Unique Inactivation Properties of NAADP-sensitive Ca\(^{2+}\) Release*  

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Calcium stores in sea urchin eggs provide an excellent model to study Ca\(^{2+}\) release mechanisms (1–4). The sea urchin egg Ca\(^{2+}\) stores contain inositol trisphosphate (InsP\(_3\)) and ryanodine receptors as the two families of intracellular Ca\(^{2+}\) release channels that have been identified, which may be regulated by separate intracellular messengers, InsP\(_3\) and cyclic adenosine 5’-diphosphate ribose, respectively. A third molecule, nicotinic acid adenine dinucleotide phosphate (NAADP), has recently been recognized as a potent Ca\(^{2+}\) releasing agent in sea urchin eggs and microsomes. We now report that non-releasing concentrations of NAADP fully and irreversibly inactivate the NAADP-sensitive Ca\(^{2+}\) release mechanism. This phenomenon occurred both in intact sea urchin eggs and in homogenates, and is not shared by either InsP\(_3\) or cyclic adenosine 5’-diphosphate ribose. The novel properties of this Ca\(^{2+}\) release mechanism, giving a one-shot Ca\(^{2+}\) release, may be suited to irreversible cellular events.

In the present study, NAADP potently released Ca\(^{2+}\) in a dose-dependent manner from sea urchin homogenates (EC\(_{50}\) approximately 32 nM) and triggered a Ca\(^{2+}\) wave in the intact sea urchin egg (Figs. 1 and 2A). Surprisingly, very low concentrations of NAADP, which evoked little or no Ca\(^{2+}\) release, were found to fully inactivate the NAADP-sensitive Ca\(^{2+}\) release mechanism both in the homogenate and the intact egg (Fig. 1, A and C, and Fig. 2, A and B). Within the intact egg NAADP (approximate cytoplasmic concentrations of 50 and 500 nM) released Ca\(^{2+}\) in a biphasic manner (Fig. 2A). The pattern of the Ca\(^{2+}\) release was an

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1 The abbreviations used are: InsP\(_3\), inositol trisphosphate; cADPR, cyclic adenosine 5’-diphosphate ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; Pipes, 1,4-piperazinedinetanesulfonic acid.

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Fig. 1. A, representative fluorimetric traces of Ca\(^{2+}\) release by 500 nM NAADP (top trace) and of homologous desensitization by sub-threshold concentrations of NAADP in 2.5% sea urchin homogenate. Homogenates were pretreated for 3 min with the illustrated concentrations of NAADP and then challenged with 500 nM NAADP. Ca\(^{2+}\) release is expressed as relative fluorescence units (R.F.U.). B, representative fluorimetric trace showing the absence of cross desensitization by NAADP on cADPR and InsP\(_3\)-induced Ca\(^{2+}\) release. Concentration of NAADP (N) and cADPR (cA) was 500 nM unless stated. InsP\(_3\) was 1 \(\mu\)M. C–E, dose-response curves to NAADP, cADPR, and InsP\(_3\) in homogenate (filled squares) and residual release by maximal concentrations of agonists (500 nM NAADP; 500 nM cADPR; 1 mM InsP\(_3\)) after 3 min preincubation with stated concentrations (open circles). Values are mean \(\pm\) standard deviation of 6–9 determinations.

