Nicotinic Acid Adenine Dinucleotide Phosphate-induced Ca\(^{2+}\) Release

INTERACTIONS AMONG DISTINCT Ca\(^{2+}\) MOBILIZING MECHANISMS IN STARFISH OOCYTES*

(Received for publication, September 7, 1999, and in revised form November 22, 1999)

Luigia Santella\(^{1}\), Keiichiro Kyoza\(^{2}\), Armando A. Genazzani\(^{3}\), Laura De Riso\(^{4}\), and Ernesto Carafoli\(^{1}\)**

From the \(^{1}\)Laboratory of Cell Biology Stazione Zoologica “A. Dohrn” Villa Comunale, I-80121, Napoli, Italy, the \(^{2}\)Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1QJ United Kingdom, and the \(^{3}\)Department of Biochemistry, University of Padova, 35121 Padova, Italy

An intracellular mechanism activated by nicotinic acid adenine dinucleotide phosphate (NAADP\(^{1}\)) contributes to intracellular Ca\(^{2+}\) release alongside 1,4,5-trisphosphate (Ins-P\(_{3}\)) and ryanodine receptors. The NAADP\(^{1}\)-sensitive mechanism has been shown to be operative in sea urchin eggs, ascidian eggs, and pancreatic acinar cells. Furthermore, mature mammalian cell types can synthesize NAADP\(^{1}\), with nicotinic acid and NADP\(^{1}\) as precursors. In this contribution, NAADP\(^{1}\)-induced Ca\(^{2+}\) release has been investigated in starfish oocytes. Uncaging of injected NAADP\(^{1}\)-induced Ca\(^{2+}\) mobilization in both immature oocytes and in oocytes matured by the hormone 1-methyladenine (1-MA). The role of extracellular Ca\(^{2+}\) in NAADP\(^{1}\)-induced Ca\(^{2+}\) mobilization, which was minor in immature oocytes, was instead essential in mature oocytes. Thus, the NAADP\(^{1}\)-sensitive Ca\(^{2+}\) pool, which is known to be distinct from those sensitive to inositol 1,4,5-trisphosphate or cyclic ADPribose, apparently migrated closer to (or became part of) the plasma membrane during the maturation process. Inhibition of both Ins-P\(_{3}\) and ryanodine receptors, but not of either alone, substantially inhibited NAADP\(^{1}\)-induced Ca\(^{2+}\) mobilization in both immature and mature oocytes. The data also suggest that NAADP\(^{1}\)-induced Ca\(^{2+}\) mobilization acted as a trigger for Ca\(^{2+}\) release via Ins-P\(_{3}\) and ryanodine receptors.

Ca\(^{2+}\) release from intracellular stores contributes to the regulation of numerous cellular functions, among them egg fertilization. In sea urchin eggs, the intracellular Ca\(^{2+}\) wave that follows the interaction with the sperm is initiated by Ca\(^{2+}\) release via Ins-P\(_{3}\)\(^{1}\) and ryanodine-sensitive receptors. The latter are activated by cADPr\((1, 2)\), an endogenous pyridine nucleotide derived from NAD\(^{+}\), which acts in a wide range of cells from unicellular organisms to plants (3).

Recently, work in sea urchin eggs has shown that another derivative of pyridine nucleotides, nicotinic acid adenine dinucleotide phosphate (NAADP\(^{1}\)), also releases Ca\(^{2+}\) from intracellular stores (4–6). NAADP\(^{1}\) releases Ca\(^{2+}\) via a mechanism distinct from those triggered by Ins-P\(_{3}\) and cADPr because: (i) the antagonists heparin and 8-NH\(_{2}\)-cADPr do not block NAADP\(^{1}\)-induced Ca\(^{2+}\) release (4); (ii) desensitization of Ins-P\(_{3}\) and ryanodine receptors does not influence NAADP\(^{1}\)-induced Ca\(^{2+}\) release (4, 5); and, (iii) NAADP\(^{1}\)-induced Ca\(^{2+}\) release appears to be additive to that induced by Ins-P\(_{3}\) or cADPr (7). These findings, in particular the latter, have prompted investigations to establish whether NAADP\(^{1}\)-induced Ca\(^{2+}\) release from a pool different from that activated by the other two messengers. This indeed appears to be the case, because depletion of endoplasmic reticulum pools by thapsigargin abolished Ins-P\(_{3}\) or cADPr-induced Ca\(^{2+}\) release but failed to influence the Ca\(^{2+}\) response to NAADP\(^{1}\) (7). Fractionation experiments have supported these observations because membranes containing Ins-P\(_{3}\)- and cADPr-sensitive pools migrated differently from those containing the NAADP\(^{1}\)-sensitive pool in Percoll gradients (4). Furthermore, NAADP\(^{1}\)-induced Ca\(^{2+}\) release in sea urchin eggs was blocked by high concentrations of L-type Ca\(^{2+}\) channels antagonists (8) which had no effect on the other two channels. An interesting property of this novel release mechanism is that minute concentrations of NAADP\(^{1}\), which would be per se unable to induce Ca\(^{2+}\) release, blocked further Ca\(^{2+}\) release by supra-maximal concentrations of NAADP\(^{1}\) (9, 10).

