

## Rapid Report

# Nicotinic acid adenine dinucleotide phosphate (NAADP) is present at micromolar concentrations in sea urchin spermatozoa

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Nicotinic acid adenine dinucleotide phosphate (NAADP) has been shown to induce  $\text{Ca}^{2+}$  release in numerous cellular models, ranging from marine invertebrates to mammals. However, endogenous levels of this pyridine dinucleotide have yet to be demonstrated. In the sea urchin egg, NAADP receptors are abundant but have the peculiarity of being inactivated at low concentrations (picomolar) and activated at higher concentrations (nanomolar) which apparently rules out any possibility of the receptor being activated by concentration rises induced by a slow enzymatic formation in the cytosol. One of the most important events of fertilization is a  $\text{Ca}^{2+}$  transient in the egg, which leads to egg activation. The mechanisms which underlie the transient are still unclear and several theories persist including the existence of a sperm receptor and that soluble factors may pass from the sperm to the egg cytosol. We have investigated the possibility that NAADP might be present in sperm. Indeed, we found that sea water-activated spermatozoa are able to synthesize NAADP and that sperm extracts contain micromolar concentrations of the messenger. Although it is unlikely that NAADP alone mediates the fertilization wave, our data suggest that transfer of NAADP from spermatozoa to egg may play a role in this phenomenon.

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Nicotinic acid adenine dinucleotide phosphate (NAADP) is a pyridine nucleotide that has been shown to induce  $\text{Ca}^{2+}$  release in numerous cellular models, ranging from marine invertebrates to mammals (Lee, 2000; Patel *et al.* 2001). Although it is structurally similar to cyclic ADP ribose (cADPR), an agonist/modulator of ryanodine receptors, NAADP activates a distinct  $\text{Ca}^{2+}$  release mechanism on intracellular membranes (Genazzani & Galione, 1997; Lee, 2000; Patel *et al.* 2001). In the sea urchin, the best characterized model in which the pioneering experiments on this molecule were performed (Clapper *et al.* 1987; Lee & Aarhus, 1995; Chini *et al.* 1995), NAADP-induced  $\text{Ca}^{2+}$ -release is insensitive to pharmacological agents that affect cADPR- or  $\text{IP}_3$ -induced responses. Furthermore, it does not show heterologous desensitization with the  $\text{IP}_3$  and cADPR responses, while it does show homologous desensitization. In the sea urchin egg, NAADP exhibits two peculiarities. Firstly, NAADP, unlike  $\text{IP}_3$  and cADPR, releases  $\text{Ca}^{2+}$  from a store distinct from the endoplasmic reticulum (Genazzani & Galione, 1996; Lee & Aarhus, 2000). Secondly, low concentrations of NAADP that are unable to release  $\text{Ca}^{2+}$  can block any further responses even to maximal concentrations of the messenger (Genazzani *et*

*al.* 1996; Aarhus *et al.* 1996). It would appear that the latter phenomenon is due to the irreversible binding of NAADP to the receptor (Aarhus *et al.* 1996; Billington & Genazzani, 2000; Patel *et al.* 2000). This inactivation process is observed both in homogenates and in intact eggs, suggesting that it might play a role in  $\text{Ca}^{2+}$  responses in the egg. Therefore, if NAADP receptors are to be activated in the egg, a rapid surge of messenger has to occur so as to bypass inactivation. One possibility is that NAADP is compartmentalized and released into the cytosol upon requirement.

