Current Biology, Vol. 13, 125–128, January 21, 2003, ©2003 Elsevier Science Ltd. All rights reserved. PII S0960-9822(03)00002-2

Sperm Deliver a New Second Messenger: NAADP

Grant C. Churchill,[1,*] John S. O'Neill,[1] Roser Masgrau,[1] Sandip Patel,[2] Justyn M. Thomas,[1] Armando A. Genazzani,[3] and Antony Galione[1]

[1]Department of Pharmacology
University of Oxford
Mansfield Road
Oxford OX1 3QT
[2]Department of Physiology
University College London
Gower Street
London WC1E 6BT
[3]Department of Pharmacology
University of Cambridge
Tennis Court Road
Cambridge CB2 1QJ
United Kingdom

Summary

NAADP is a highly potent mobilizer of Ca^{2+} [1, 2], which in turn triggers Ca^{2+}-induced Ca^{2+} release pathways [3–6] in a wide range of species [2, 7]. Nevertheless, NAADP is not presently classified as a second messenger because it has not been shown to increase in response to a physiological stimulus. We now report a dramatic increase in NAADP during sea urchin egg fertilization that was largely due to production in sperm upon contacting egg jelly. The NAADP bolus plays a physiological role upon delivery to the egg based on its ability to induce a cortical flash, a depolarization-induced activation of L-type Ca^{2+} channels. Moreover, the sperm-induced cortical flash was eliminated in eggs desensitized to NAADP. We conclude that an NAADP increase plays a physiologically relevant role during fertilization and provides the first conclusive demonstration that NAADP is a genuine second messenger.

Results and Discussion

Based on the archetype cyclic AMP, a molecule must meet five criteria to be unambiguously designated a second messenger [8]. Taking results from different cell systems, the Ca^{2+}-mobilizing agent NAADP (nicotinic acid adenine dinucleotide phosphate) currently meets four of these criteria. First, NAADP applied intracellularly mimics the effect of an extracellular stimulus [4, 5]; second, it can be synthesized and metabolized in a range of tissues from many species [2, 7, 9–11]; third, antagonism of the action of the messenger blocks the effects of the extracellular messenger [4, 5]; and, fourth, specific intracellular binding sites are present [9, 12–14]. For NAADP to achieve full second messenger status, however, the level of NAADP must be shown to change in response to a physiologically relevant stimulus.

To determine whether NAADP could fulfill all five criteria for a second messenger in a single biological system, we investigated the role of NAADP in sea urchin egg fertilization. We first investigated the effect of fertilization on NAADP levels. To determine NAADP levels, we developed a sensitive and specific radioreceptor assay based on the sea urchin binding protein and radiolabeled NAADP. The specificity of this protein for NAADP is high (see Figure S1 in the Supplementary Material available with this article online), as has been reported previously [12, 13, 15]. NAADP was endogenously present in eggs at a concentration of 4.0 ± 0.96 pmol/mg protein ($n = 8$). Fertilization resulted in an increase in NAADP levels (Figure 1A). Asynchronous fertilization resulted in shifts in the exact time of the maximal increase in NAADP; however, the profiles of the increases over time were similar, with an early peak followed by a later peak (Figure 1A). The average fold increase in NAADP was 4.3 ± 0.72 ($n = 5$). In eggs that were not fertilized, but were treated with the same experimental procedure, there was no significant change in NAADP levels (Figure 1A).

NAADP synthesis could have occurred in either the egg or sperm. Sperm diluted into artificial seawater became motile, as determined by phase-contrast microscopy, but did not show an increase in NAADP (Figure 1B). In contrast, sperm incubated in artificial seawater containing egg jelly showed an increase in NAADP (Figure 1B). On the basis of NAADP per milligram of protein, basal NAADP was ~10-fold higher in sperm than in eggs (Figure 1A versus Figure 1B); this increase can be accounted for by dilution of the sperm-produced NAADP by egg protein given the relative amounts of protein in the sample provided by the eggs (3.6 mg/ml) and sperm (0.38 mg/ml). The increase in NAADP upon sperm activation was approximately 5-fold, which is similar to the fold increase with fertilization (Figure 1A). These results indicate that the first NAADP increase detected during fertilization was mostly, if not entirely, due to NAADP production in sperm, whereas the origin of the second increase is not clear at present.

