).

Pyridine nucleotides have also been implicated in the organic peroxide-induced Ca\(^{2+}\) release from mitochondria. It was proposed that NAD- or NADP-dependent ADP-ribosylation of a mitochondrial protein mediates hydroperoxide-induced Ca\(^{2+}\) release (23). This mechanism is unlikely to explain the pyridine nucleotide-induced stimulation of Ca\(^{2+}\) release in sea urchin egg homogenates since: 1) Ca\(^{2+}\) release does not correlate with the presence of mitochondria (Table I); 2) the addition of an organic peroxide is not needed for pyridine nucleotide-induced Ca\(^{2+}\) release; and 3) ADP-ribosylation is probably not involved, since NAD is converted into a soluble metabolite (E-NAD) that is neither nicotinamide nor ADP-ribose.

Another important conclusion of this study is that sea urchin eggs possess multiple systems for releasing Ca\(^{2+}\) from internal stores. Two lines of evidence support this conclusion. First, repeated additions of either IP\(_3\), E-NAD, or A-NADP can induce desensitization. Microsomes desensitized to any two of the activators will release Ca\(^{2+}\) in response to the third (e.g. Figs. 2, 3, and 7), indicating all three activators act independently. Second, Percoll density gradient centrifugation allowed a partial separation of the A-NADP-sensitive Ca\(^{2+}\) store from those sensitive to IP\(_3\) and E-NAD (Table I). All three stores appear to be nonmitochondrial since their
distributions in the Percoll density gradient are distinct from the cytochrome c oxidase activity. Definitive proof of their identity, however, must await detailed marker enzyme characterization of the gradient.

It is attractive to think that each of these three activators is acting on a physically distinct set of microsomes. These microsomes need not distribute homogeneously throughout the cell. They could be concentrated at specific regions within the cell, so that activation by an appropriate signal could produce localized increase in Ca\textsuperscript{2+} within the cell. Indeed, spatial inhomogeneity of Ca\textsuperscript{2+} has been observed in sea urchin eggs during fertilization (8, 24), in several cell types associated with mitotic events (24–26), in growing processes of nerve cells (27), and in neutrophils during phagocytosis and chemotaxis (28). Whether the NAD system described in this study is involved in any of these cellular events remains to be determined. However, this new Ca\textsuperscript{2+} release system does offer many exciting possibilities and warrants further investigation.

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