In the present study, we have investigated the possibility that the separate compartment might be the spermatozoa. It is well known that, upon sperm–egg fusion, a  $\text{Ca}^{2+}$  wave initiates from the point of contact (Stricker, 1999). Furthermore, it is known that the spermatozoa release the contents of their cytosol (including the genetic material) into the egg. The association of these two phenomena is debatable, and while there is evidence that the  $\text{Ca}^{2+}$  wave might be dependent on soluble sperm factors, contradictory evidence associates the wave with the activation of a sperm receptor on the plasma membrane (Hogben *et al.* 1998). Here, we show that NAADP can be

synthesized in sperm extracts, and more importantly, that micromolar NAADP levels are detectable in sea water-activated sperm cytosol. This evidence suggests that, at fertilization, NAADP receptors neighbouring the point of sperm–egg fusion could be activated by NAADP from the spermatozoa. This, coupled with the large volume of data showing the abundance of the NAADP-sensitive  $\text{Ca}^{2+}$  release mechanism in sea urchin eggs, suggests that the transfer of NAADP from spermatozoa to egg may play a role in the  $\text{Ca}^{2+}$  transient seen at egg fertilization.

## METHODS

### Collection of spermatozoa and eggs

Eggs and spermatozoa of *Lytechinus pictus* (Marinus Inc., Long Beach, CA, USA) were obtained by intracoelomic injection of 0.5 M KCl and collected in artificial sea water (ASW; 435 mM NaCl, 40 mM  $\text{MgCl}_2$ , 15 mM  $\text{MgSO}_4$ , 11 mM  $\text{CaCl}_2$ , 10 mM KCl, 2.5 mM  $\text{NaHCO}_3$ , 1 mM EDTA, pH 8.0). Egg homogenates were prepared as described previously (Dargie *et al.* 1990). Spermatozoa were treated in one of three ways prior to homogenization, so as to rule out possible artefacts of a single preparation. (1) Spermatozoa were collected in ASW and homogenized. (2) Spermatozoa were collected in ASW and centrifuged at 10 000 g. They were then resuspended and pelleted three times in 0  $\text{Ca}^{2+}$  ASW before homogenization in intracellular medium (IM; 250 mM potassium gluconate, 250 mM *N*-methyl glucamine, 20 mM Hepes, 1 mM  $\text{MgCl}_2$ , pH 7.2) + 25  $\mu\text{g ml}^{-1}$  leupeptin, 10  $\mu\text{g ml}^{-1}$  aprotinin and 50  $\mu\text{g ml}^{-1}$  SBTI. (3) Spermatozoa were collected in ASW and pelleted at 10 000 g. They were then resuspended and pelleted 3 times in low osmolarity buffer (10 mM KCl, 20 mM Hepes, 100  $\mu\text{M}$  EGTA, pH 7.5). Prior to preparation, spermatozoa were counted.

The spermatozoa were then freeze thawed 5 times in liquid  $\text{N}_2$  before homogenization in an Ultra Turrax homogenizer. Homogenates were aliquoted and frozen in liquid  $\text{N}_2$  for long term storage at  $-80^\circ\text{C}$ .

Sperm extract (cytosol) was prepared by a centrifugation protocol. Homogenates were spun at 20 000 g for 10 min at  $4^\circ\text{C}$  and the supernatant was boiled for 10 min. After centrifugation at 100 000 g for 1 h, the supernatant was kept and the pellet discarded.

### $^{32}\text{P}$ NAADP binding assays

$^{32}\text{P}$ NAADP and  $^{32}\text{P}$ NADP synthesis was conducted as described previously (Aarhus *et al.* 1996; Billington & Genazzani, 2000). Binding reactions were carried out on ice in IM for 20 min. Reactions, final volume 200  $\mu\text{l}$ , were initiated by the addition of egg homogenate (50  $\mu\text{g}$  protein) to IM, 50 pM  $^{32}\text{P}$ NAADP and sperm extract. After 20 min, reactions were terminated by centrifugation at 20 000 g, the pellet was washed in 1 ml ice-cold IM and resuspended in scintillation fluid before standard liquid scintillation counting. Preliminary experiments on spermatozoal membranes revealed that there was no detectable  $^{32}\text{P}$ NAADP binding.

Desensitization experiments were performed as follows; 50  $\mu\text{g}$  of homogenate was treated with sperm extract at concentrations below half-maximal for 20 min (final volume 40  $\mu\text{l}$ ). This was then diluted in 360  $\mu\text{l}$  IM containing 50 pM  $^{32}\text{P}$ NAADP to decrease the extract concentration to non-inhibitory concentrations. Reactions then proceeded as above.