Does a single sperm contain enough NAADP to have a physiological role at fertilization? The cytosolic concentration of NAADP in resting eggs is calculated to be 0.29 μM based on 72 mg protein/ml of eggs and 4 pmol NAADP/mg protein. As this concentration would be sufficient to deplete all NAADP-sensitive intracellular Ca^{2+} stores, the value is an overestimate, likely representing compartmentalization of NAADP away from receptor sites, as has been suggested for IP_3 [16]. Although these values are estimates, they remain useful until the spatial distribution of NAADP can be determined. If sperm are assumed to have an aqueous volume of 7 fl [17], a protein content of 0.19 mg per 1×10^8 sperm, and 200 pmol NAADP/mg protein, then the concentration of NAADP is 54 μM. If the egg volume is 500 pl and all the NAADP in a sperm (3.8×10^{-19} mol) enters the egg

*Correspondence: grant.churchill@pharm.ox.ac.uk

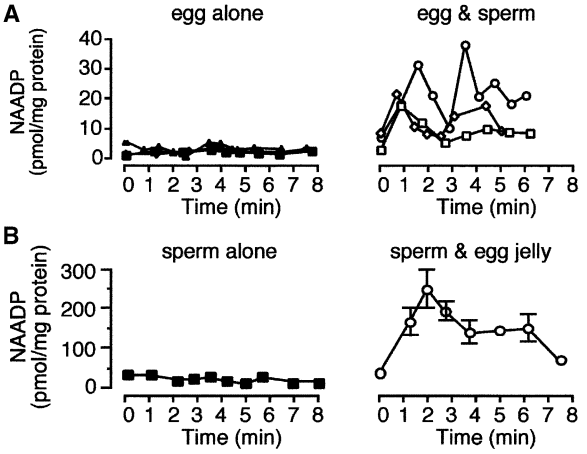

Figure 1. NAADP Levels

(A) NAADP levels increase in eggs and sperm (right panel), but not in eggs alone (left panel). Results are shown for three individual experiments for each treatment, with each curve distinguished by a different symbol.

(B) NAADP levels increase during sperm activation. Sperm were diluted into artificial seawater in the presence (right panel) or absence (left panel) of egg jelly. Points represent the mean ± standard error of the mean from 3–6 determinations.

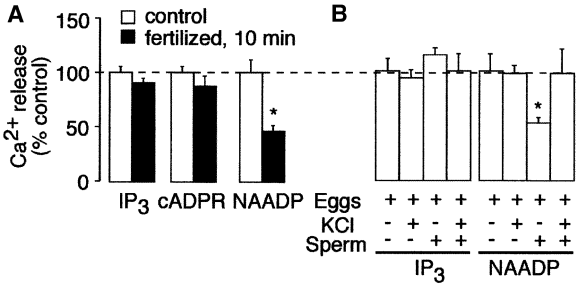

Figure 2. Fertilization Depletes NAADP-Sensitive Ca^{2+} Stores in Sea Urchin Homogenates

(A) Effect of fertilization on the amount of Ca^{2+} released by IP_3 (1 μM), cADPR (0.5 μM), and NAADP (0.5 μM).

(B) Effect of blocking sperm-egg fusion by KCl-mediated (0.5 M) egg depolarization on the amount of Ca^{2+} released by IP_3 (1 μM) and NAADP (0.5 μM). Eggs were homogenized as described in the Supplementary Experimental Procedures 10 min after the addition of sperm or artificial seawater.

cytosol, then, at the site of sperm-egg fusion, the local NAADP concentration would be ~54 μM initially and 0.76 nM when evenly distributed. These numbers suggest that sperm could deliver a bolus of NAADP sufficient to mobilize Ca^{2+} from intracellular stores and form a gradient in the egg from supramaximal (EC_{50} = 30 nM) to inactivating (IC_{50} = 0.3 nM) concentrations. Combining this result with the ability of NAADP to form gradients more readily than other messengers [3] to form a memory over time and space [18] suggests that the sperm may use NAADP to encode spatial information about its contact site.

