Ca²⁺ release triggered by nicotinate adenine dinucleotide phosphate in intact sea urchin eggs

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Nicotinate adenine dinucleotide phosphate (NAADP) was recently identified [Lee and Aarhus (1995) *J. Biol. Chem.* **270**, 2152–2157; Chini, Beers and Dousa (1995) *J. Biol. Chem.* **270**, 3116–3223] as a potent Ca²⁺-releasing agent in sea urchin egg homogenates. NAADP triggered Ca²⁺ release by a mechanism that was distinct from inositol 1,4,5-trisphosphate (InsP₃) and cyclic ADP-ribose (cADPR)-induced Ca²⁺ release. When NAADP was microinjected into intact sea urchin eggs it induced a dose-dependent increase in cytoplasmic free Ca²⁺ which was independent of the extracellular [Ca²⁺]. The Ca²⁺ waves elicited by microinjections of NAADP originated at the site of injection and swept across the cytosol. As previously found in sea urchin

egg homogenates, NAADP-induced Ca²⁺ release in intact eggs was not blocked by heparin or by prior desensitization to InsP₃ or cADPR. Thio-NADP, a specific inhibitor of the NAADP-induced Ca²⁺ release in sea urchin homogenates [Chini, Beers and Dousa (1995) *J. Biol. Chem.* **270**, 3116–3223] blocked NAADP (but not InsP₃ or cADPR) injection-induced Ca²⁺ release in intact sea urchin eggs. Finally, fertilization of sea urchin eggs abrogated subsequent NAADP-induced Ca²⁺ release, suggesting that the NAADP-sensitive Ca²⁺ pool may participate in the fertilization response. This study demonstrates that NAADP acts as a selective Ca²⁺-releasing agonist in intact cells.

INTRODUCTION

Ca²⁺ release from intracellular stores is an important component in several signalling pathways in diverse cell types [1,2]. For example, during fertilization of sea urchin eggs, a transient rise in the intracellular Ca²⁺ concentration ([Ca²⁺]_i) starts at the sperm-egg contact site and sweeps across the cytoplasm [3,4]. In generating this Ca²⁺ wave, which is both necessary and sufficient for metabolic activation of the quiescent egg [3], two major signals are known to trigger Ca²⁺ release from intracellular stores: inositol 1,4,5-trisphosphate (InsP₃)-triggered Ca²⁺ release via the InsP₃ receptor/channel, and Ca²⁺-induced release via the ryanodine receptor/channel [1,2]. Recent observations indicate that cyclic ADP-ribose (cADPR) is the endogenous regulator of the ryanodine receptor in sea urchin eggs [5–10]. It now appears that sea urchin eggs contain an additional Ca²⁺-release system which is triggered by the recently identified agonist nucleotide nicotinate adenine dinucleotide phosphate (NAADP) [11,12]. NAADP, in nanomolar concentrations, triggered Ca²⁺ release in sea urchin egg homogenates via a mechanism which was clearly distinct from those controlled by InsP₃ and cADPR [11,12]. Experimental evidence for the identity of the NAADP-induced Ca²⁺-release mechanism in homogenates included: (1) the absence of cross-desensitization with InsP₃ and cADPR; (2) specific inhibition by nanomolar concentrations of thio-NADP [12]; and (3) the lack of inhibition of NAADP-triggered Ca²⁺ release by known antagonists of cADPR and InsP₃ [11,12].

In the present study, we microinjected NAADP into intact sea urchin eggs (1) to characterize its effects in living cells, (2) to determine the dependence of the NAADP response on extracellular Ca²⁺, and (3) to define the possible participation of the NAADP-sensitive intracellular Ca²⁺ pathway in the fertilization response. Our work confirms the results obtained in sea urchin

egg homogenates and shows that the properties of NAADP-releasing system observed in homogenates are fully functional in intact sea urchin eggs. In addition, fertilized eggs are desensitized to NAADP, suggesting that the intracellular NAADP-induced Ca²⁺-release system may play a role in sea urchin eggs during fertilization.

MATERIAL AND METHODS

Gametes

Eggs and sperm of the sea urchin *Lytechinus pictus* were obtained by injection of 1 ml of KCl (0.5 M) into the coelomic cavity. Eggs were shed in artificial sea water (ASW). Jelly coats were removed from the eggs by several passages through fine-mesh silk and two washes in ASW. Eggs were kept in suspension by constant stirring until used for fluorimetric or confocal monitoring of Ca²⁺ within 4 h of shedding. Sea urchin sperm was collected and stored at 4 °C.