### Enzyme assays

Assays for the degradation of the active factor by alkaline phosphatase (AP; Sigma) and nucleotide pyrophosphatase (NPP; Sigma) were carried out in buffer consisting of 20 mM Tris and 2.5 mM  $\text{Mg}^{2+}$ , pH 10.5 for AP and pH 7.5 for NPP. Sperm extract was incubated at  $37^\circ\text{C}$  with either AP at a final concentration of 1 unit  $\text{ml}^{-1}$  or NPP at 0.01 units  $\text{ml}^{-1}$ . Aliquots were removed at various time points and assayed for remaining inhibitory ability.

### HPLC of sperm extract and production of NAADP

A 1 ml sample of sperm extract was separated by HPLC on AG-MP1 resin (Bio-Rad) as described previously (Aarhus *et al.* 1995). Fractions were collected at 1 min intervals and then completely dried in a vacuum drier. Each fraction was resuspended in 100  $\mu\text{l}$  double-distilled  $\text{H}_2\text{O}$  and frozen at  $-80^\circ\text{C}$  until needed for binding experiments.

To assess production of NAADP by sperm extracts, 800  $\mu\text{g}$  of sperm protein (determined using the Bradford assay) were used and incubated under different conditions at room temperature. The subsequent reaction was analysed by HPLC as above and compared to standards of authentic nucleotides.

## RESULTS AND DISCUSSION

$^{32}\text{P}$ NAADP binding to sea urchin egg homogenates has been characterized by several laboratories using a competition assay (Aarhus *et al.* 1996; Billington & Genazzani, 2000; Patel *et al.* 2000) and we have used this assay to test extracts of sea water-activated sperm for the presence of compounds able to inhibit  $^{32}\text{P}$ NAADP binding. We found that sperm extracts were able to inhibit NAADP binding to egg membranes. Three different preparations (prepared as detailed in Methods) were tested and were found to have differing amounts of the active compound(s) (Fig. 1A). This can be explained, in part, by the different spermatozoa concentrations of the preparations (approximately  $20 \times 10^8$ ,  $0.13 \times 10^8$  and  $5 \times 10^8$  spermatozoa  $\text{ml}^{-1}$  for Aug 00, Jul 01 and Aug 01 preparations, respectively) as the concentration of the inhibitory agent in each preparation closely mirrored the spermatozoa concentration.

Using the most concentrated preparation (Aug 00), it was possible to perform a competition binding curve (Fig. 1B). The competition binding curve obtained with the sperm extract and with authentic NAADP were superimposable, since both showed a Hill slope of 1.

NAADP binding is virtually irreversible and therefore pre-incubation of egg homogenate with authentic NAADP results in the abolition of further binding (Aarhus *et al.* 1996; Billington & Genazzani, 2000; Patel *et al.* 2000). Nonetheless, high salt concentrations can both displace NAADP in competition binding (Patel *et al.* 2000) and can dissociate pre-bound NAADP (R. A. Billington & A. A. Genazzani, unpublished data). To test if the compound in sperm extract could be NAADP, experiments were performed based on the ability of the sea urchin receptor to bind NAADP practically irreversibly. When egg