The above measurements and calculations suggest that sperm produce NAADP during the sperm-egg interaction and that the transfer of this to the egg could have profound effects on calcium mobilization. To further assess whether there was an increase in NAADP, we employed NAADP's unique properties of inactivation [12, 19] and irreversible binding [12, 13, 15]. In these experiments, we homogenized eggs 10 min after the addition of sperm. Fertilization did not affect the amount of Ca^{2+} released by IP_3 or cADPR, but it decreased the amount of Ca^{2+} mobilized by NAADP (Figure 2A). Moreover, the decrease in Ca^{2+} released by NAADP was dependent on the presence of sperm and was inhibited by KCl (Figure 2B), which prevents fertilization [20]. The reduction in the NAADP response after fertilization is consistent with a physiological increase in NAADP and prolonged homologous NAADP desensitization, possibly at the level of the NAADP receptor [12, 13, 15].

The previous data raise the possibility that sperm appear to provide the egg with NAADP as a preformed messenger (Figure 1B) and suggest a role for NAADP in the early Ca^{2+} responses at fertilization. One of the first events that occurs upon sperm-egg fusion is the cortical flash, which accompanies the so-called fast block to polyspermy [20, 21]. Therefore, we investigated the role of NAADP in mediating the cortical flash. Mechanistically, the cortical flash is a sperm-induced depolarization of the egg that is then amplified throughout the egg by an action potential mediated by L-type Ca^{2+} channels and is thus detected as a Ca^{2+} increase that is restricted to the cortex [22]. Photorelease of a relatively large amount of NAADP (~100 μW UV) resulted in a global Ca^{2+} wave (Figure 3A), as reported previously [3], and an almost immediate Ca^{2+} increase on the side of the egg opposite to the region of photolysis (Figure 3A). This increase is specific for NAADP, because no cortical flash was seen following the photorelease of Ca^{2+}, cADPR, or IP_3 (Figure 3A and Table S1). In starfish oocytes, the NAADP-mediated Ca^{2+} increase is entirely dependent on extracellular Ca^{2+} rather than mobilization from intracellular stores [6]. In sea urchin eggs, the ability of NAADP to induce a cortical flash and propagate a calcium wave suggests that NAADP regulates both Ca^{2+} mobilization from intracellular stores and influx. Upon photorelease of a relatively small amount of NAADP (~10 μW UV), the Ca^{2+} increase in the center of the egg was largely confined to the region of photolysis, but the Ca^{2+} increase in the cortex was global (Figure 3B). Removing Ca^{2+} from the artificial seawater or adding the Ca^{2+} channel-blocking metal ion Cd^{2+} to the extracellular medium eliminated the NAADP-induced cortical flash (Figure 3C), demonstrating the need for Ca^{2+} influx.

One of the criteria for a compound to be a second messenger is that antagonism of the action of the messenger blocks the effects of the extracellular messenger [8]. We used the property of NAADP self-antagonism, both of the cortical flash (Figure 3D, blue trace, UV3) and Ca^{2+} mobilization (Figure 3D, black trace) [12, 19], to investigate the role of NAADP in the sperm-induced cortical flash and Ca^{2+} transient. In eggs desensitized to NAADP, fertilization still occurred, but the cortical flash was present in only 3/18 eggs (Figure 3E) compared to 19/20 control eggs ($p < 0.0001$, Fisher's exact test; see the Supplementary Results and Discussion). NAADP desensitization also shortened the duration of the fertilization Ca^{2+} transient to 93 ± 12 s ($n = 6$) com-