Microinjection procedures

Pipettes for microinjection were pulled on a Narishige PE-2 puller from 1 mm (outer diam.) × 0.8 mm (internal diam.) pyrex glass tubing heated to 200 °C and silane-treated by exposure to hexamethyldisilazane vapours. Eggs were immobilized on a poly(L-lysine)-treated glass coverslip and impaled with a micro-pipette. A pulse of N₂ delivered into a Narishige IM200 pneumatic microinjector expelled solution into the eggs. An estimate of the injection volume of the agonists was made by using 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF)-dextran to calibrate the dilution of various compounds. InsP₃, cADPR and NAADP were injected with 10 mg/ml BCECF-dextran and the

Abbreviations used: ASW, artificial sea water; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein; cADPR, cyclic ADP-ribose; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; NAADP, nicotinate adenine dinucleotide phosphate.

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fluorescence measured at 535 nm (excitation 440 nm). The emission intensity was compared with that of a BCECF-dextran standard at various dilutions in intracellular buffer [9,13]. The injection volume ranged from 0.5 to 1.0% of the total volume of the egg (approx. 0.7 nl). In most cases eggs were injected with 5 mM fluo-3 or 10 mM fura-2 (final estimated concentrations 10 μ M and 20 μ M respectively), in the presence or absence of heparin or thio-NADP, and subsequently with Ca^{2+} -release agonists within 10 min using separate micropipettes.

Fluorimetric monitoring of $[\text{Ca}^{2+}]_i$ in intact sea urchin eggs

Changes in $[\text{Ca}^{2+}]_i$ in injected eggs were monitored by epifluorescence as described [13]. The excitation light source was a 75 W xenon lamp shielded by a neutral density filter to prevent photobleaching. Excitation filters for BCECF-dextran and fura-2 were 440 DF10, 490 DF10, 340 DF11 and 380 DF13 bandpass filters (Omega Optical, Brattleboro, VT, U.S.A.) respectively in a Nikon Diaphot inverted microscope. Fluorescence from the egg was measured and quantified via a photomultiplier tube (Model 9524B; Thorn EMI, Fairfield, NJ, U.S.A.). Data collection, data processing and the stepper motor were controlled by an AT computer with UMANS software (written by Dr. C. M. Regan, Bio-Rad). The 340/380 nm ratio for fura-2 was collected at 2 Hz and calibrated by determining $R_{\min}$ and $R_{\max}$ [13]. All recordings were made at 18–21 $^{\circ}\text{C}$.

Confocal laser scanning microscopy

Fluo-3-loaded eggs were imaged with a Zeiss LSM-410 laser scanning confocal microscope using the 488 nm line of an argon/krypton laser. Two-dimensional confocal images were acquired by scanning an image of 256×256 pixels at 3 s intervals. The effective thickness of the confocal optical section was controlled by varying the opening of a pinhole aperture in the detection path. The limiting z -plane resolution was 7 μm . An oil immersion lens (Zeiss 16 $\times$) with a numerical aperture of 0.5 was used in all experiments. Analysis of confocal images were performed using ANALYZE software (Mayo Foundation, Rochester, MN, U.S.A.) on a Silicon Graphics Iris computer.

Materials and solutions

Sea urchins (*Lytechinus pictus*) were purchased from Marinus (Long Beach, CA, U.S.A.). ASW was composed of (in mM): NaCl 470, KCl 10, CaCl_2 11, MgSO_4 29, MgCl_2 27, NaHCO_3 2.5, pH 8. Injection buffer contained (in mM): potassium acetate 220, glycine 500, NaCl 40, Tris 20, plus 100 μM EGTA, pH 6.9. Pyrex glass tubing was obtained from Drummond Scientific (Broomall, PA, U.S.A.). Fluo-3, fura-2 and BCECF-dextran (M_r 40000) were purchased from Molecular Probes (Eugene, OR, U.S.A.). InsP_3 was obtained from Calbiochem (San Diego, CA, U.S.A.). NAADP and cADPR were synthesized as described in [12] and [14] respectively.

Statistics

Results are expressed as means $\pm$ S.E.M. Statistical comparisons were carried out by Student's unpaired t test data analysis.