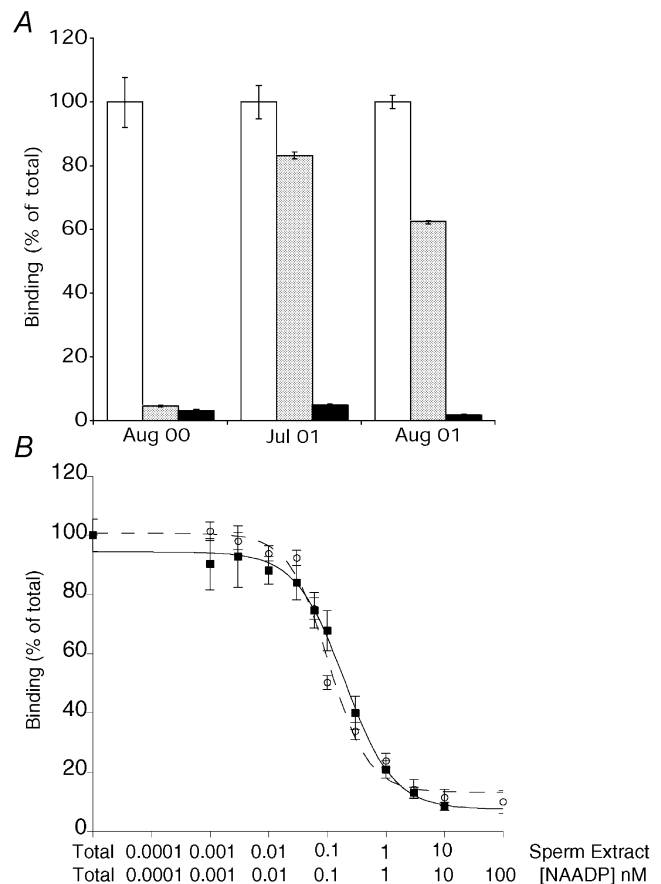
homogenates were pre-treated with sperm extract or with a concentration of NAADP with identical inhibitory potential, subsequent binding of [ $^{32}$ P]NAADP was abolished to the same extent (Table 1). Furthermore, unlike high salt concentrations but like authentic NAADP, the sperm extract was unable to displace specifically pre-bound [ $^{32}$ P]NAADP (Table 1). These results are consistent with the presence of a pyridine nucleotide in the sperm extract since the only compounds known to display these peculiarities are NAADP and NADP (although it has been shown that the latter effect could be due to contaminating NAADP (Dickey *et al.* 1998). The inhibitory agent was unlikely to be a protein, since boiling of the samples for 5–10 min did not alter the capacity of the extract to compete for binding (R. A. Billington & A. A. Genazzani, unpublished data). In order to verify that the active factor was a phosphate-containing nucleotide, the extract was incubated with alkaline phosphatase or nucleotide pyrophosphatase and then assayed for its ability to inhibit binding. After incubation with alkaline phosphatase for 1 h, 76.7  $\pm$  1.29% of the inhibitory activity had been lost. Similarly, when the extract was incubated with nucleotide pyrophosphatase, the inhibitory activity was completely abolished within 1 h, strengthening the idea that the active factor was indeed a nucleotide.

To investigate if the sperm extract contained NAADP, the extract was fractionated by HPLC using an anion-exchange protocol routinely used for separation of pyridine nucleotides (Aarhus *et al.* 1995). One minute fractions were collected and assayed for their ability to inhibit [ $^{32}$ P]NAADP binding. In all three preparations, fractions which were able to inhibit [ $^{32}$ P]NAADP binding to egg membranes co-eluted with authentic NAADP (Fig. 2) or with [ $^{32}$ P]NAADP. These fractions were also able to completely desensitize the NAADP-induced  $Ca^{2+}$  release system in egg homogenates (R. A. Billington & A. A. Genazzani, unpublished data). The majority of the displacing factor eluted in one peak, but activity could also be observed in a smaller peak eluting earlier. The identity of this compound was not characterized, but it accounts for 18.7% of total activity. The concentration of NAADP in spermatozoa can be calculated using the published value for the water volume of sea urchin spermatozoa of 0.71  $\mu$ l per  $10^8$  spermatozoa (Christen *et al.* 1982). The concentration of NAADP in the extract can therefore be obtained by comparing the  $IC_{50}$  for authentic NAADP to the volume of extract necessary to inhibit 50% of binding and by accounting for the fact that 18.7% of the activity is not attributable to NAADP. Using 193 pM as the  $IC_{50}$  of NAADP (Billington & Genazzani, 2000), the calculated concentration of NAADP in the sperm cytosol from the three preparations was  $4.0 \pm 2.3 \mu$ M (values for the preparations ranged from 7.8 to 0.27  $\mu$ M). The variation could be dependent on seasonal variations or different methods used for preparation of the extracts. Nonetheless,