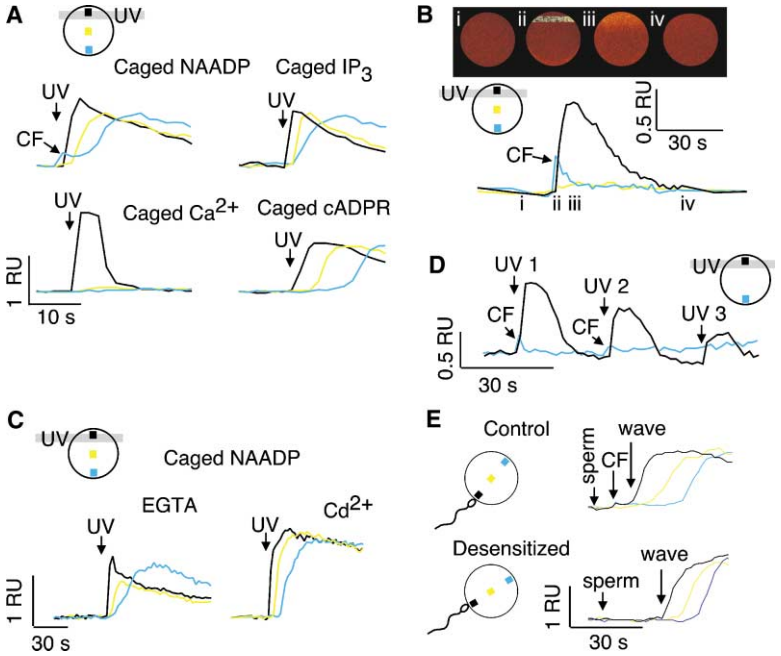

Figure 3. NAADP Is Involved in the Cortical Flash

(A) Photorelease (arrow marked UV) of NAADP, but not Ca^{2+}, IP_3, or cADPR, activates a cortical flash (arrow marked CF) in sea urchin eggs.

(B) A small and local photorelease of NAADP is sufficient to induce a global cortical flash. An increase in calcium is seen only at the bottom of the image due to the way a scanning-confocal image is formed with the top part of the image representing earlier times than the bottom. Thus, this figures shows the time taken for the image to form from top to bottom and the time taken for the response to fully develop after the UV pulse.

(C) The NAADP-induced cortical flash requires an influx of Ca^{2+}. Eggs were incubated in artificial seawater without added Ca^{2+}, but with 1 mM EGTA, or with normal Ca^{2+} and 5 mM $CdCl_2$.

(D) The NAADP-induced cortical flash exhibits desensitization in response to repeated photorelease.

(E) NAADP desensitization inhibits the sperm-induced cortical flash. Eggs were desensitized by coinjection of free NAADP (5 nM intracellular) and caged NAADP (0.5 μM intracellular). Desensitization to NAADP was confirmed by the lack of a response to a test photolysis (UV intensity of 100 μW). In each panel, the inset diagram of an egg shows the locations of the UV illumination, the point of sperm attachment, and the regions of interest, which are color coded to match the traces. The intracellular concentrations are as follows: OGBD, 10 μM; caged NAADP, 0.5 μM; and caged Ca^{2+} (nitrophenylethyl-EGTA), 500 μM. Data are representative of 5–21 similar experiments.

pared to 162 ± 6.6 s (n = 14) for the control (p = 0.0005, t test; Figure 4A). Reciprocally, fertilization shortened the duration of the NAADP-induced Ca^{2+} transient to 46 ± 5.9 s (n = 6) from 123 ± 18 s (n = 12) in the unfertilized eggs (p = 0.009, t test; Figure 4B). Thus, NAADP affects fertilization and vice versa, providing additional evidence for a physiological role for NAADP at fertilization.