RESULTS

NAADP triggers an increase in $[\text{Ca}^{2+}]_i$ in intact sea urchin eggs

Microinjection of purified NAADP into intact sea urchin eggs increased $[\text{Ca}^{2+}]_i$ as monitored by conventional epifluorescence. Injections of 10 and 100 μM NAADP ($\sim$ 20 and 100 nM final

Figure 1 Dose-dependence of NAADP-induced Ca^{2+} release

$[\text{Ca}^{2+}]_i$ was measured after the injection of $\sim$ 5, 20 and 100 nM NAADP (estimated final intracellular concentrations) into sea urchin eggs loaded with fura-2 (estimated final intracellular concentration $\sim$ 20 μM). Microinjection of injection buffer alone did not induce significant $[\text{Ca}^{2+}]_i$ changes.

intracellular concentrations respectively) evoked a transient increment of $[\text{Ca}^{2+}]_i$ from a resting concentration of $0.13 \pm 0.01 \mu\text{M}$ ($n = 7$) to $1.6 \pm 0.05 \mu\text{M}$ ($n = 4$) and $2.2 \pm 0.1 \mu\text{M}$ ($n = 3$) respectively (Figure 1). A fertilization envelope developed in all eggs following microinjection (18/18 cells). NAADP at concentrations below or equal to 5 nM did not produce a significant change in $[\text{Ca}^{2+}]_i$ (Figure 1) nor cause development of a fertilization envelope. Injection of deamido-NAD, a dephosphorylated metabolite of NAADP, into intact sea urchin eggs did not significantly change $[\text{Ca}^{2+}]_i$. Importantly, extracellular Ca^{2+} was not required for NAADP-induced Ca^{2+} release. No differences on injection of NAADP were observed between eggs placed in solutions containing low $[\text{Ca}^{2+}]$ (approx. 500 nM) or high $[\text{Ca}^{2+}]$ (11 mM). Also, it is unlikely that NAADP acted as a Ca^{2+} ionophore, since injections of $[\text{NAADP}]$ above 10 μM in intact *Xenopus* oocytes did not result in changes in $[\text{Ca}^{2+}]_i$ up to 10 min following microinjection in 13/15 oocytes (results not shown). Microinjections of $\sim$ 1 μM heat-inactivated NAADP (boiled for 30 min) did not elicit responses in intact sea urchin eggs.

Confocal images of intact sea urchin eggs

For more precise spatio-temporal resolution of the $[\text{Ca}^{2+}]_i$ changes, images from intact eggs were obtained by laser scanning confocal microscopy. Sea urchin eggs microinjected with 10 μM NAADP (approx. 20 nM intracellular concentration) exhibited a rapid rise in $[\text{Ca}^{2+}]_i$ which propagated from the site of injection across the cytoplasm (Figure 2).

Characteristics of NAADP-induced Ca^{2+} release in intact sea urchin eggs

Effect of Ca^{2+} -release antagonists

Pretreatment of the egg with the InsP_3 receptor antagonist heparin (0.5 g/ml) [1] did not inhibit NAADP-induced Ca^{2+} release, but significantly inhibited Ca^{2+} release triggered by InsP_3

Figure 2 Confocal images of the NAADP-induced response

[Ca²⁺]_i in intact sea urchin eggs (80 μm) loaded with the Ca²⁺-sensitive dye fluo-3 (intracellular concentration ~ 10 μM) was measured confocally. Microinjection of 10 μM NAADP (~ 20 nM intracellular concentration) immediately produced an increase in [Ca²⁺]_i and a propagating Ca²⁺ wave. The wave started at the site of injection and crossed the egg over a period of 30 s. The vertical bar represents the relative fluorescence scale (yellow indicates the highest Ca²⁺ concentration).

(Figures 3a and 3b). cADPR-induced Ca²⁺ release is specifically antagonized by 8-NH₂-cADPR [8]. However, 8-NH₂-cADPR is not feasible to use for microinjection into intact sea urchin eggs due to its chemical instability. In order to test the specificity of the NAADP effect, we employed an NADP analogue which has been found to block specifically the response to NAADP in sea urchin egg homogenates [12]. Microinjection of 600 μM thio-NADP (~ 0.5–1 μM final concentration) into intact sea urchin eggs partially prevented Ca²⁺ release induced by ~ 20–50 nM NAADP (estimated intracellular concentration; Table 1). In contrast, thio-NADP had no inhibitory effect upon Ca²⁺ release triggered by microinjections of 100 μM cADPR (50–100 nM intracellular concentration) or 100 μM InsP₃ (100–200 nM intracellular concentration) (Table 1). These results show that NAADP-induced Ca²⁺ release in intact sea urchin eggs was clearly distinct from InsP₃- and cADPR-induced Ca²⁺ release.