**Table 1. Effect of sperm extract and NAADP on [ $^{32}$ P]NAADP bound to egg homogenate**

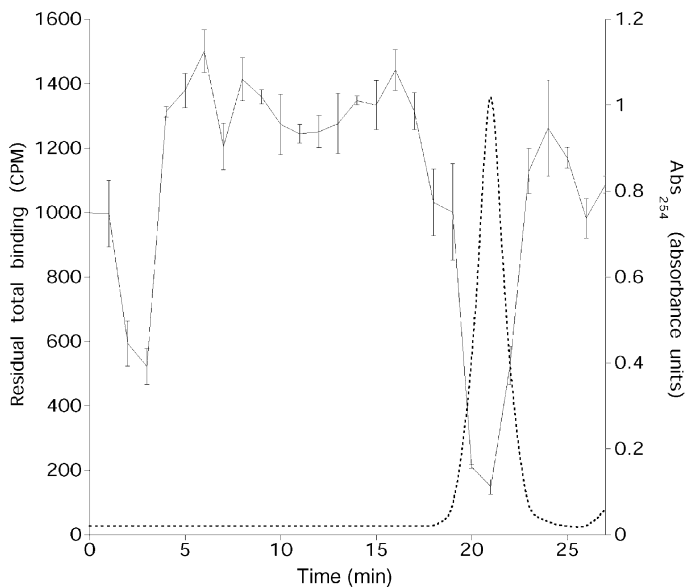
Displacement of bound [ $^{32}$ P]NAADP (% of residual)		Inhibition of [ $^{32}$ P]NAADP binding (% of total)	
[ $^{32}$ P]NAADP $\rightarrow$ agent		Agent $\rightarrow$ [ $^{32}$ P]NAADP	
Buffer	100 $\pm$ 3.53	Buffer	100 $\pm$ 2.93
Sperm extract	70.51 $\pm$ 3.19	Sperm extract	15.37 $\pm$ 2.02
NAADP 1 $\mu$ M	78.73 $\pm$ 1.98	NAADP 1.5 nM	11.23 $\pm$ 2.50

In the left columns, [ $^{32}$ P]NAADP was pre-incubated with egg homogenate then challenged in an attempt to induce dissociation. In the right columns, the agent was incubated with homogenate and then [ $^{32}$ P]NAADP was added in a large enough volume to decrease the agent to non-inhibitory concentrations. Values are given as means  $\pm$  S.E.M.  $n = 6-8$ .



**Figure 1. A factor in sperm extracts is able to compete for [ $^{32}$ P]NAADP binding**

A, inhibition of [ $^{32}$ P]NAADP binding to egg homogenates by the three different sperm homogenate preparations and authentic NAADP. B, representative concentration–response curves obtained from competition binding experiments with sperm extract (Aug 00,  $\blacksquare$ ) and NAADP ( $\circ$ ). Units for sperm homogenate are expressed as % volume/200  $\mu$ l binding reaction. Data are presented as means  $\pm$  S.E.M.;  $n = 3-9$ .



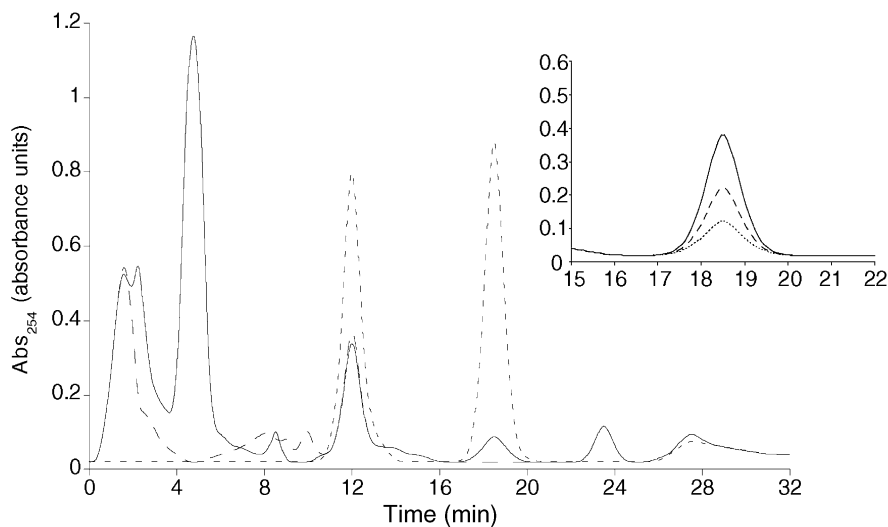
**Figure 2. The competing factor co-elutes with NAADP on HPLC**