The NAADP introduced by the sperm into the egg may be the endogenous trigger for the cortical flash. NAADP may then release Ca^{2+} from physically separate stores to load the Ca^{2+}-induced Ca^{2+} release (CICR) stores, which are important for propagating the regenerative Ca^{2+} wave. Evidence for transfer of Ca^{2+} from NAADP-sensitive to CICR stores comes from our previous study showing that thapsigargin-sensitive stores are preferentially filled following NAADP-mediated Ca^{2+} release [23]. These two roles for NAADP are a variation of the conduit and content models proposed to explain how a sperm activates an egg [24]. In the sperm conduit model, Ca^{2+} flows through the sperm (conduit) into the egg, thereby loading the CICR stores in preparation for the global regenerative Ca^{2+} wave [24]. The content model suggests that the sperm delivers a factor to the egg [24]. Our results suggest that NAADP is an egg-induced sperm factor (content) that then participates in loading the CICR stores (conduit) by shuttling Ca^{2+} from separate NAADP-sensitive stores into the CICR stores [23].

In summary, NAADP increases in sperm upon contact with egg jelly and thus plays a role in fertilization of sea urchin eggs. These data provide the first demonstration of an increase in NAADP in any tissue and therefore provide the last piece of evidence necessary to demonstrate that NAADP is a true second messenger. Moreover, we have demonstrated in a single system (sea urchin) that NAADP meets all five of the criteria [8] required to be designated a true second messenger.

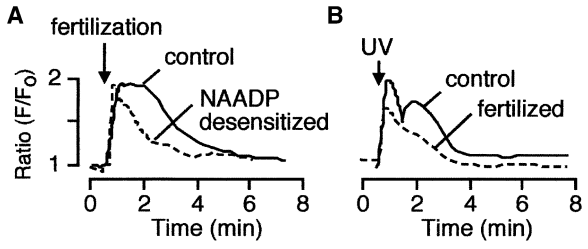

Figure 4. NAADP Is Involved in the Fertilization Ca^{2+} Transient

Reciprocal shortening of the duration of the Ca^{2+} transient elicited by either NAADP or fertilization pretreatment.

(A) The Ca^{2+} transient elicited by fertilization is shortened following NAADP pretreatment.

(B) The Ca^{2+} transient elicited by NAADP is shortened following fertilization. The duration of the Ca^{2+} transient was calculated as the time that Ca^{2+} remained above 1/2 its peak amplitude. Data are representative of 6–14 similar experiments.

Supplementary Material

Supplementary Material including the Experimental Procedures and additional Results and Discussion is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

We thank Anthony Morgan for insightful discussion. This work was supported by the Wellcome Trust.

Received: August 13, 2002
Revised: September 25, 2002
Accepted: November 5, 2002
Published: January 21, 2003