Absence of heterologous desensitization of Ca²⁺ release

To further determine whether the NAADP-induced Ca²⁺-release mechanism in intact sea urchin eggs is distinct from the cADPR- and InsP₃-induced Ca²⁺-release mechanisms, we tested for possible agonist cross-desensitization. It has been shown previously that sea urchin eggs develop homologous desensitization

to sequential additions of saturating concentrations of the same Ca²⁺-releasing agent, but neither InsP₃ nor cADPR cross-desensitizes the response [15]. As shown in Figure 4, microinjections of 100 μM cADPR (60–100 nM intracellular concentration) or 200 μM InsP₃ (400–500 nM intracellular concentration) homologously desensitized the sea urchin egg Ca²⁺-release response to subsequent microinjections of cADPR or InsP₃ respectively, but had no effect on Ca²⁺ release in response to 10 μM NAADP (20–50 nM intracellular concentration). In fact, the response to NAADP was actually enhanced when the egg was first desensitized to InsP₃ (Figure 4b). The NAADP-induced Ca²⁺ peak transient amplitude was 1.7 ± 0.10 μM in eggs without prior exposure to InsP₃ and 3.1 ± 0.11 μM in eggs desensitized to InsP₃ ($n = 4$). The reverse experiment showed that desensitization of the sea urchin egg to subsequent additions of NAADP did not alter Ca²⁺ release by cADPR or InsP₃ (results not shown). These results further support the hypothesis that NAADP elicits its effect through a mechanism distinct from the InsP₃- and ryanodine-sensitive Ca²⁺-release mechanisms.

NAADP-induced Ca²⁺ release in fertilized eggs

One of the most important events in sea urchin fertilization is a transient increase in [Ca²⁺]_i which regulates the metabolic and

Figure 3 Heparin does not block NAADP-induced Ca^{2+} release

$[Ca^{2+}]_i$ was measured in sea urchin eggs using fura-2 as the Ca^{2+} indicator. (a) $InsP_3$ (100–200 nM intracellular concentration)-induced Ca^{2+} release was blocked by microinjection of $\sim 500 \mu g$ of heparin. (b) Ca^{2+} release was elicited by microinjection of $10 \mu M$ NAADP (~ 20 nM intracellular concentration) into an egg preinjected with heparin ($500 \mu g$). The arrow indicates the time of microinjection.

Table 1 Inhibition of NAADP-induced Ca^{2+} release by thio-NADP

Sea urchin eggs were first injected with fura-2 in the presence or absence of thio-NADP (0.5 – $1 \mu M$ intracellular concentration). After 10 min, eggs were microinjected with the following agonists (estimated intracellular concentrations given): ~ 20 – 50 nM NAADP, ~ 50 – 100 nM cADPR or ~ 100 – 200 nM $InsP_3$, and $[Ca^{2+}]_i$ was monitored by the 340/380 nm fluorescence ratio of the fura-2 loaded eggs. The values are means $\pm$ S.E.M. of five experiments.

Agonist	$[Ca^{2+}]_i$ (μM)	
	– Thio-NADP	+ Thio-NADP
NAADP	1.59 ± 0.07	0.48 ± 0.06
cADPR	1.15 ± 0.06	1.47 ± 0.24
$InsP_3$	1.70 ± 0.16	2.09 ± 0.03

developmental activation of the egg [16,17]. Both $InsP_3$ - and cADPR-sensitive Ca^{2+} -release mechanisms participate in this dynamic fertilization response [4,8,18].

To test whether intracellular Ca^{2+} stores regulated by NAADP could also contribute to Ca^{2+} transients observed during

Figure 4 Desensitization of the sea urchin eggs

$[Ca^{2+}]_i$ changes in an intact sea urchin egg were desensitized to subsequent cADPR or $InsP_3$ microinjections but not to NAADP. Arrows represent the sequential addition of ~ 60 – 100 nM cADPR, ~ 20 – 50 nM NAADP and ~ 400 – 500 nM $InsP_3$ (intracellular concentrations). The sea urchin egg was preloaded with $\sim 20 \mu M$ fura-2.

Figure 5 Desensitization of NAADP-induced Ca^{2+} release in fertilized eggs

(a) $[Ca^{2+}]_i$ increases were induced by sequential microinjections of NAADP (~ 60 nM estimated final concentration). (b) Eggs were fertilized by addition of sperm at the time indicated by the arrow, which induced a Ca^{2+} transient. After reuptake of Ca^{2+} the egg was microinjected with ~ 60 nM NAADP. Under these conditions, NAADP induced Ca^{2+} release which was $\sim 35\%$ of the total Ca^{2+} released by NAADP in non-fertilized eggs.