Fig. 2. Pseudocolor images of Ca\(^{2+}\) levels, measured using Fura-2, in sea urchin eggs. A, injection of NAADP into sea urchin eggs resulted in a Ca\(^{2+}\) release that spread in a wave form across the egg followed by a slower, smaller amplitude Ca\(^{2+}\) release. Subsequent injection of a higher concentration of NAADP could not elicit substantial Ca\(^{2+}\) release in the egg. B, lower concentrations (5 nM) of NAADP resulted in less Ca\(^{2+}\) release, but these too prevented Ca\(^{2+}\) release by a subsequent NAADP injection. C, InsP\(_3\) injections released Ca\(^{2+}\) but this did not desensitise a subsequent Ca\(^{2+}\) release response to higher concentrations of InsP\(_3\) applied some 1500 s later. The time course of the average Ca\(^{2+}\) change within the egg (measured with Fura-2, Molecular Probes) and the accompanying ratios of 340 and 380 nm excitation fluorescence images are shown. Open symbols on the time course represent the average Ca\(^{2+}\) levels within the egg determined from the numbered images shown below the time course.
initial Ca\textsuperscript{2+} peak, followed by a partial recovery, and a later, slower and smaller amplitude release as previously observed by others (6) (peak [Ca\textsuperscript{2+}] was 1897 ± 268 nm, n = 5). We were able to demonstrate the inactivation phenomenon of NAADP-induced Ca\textsuperscript{2+} release in the whole egg by first injecting into the egg as little as 5 nM NAADP, which then prevented any further response of the egg to larger injections of NAADP. 5 nM NAADP, in itself produced only a small rise in Ca\textsuperscript{2+} (but significantly reduced Ca\textsuperscript{2+} release by a subsequent injection of 500 nM NAADP even after waiting at least 1500 s (267 ± 66 nM, n = 4). This persistent inactivation contrasted with the inactivation of InsP\textsubscript{3} and cADPR receptors, which underwent a transient desensitization (data not shown), but recovered within 30 min (Fig. 2C). In fact, InsP\textsubscript{3} (2 μM) injections released Ca\textsuperscript{2+} in the whole egg in a monophasic manner (1102 ± 61 nmM, n = 3) but in contrast to NAADP, a higher concentration of InsP\textsubscript{3} (20 μM) was still able to release maximal Ca\textsuperscript{2+} at least 1500 s later (1982 ± 163 nmM, n = 3). Like InsP\textsubscript{3}, preapplication of 1 μM cADPR to the egg (giving 1094 ± 307 nM Ca\textsuperscript{2+} release, n = 3) did not block the Ca\textsuperscript{2+} response to 10 μM cADPR at least 1500 s later (mean Ca\textsuperscript{2+} 1845 ± 78 nmM, n = 3; data not shown).

In the homogenate, the extent of the inactivation was both concentration (Fig. 1, A and C) and time-dependent (Fig. 3).

The potenti N-type voltage-gated Ca\textsuperscript{2+} channel blocker ω-conotoxin on Ca\textsuperscript{2+} release.

**Fig. 3.** Time course of the inactivation by various concentrations of NAADP in 2.5% sea urchin homogenate. The homogenate was challenged with NAADP (500 nm) after addition at time zero of sub-threshold concentrations (as indicated) of NAADP. Values are median of 3–6 replicates from two experiments. Ca\textsuperscript{2+} release by NAADP (500 nm) in the absence of any NAADP pretreatment was 10.5 ± 0.65 nmol.

**Fig. 4.** A, representative fluorimetric traces of the inhibition by diltiazem on NAADP-induced Ca\textsuperscript{2+} release. B, effect of classical L-channel Ca\textsuperscript{2+}-modulators and ω-conotoxin on Ca\textsuperscript{2+} mobilization by NAADP, cADPR, or InsP\textsubscript{3}. Antagonists were added 20 s prior to agonists. Values are mean ± standard deviation of 3–9 determinations from 1–3 separate experiments. Diltiazem, nifedipine, verapamil, and BAY K8644 were diluted in dimethyl sulfoxide (Me\textsubscript{2}SO), and 5 μL of Me\textsubscript{2}SO were added to the control.

Cross-desensitization to InsP\textsubscript{3} and cADPR by NAADP (20 nM to 1 μM) did not occur (Fig. 1B). Instead full inactivation occurred by pretreatment with 1–2 nM NAADP, but substantial inactivation still occurred with concentrations as low as 100 pm (Fig. 1, A and C). Within 60 s full inactivation occurred with a non-stimulating concentration of 5 nM NAADP, but took longer and was less extensive by pretreatment with lower concentrations (Fig. 3). Inactivation was also apparently irreversible, since the receptor did not resensitize after 12 h of exposure to 2 nM NAADP (data not shown), and after reconstituting purified microsomes prepared from desensitized homogenate in fresh buffer not containing NAADP (data not shown). In the homogenate, Ca\textsuperscript{2+} release by InsP\textsubscript{3} and cADPR desensitized only after treatment with stimulating concentrations and in a manner paralleling the extent of channel activation (Fig. 1, D and E; see also Ref. 13).