References

1. Lee, H.C., and Aarhus, R. (1995). A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. J. Biol. Chem. *270*, 2152–2157.
2. Patel, S., Churchill, G.C., and Galione, A. (2001). Coordination of Ca^{2+} signalling by NAADP. Trends Biochem. Sci. *26*, 482–489.
3. Churchill, G.C., and Galione, A. (2000). Spatial control of Ca^{2+} signaling by nicotinic acid adenine dinucleotide phosphate diffusion and gradients. J. Biol. Chem. *275*, 38687–38692.
4. Cancela, J.M., Churchill, G.C., and Galione, A. (1999). Coordination of agonist-induced Ca^{2+}-signalling patterns by NAADP in pancreatic acinar cells. Nature *398*, 74–76.
5. Berg, I., Potter, B.V., Mayr, G.W., and Guse, A.H. (2000). Nicotinic acid adenine dinucleotide phosphate $NAADP^{+}$ is an essential regulator of T-lymphocyte Ca^{2+}-signaling. J. Cell Biol. *150*, 581–588.
6. Santella, L., Kyozuka, K., Genazzani, A.A., De Riso, L., and Carafoli, E. (2000). Nicotinic acid adenine dinucleotide phosphate-induced Ca^{2+} release. Interactions among distinct Ca^{2+} mobilizing mechanisms in starfish oocytes. J. Biol. Chem. *275*, 8301–8306.
7. Lee, H.C. (2001). Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. Annu. Rev. Pharmacol. Toxicol. *41*, 317–345.
8. Robison, G.A., Butcher, R.W., Sutherland, E.W., Posternak, T., and Hardman, J.G. (1971). Cyclic AMP (New York: Academic Press).
9. Bak, J., Billington, R.A., Timar, G., Dutton, A.C., and Genazzani, A.A. (2001). NAADP receptors are present and functional in the heart. Curr. Biol. *11*, 987–990.
10. Chini, E.N., and Dousa, T.P. (1995). Enzymatic synthesis and degradation of nicotinate adenine dinucleotide phosphate (NAADP), a Ca^{2+}-releasing agonist, in rat tissues. Biochem. Biophys. Res. Commun. *209*, 167–174.
11. Berridge, G., Cramer, R., Galione, A., and Patel, S. (2002). Metabolism of the novel Ca^{2+}-mobilizing messenger nicotinic acid-adenine dinucleotide phosphate via a 2'-specific Ca^{2+}-dependent phosphatase. Biochem. J. *365*, 295–301.
12. Aarhus, R., Dickey, D.M., Graeff, R.M., Gee, K.R., Walseth, T.F., and Lee, H.C. (1996). Activation and inactivation of Ca^{2+} release by $NAADP^{+}$. J. Biol. Chem. *271*, 8513–8516.
13. Billington, R.A., and Genazzani, A.A. (2000). Characterization of $NAADP^{+}$ binding in sea urchin eggs. Biochem. Biophys. Res. Commun. *276*, 112–116.
14. Patel, S., Churchill, G.C., Sharp, T., and Galione, A. (2000). Widespread distribution of binding sites for the novel Ca^{2+}-mobilizing messenger, nicotinic acid adenine dinucleotide phosphate, in the brain. J. Biol. Chem. *275*, 36495–36497.
15. Patel, S., Churchill, G.C., and Galione, A. (2000). Unique kinetics of nicotinic acid-adenine dinucleotide phosphate (NAADP) binding enhance the sensitivity of NAADP receptors for their ligand. Biochem. J. *352*, 725–729.
16. Horstman, D.A., Takemura, H., and Putney, J.W., Jr. (1988). Formation and metabolism of [3H]inositol phosphates in AR42J pancreatoma cells. Substance P-induced Ca^{2+} mobilization in the apparent absence of inositol 1,4,5-trisphosphate 3-kinase activity. J. Biol. Chem. *263*, 15297–15303.
17. Christen, R., Schackmann, R.W., and Shapiro, B.M. (1982). Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, *Strongylocentrotus purpuratus*. J. Biol. Chem. *257*, 14881–14890.
18. Churchill, G.C., and Galione, A. (2001). Prolonged inactivation of NAADP-induced Ca^{2+} release mediates a spatiotemporal Ca^{2+} memory. J. Biol. Chem. *276*, 11223–11225.
19. Genazzani, A.A., Empson, R.M., and Galione, A. (1996). Unique inactivation properties of NAADP-sensitive Ca2+ release. J. Biol. Chem. *271*, 11599–11602.
20. Jaffe, L.A. (1976). Fast block to polyspermy in sea urchin eggs is electrically mediated. Nature *261*, 68–71.
21. McCulloh, D.H., and Chambers, E.L. (1992). Fusion of membranes during fertilization. Increases of the sea urchin egg's membrane capacitance and membrane conductance at the site of contact with the sperm. J. Gen. Physiol. *99*, 137–175.
22. McDougall, A., Gillot, I., and Whitaker, M. (1993). Thimerosal reveals calcium-induced calcium release in unfertilised sea urchin eggs. Zygote *1*, 35–42.
23. Churchill, G.C., and Galione, A. (2001). NAADP induces Ca^{2+} oscillations via a two-pool mechanism by priming IP_3- and cADPR-sensitive Ca^{2+} stores. EMBO J. *20*, 2666–2671.
24. Creton, R., and Jaffe, L.F. (1995). Role of calcium influx during the latent period in sea urchin eggs. Dev. Growth Differ. *37*, 703–709.