fertilization, we examined the response of fertilized sea urchin eggs to NAADP. Figure 5(a) shows that the first microinjection of $100 \mu M$ NAADP (~ 60 nM intracellular concentration) into unfertilized eggs stimulated a large increase in $[Ca^{2+}]_i$ ($0.11 \pm 0.07 \mu M$ to $1.7 \pm 0.09 \mu M$, $n = 4$), while subsequent

injections of the same concentration of NAADP resulted in an attenuated response ($0.8 \pm 0.2 \mu\text{M}$, $n = 4$). Injection of NAADP into eggs after fertilization stimulated the Ca²⁺ transient to only $0.6 \pm 0.2 \mu\text{M}$ ($n = 5$; Figure 5b), indicating a desensitization to NAADP similar to that caused by repeated injections of NAADP into intact sea urchin eggs. We interpret the attenuation of the Ca²⁺-release response to NAADP as an indicator of the participation of the NAADP-sensitive Ca²⁺ stores in the fertilization response. Although this result suggests that an NAADP-sensitive Ca²⁺ pool may play a role in the fertilization process, it does not prove that the NAADP molecule is an endogenous agonist during the fertilization response.

DISCUSSION

NAADP induces Ca²⁺ release in intact sea urchin eggs

We have demonstrated that the NAADP-sensitive Ca²⁺-release system is present in intact sea urchin eggs. Microinjection of NAADP into intact sea urchin eggs caused Ca²⁺ release similar to that in the cell-free egg homogenate system: (a) transient Ca²⁺ release was elicited by NAADP in a dose-dependent manner at nanomolar concentrations; (b) NAADP was more potent in releasing Ca²⁺ than InsP₃ and comparable in potency to cADPR; (c) the Ca²⁺-release response was not inhibited by an antagonist of the InsP₃ system; (d) the response was inhibited by thio-NADP, which did not diminish the Ca²⁺-release response to cADPR or InsP₃; and (e) the agonist-specificity of the NAADP-evoked Ca²⁺-release response was further affirmed by homologous desensitization after repeated injections of NAADP. The rapid onset of the time course of the response to NAADP after microinjection suggests that the Ca²⁺ release was not due to a metabolite of NAADP. These results constitute pharmacological evidence for the specificity of NAADP's action in intact sea urchin eggs.

NAADP stimulated Ca²⁺ release from sea urchin eggs desensitized to InsP₃ and cADPR. An interesting observation is that when the eggs were desensitized to InsP₃, the response to a subsequent application of NAADP was actually higher. This result suggests communication between stores, or the possibility that InsP₃ and/or the Ca²⁺ released by InsP₃ acts as a co-agonist with NAADP.

Role of NAADP-sensitive Ca²⁺ stores in sea urchin eggs

While participation of InsP₃ and cADPR as Ca²⁺-releasing agents during sea urchin fertilization has been documented [8,18], our observation suggests that an NAADP-dependent Ca²⁺ store may also contribute to the Ca²⁺ transient response in fertilization. This notion was supported by our observation that NAADP-induced Ca²⁺ release was decreased after fertilization. We surmise that this phenomenon represents homologous desensitization. Antagonists to InsP₃ and cADPR receptors block the initiation and propagation of Ca²⁺ transients induced by sperm in sea urchin eggs [8,18]. We failed to block the fertilization response

with $0.5 \mu\text{M}$ thio-NADP. However, these observations do not rule out the possibility that an NAADP-sensitive Ca²⁺-release pathway could also be involved in Ca²⁺ mobilization during fertilization after the initial response. The NAADP-induced Ca²⁺-release pathway could mediate Ca²⁺-induced Ca²⁺ release permitting the generation of complex spatio-temporal changes in [Ca²⁺]_i. In support of this notion, partially activated eggs in which [Ca²⁺]_i was elevated showed an enhanced sensitivity to NAADP when compared with non-activated eggs.

The existence of distinct Ca²⁺-mobilization pathways is not surprising, given the importance of [Ca²⁺]_i in regulating various cellular functions. However, whether NAADP is an endogenous regulator of Ca²⁺ release in sea urchin eggs is unresolved. NAADP may be a pharmacological modulator of this novel Ca²⁺-release mechanism in sea urchin eggs or a distinct second messenger for modulation of intracellular Ca²⁺ release.

In summary, the results of the experiments presented here demonstrate that the NAADP-dependent Ca²⁺-release system is fully functional in intact cells and clearly triggers Ca²⁺ release from intracellular stores. The evidence supports the hypothesis that intracellular Ca²⁺ release in sea urchin eggs is under the control of at least three systems: those induced by InsP₃, cADPR and the newly discovered NAADP. Thus NAADP can be used as a selective pharmacological agent to further characterize this novel Ca²⁺-release system. To answer the question of whether NAADP is an endogenous agonist for a new Ca²⁺-release pool will require the characterization of the native biosynthetic and degradative NAADP pathways and determination of the site of action of NAADP.

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