Inhibition of [ $^{32}$ P]NAADP binding to egg homogenate by HPLC fractionated sperm extract (Aug 01; continuous line). The dashed line represents absorbance at 254 nm for authentic NAADP run on HPLC. Binding data are represented as means  $\pm$  S.E.M.;  $n = 4$ . Similar data were obtained with the other two preparations.

at present it is not possible to state whether NAADP levels were similar in non-activated sperm or whether sea water activation of spermatozoa induced a surge in levels of this compound.

It is indeed possible that the NAADP measured does not represent the true endogenous concentration since synthesis or degradation might occur during preparation. To exclude this possibility, we prepared extracts as described above in the presence of [ $^{32}$ P]NADP. Since the only known route of NAADP synthesis is a base-exchange reaction starting from NADP and nicotinic acid (Aarhus *et al.* 1995; see below), we would expect that, if production

did occur, part of the tracer would be converted to NAADP. After the separation of the extract by HPLC and subsequent determination of the radioactivity of the fractions,  $92.9 \pm 1.3\%$  of the radioactivity was recovered in a peak that co-eluted with NADP, while the remaining radioactivity was recovered in the membrane fraction ( $3.4 \pm 0.2\%$ ) and in the flow-through. No radioactivity co-eluted with NAADP, demonstrating that NAADP synthesis does not occur. To investigate whether NAADP degradation occurs, we performed identical experiments in the presence of [ $^{32}$ P]NAADP. After HPLC separation,  $83.8 \pm 1.9\%$  of the radioactivity was recovered in a peak co-eluting with NAADP, with the remaining radioactivity



**Figure 3. Sperm homogenates can synthesise NAADP**

Representative HPLC traces ( $n = 6-8$ ) showing production of NAADP by sperm homogenates after 1 h in the presence of 2 mM NADP and 6 mM nicotinic acid (NA) at pH 7.0 (continuous line), in the absence of NA (longer dashed line). Shorter dashed lines represent elution peaks of authentic NADP and NAADP standards. Other peaks on the trace have not been characterized. Inset, expanded view of the peak co-eluting with NAADP showing production of NAADP at pH 7 (dotted line), pH 6 (dashed line) and pH 5 (continuous line). Representative traces ( $n = 4$ ) illustrated.



in the membrane fraction ( $4.2 \pm 0.4\%$ ), and in small non-identifiable peaks. Therefore, it is likely that our determination of NAADP levels in sperm represents a conservative under-estimate of the content but is within our calculated standard error.