The graded release of Ca\textsuperscript{2+} induced by NAADP (Fig. 1C) suggests that release by this agent is quantal (14). Changes in luminal or cytosolic Ca\textsuperscript{2+} concentrations or pool depletion have been proposed to explain this phenomenon for both InsP\textsubscript{3}R and RyRs (see Ref. 14 for a review). However, it seems unlikely that NAADP-induced Ca\textsuperscript{2+} release relies on these mechanisms since it is terminated by desensitization and can occur at concentrations that are non-stimulating. Instead, it appears to be an intrinsic property of the receptor mechanism as has also been proposed for the InsP\textsubscript{3}R (13).

Various experiments suggest that the inactivation mechanism is independent of enzymatic activity. First, in Percoll gradient-purified microsomes (1, 15), full desensitization at either non-stimulating or stimulating NAADP concentrations was identical to that seen in whole egg homogenates (data not shown), ruling out a requirement for cytosolic components. Second, the general kinase inhibitor staurosporine (10 μM) had no effect on the extent of inactivation (data not shown) in the homogenate. Third, the release and inactivation were unaltered by performing the experiments at 4 °C, consistent with NAADP regulating intracellular Ca\textsuperscript{2+} fluxes via a channel mechanism.

The inactivation event was independent of small electrochemical or pH gradient changes across the membrane since pretreatment for 1 h with gramicidin, valinomycin, or nigericin (all at 1 μM) did not significantly alter Ca\textsuperscript{2+} release by NAADP or InsP\textsubscript{3} (data not shown; see also Ref. 16).

The nature of the NAADP Ca\textsuperscript{2+} release mechanism is unknown but is clearly separate from the InsP\textsubscript{3}R and cADPR mechanisms. Diltiazem, nifedipine, BAY K8644, and verapamil, classical modulators of L-type voltage-gated Ca\textsuperscript{2+} channels (17), fully blocked maximal Ca\textsuperscript{2+} release by NAADP (Fig. 4) but not that by cADPR or InsP\textsubscript{3} (Fig. 4) and did not alter the NAADP-induced inactivation phenomenon (data not shown). The potent N-type voltage-gated Ca\textsuperscript{2+} channel blocker ω-cono-
toxin (17) was without any effect (Fig. 4).

The desensitization of the NAADP receptor shares similarities with neuronal nicotinic receptors (18). However, nicotinic receptor desensitization by non-stimulating concentrations of agonists is never complete; it is also reversible and may involve phosphorylation. None of these features are shown by the NAADP-sensitive mechanism.

Recent reports have indicated that mammalian cells possess the metabolic machinery to use NAADP as an intracellular messenger. First, NAADP is generated and degraded in various rat tissues, including brain, liver, and spleen (19). Second, it has been shown that ADP-ribosyl cyclase, the enzyme responsible for the cyclization of NAD\(^+\) to ADPR, and CD38, a lymphocyte differentiation antigen, can also synthesize NAADP (20). \(\text{Ca}^{2+}\) mobilizing effects of NAADP have not yet been reported for other cell types, but an agonist-stimulated \(\text{Ca}^{2+}\) release pathway that is blocked by nifedipine and diltiazem has been described in neutrophils (21) and low affinity binding sites for L-type \(\text{Ca}^{2+}\)-channel blockers are present in cardiac sarcoplasmic reticulum (22).

Multiple \(\text{Ca}^{2+}\) release mechanisms may contribute to complex patterns of \(\text{Ca}^{2+}\) signals widely observed during intracellular signaling (23). The characteristics of NAADP \(\text{Ca}^{2+}\) release described here suggest that the receptor may function as an irreversible biochemical switch activated in a one-off manner by a rapid surge in intracellular NAADP concentrations. Such a response may be suited to irreversible events such as fertilization, as recently suggested (7), cell division or cell death.

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