It has been previously shown that cADPR can be synthesized in sea urchin spermatozoa (Chini *et al.* 1997). Since it has been shown that similar or identical enzymes can also catalyse the synthesis of NAADP (Aarhus *et al.* 1995), and since we found that NAADP is present in spermatozoa, it follows that we should be able to detect NAADP production in our homogenates. Indeed, production of NAADP was detected by HPLC (a less sensitive method than the commonly used bio-assay) in the presence of exogenous NADP (2 mM) and nicotinic acid (6 mM) at pH 7 (Fig. 3). NAADP production in sperm extracts was over 350 times greater than in egg extracts. Eggs synthesized NAADP at a rate of  $0.026 \pm 0.02$  pmol  $\text{mg}^{-1} \text{min}^{-1}$ , while sperm synthesized NAADP at a rate of  $9.7 \pm 1.0$  pmol  $\text{mg}^{-1} \text{min}^{-1}$ . NAADP production was not detected in the absence of nicotinic acid (Fig. 3), indicating that the base-exchange reaction is the mechanism responsible. Furthermore, as expected, NAADP production was time dependent with maximal production observed at 1 h. Following on from earlier data (Aarhus *et al.* 1995), the pH dependence of NAADP production was investigated and it was found that NAADP production increased as the pH dropped (from 8 to 5), with maximal production at pH 5 (Fig. 3 inset). When the sperm homogenate was fractionated, NAADP synthesis was localized largely to the membrane fraction while little activity was detected in the cytosol (R. A. Billington & A. A. Genazzani, unpublished data). This distribution is similar to the one described in the sea urchin egg (Wilson & Galione, 1998).

We then addressed the question of whether NAADP had a target receptor in the spermatozoa. When [ $^{32}\text{P}$ ]NAADP binding was performed, no sites were detected on sperm membranes, consistent with the idea that NAADP is released by sperm and binds to its target receptors in the egg. Nonetheless, because of the inactivation properties of the receptor, binding sites on sperm membranes might have been occupied by unlabelled NAADP in the intact sperm.

In conclusion, we have shown that NAADP can be produced in sea urchin spermatozoa and that sperm cytosol contains micromolar concentrations of this pyridine nucleotide. Furthermore, the rate of synthesis in sperm is over 350 times that in eggs. This represents the first report of NAADP levels in any tissue and strongly supports the notion that NAADP is an endogenous modulator of  $\text{Ca}^{2+}$  release. Considering that the sperm cytosol will be diluted more than 10 000-fold when its contents are released into the egg, it is unlikely that the

NAADP concentrations reported here will produce a wave across the whole egg at fertilization. On the other hand, the NAADP from sperm will be at a high enough concentration around the site of sperm-egg fusion to activate NAADP receptors ( $\text{EC}_{50} \sim 25$  nM for  $\text{Ca}^{2+}$  release (Genazzani *et al.* 1996)), while more distal sites will, presumably, be inactivated ( $\text{IC}_{50} \sim 250$  pM for inactivation (Genazzani *et al.* 1996)). This model is substantiated by three previous observations: (i) the fertilization wave in sea urchin eggs can be abolished by co-injection of antagonists of the  $\text{IP}_3$  and ryanodine receptors, but residual  $\text{Ca}^{2+}$  release after fusion of the sperm still occurs (Galione *et al.* 1993); (ii) the NAADP response in fertilized eggs and homogenates is significantly reduced compared to unfertilized controls (Perez-Terzic *et al.* 1995; Genazzani, 1997) and (iii) the NAADP-sensitive store in starfish oocytes, a closely related echinoderm system, seems to be located just below the plasma membrane (Lim *et al.* 2001) and appears to release  $\text{Ca}^{2+}$  prior to the other two systems (Nusco *et al.* 2002). The experiments performed in starfish have revealed that while injection of NAADP leads to  $\text{Ca}^{2+}$  release from under the plasma membrane which spreads slowly to the centre of the egg, injection of  $\text{IP}_3$  leads to  $\text{Ca}^{2+}$  release and a subsequent fast moving wave emanating from the centre of the egg (Lim *et al.* 2001). Interestingly, it has been shown recently that release of  $\text{Ca}^{2+}$  by NAADP may prime the  $\text{IP}_3$  and cADPR sensitive stores for  $\text{Ca}^{2+}$  release (Churchill & Galione, 2001), providing a putative role for sperm NAADP. It has been shown previously that both  $\text{IP}_3$  and ryanodine receptors are activated at fertilization (Galione *et al.* 1993; Lee *et al.*, 1993) and, therefore, NAADP could act to fine-tune the  $\text{Ca}^{2+}$  wave.

For a fast-desensitizing messenger such as NAADP in the egg, the fact that it is produced in a separate compartment to the location of the receptor would validate its role.

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