NAADP\(^{1}\) was also found to release Ca\(^{2+}\) from ascidian eggs (11). At variance with sea urchins, in ascidians eggs NAADP\(^{1}\) did not display the low threshold inactivation property. Furthermore, in these eggs NAADP\(^{1}\) blocked the post-fertilization Ca\(^{2+}\) oscillations (11), compellingly supporting its physiological role. Because sea urchin and ascidian diverged in evolution millions of years ago, Ca\(^{2+}\) signaling by NAADP\(^{1}\) could be a widespread mechanism. In line with this suggestion, mammalian cells possess the enzymatic machinery to synthesize and degrade NAADP\(^{1}\) (12, 13).

To increase the understanding of the process of Ca\(^{2+}\) release by NAADP\(^{1}\), which is still relatively novel, we have chosen to investigate a well characterized process, the Ca\(^{2+}\) mobilization linked to the process of oocyte maturation in starfish (14–16), which is triggered by the hormone 1-methyladenine (1-MA) (17). During meiosis, the oocytes undergo physiological changes that result in an effective cortical reaction and in the appearance of the fertilization envelope (18). The findings described in
Fig. 1. Ca\(^{2+}\) mobilization induced by the uncaging of NAADP\(^{+}\) in immature oocytes. a, Ca\(^{2+}\) release triggered by the photolysis of caged NAADP\(^{+}\) injected into the cytoplasm of an immature oocyte. The arrow indicates the beginning of the 5-s uncaging process. b, heparin injected prior to the uncaging of NAADP\(^{+}\); c, 8-NH\(_2\)-cADPr injected prior to the uncaging of NAADP\(^{+}\); d, both heparin and 8-NH\(_2\)-cADPr injected into the cytoplasm prior to the injection and uncaging of NAADP\(^{+}\); e, uncaging of injected NAADP\(^{+}\) in seawater containing 250 \(\mu\)M verapamil; f, uncaging
this contribution have shown that NAADP$^+$ indeed elicited a Ca$^{2+}$ increase in both mature and immature oocytes. Importantly, however, the properties of the Ca$^{2+}$ mobilization process differed very significantly in the two stages of maturation. In addition, the results have shown a close interplay between the NAADP$^+$-sensitive Ca$^{2+}$ release process, those triggered by the two other conventional intracellular Ca$^{2+}$ channels, and the process of Ca$^{2+}$ influx.

**RESULTS**

In all experiments described below, caged NAADP$^+$ (100 μM) was injected into the cytoplasm of the oocytes with the Ca$^{2+}$ indicator calcium green dextran (5 mg/ml). Its final concentration in the oocyte cytosol varied between 1 and 2 μM (see “Materials and Methods”). No Ca$^{2+}$ release followed the injection of caged NAADP$^+$ into the cytoplasm of either immature or mature oocytes if flash photolysis was not applied. Furthermore, addition of uncaged NAADP$^+$ to the external medium at a concentration between 100 μM and 1 mM failed to elicit Ca$^{2+}$ mobilization (data not shown).

**Immature Oocytes**—Fig. 1 shows that photolysing caged NAADP$^+$ with a brief (2.5 s) exposure to UV light (340 nm) promptly produced a rapid elevation of intracellular Ca$^{2+}$. The cytosolic transient reached a maximum of about 0.5 arbitrary units in a few sec and decayed to a level slightly higher than the baseline in about 10 min (n = 11; 0.54 ± 0.1). A damped oscillation followed the rapid decay of the transient (Fig. 1a).

To establish whether NAADP$^+$-induced release occurred via Ins-P$_3$- and cADPr-independent mechanisms, specific inhibitors were injected together with caged NAADP$^+$. Neither heparin (inhibitor of Ins-P$_3$ receptors; Fig. 1b) nor 8-NH$_2$cADPr (inhibitor of the ryanodine receptor (20); Fig. 1c) blocked the response elicited by NAADP$^+$. In fact, in the presence of either of the two inhibitors, the Ca$^{2+}$ release induced by NAADP$^+$ reached a significantly higher peak (with heparin, n = 4; 0.92 ± 0.1, with 8-NH$_2$cADPr, n = 4; 0.72 ± 0.1) compared with the controls injected with NAADP$^+$ alone. A similar observation on NAADP$^+$-induced Ca$^{2+}$ release had been reported by Perez-Terzic et al. (21) in sea urchin eggs for Ins-P$_3$ receptors. As expected, the preinjection of heparin or 8-NH$_2$cADPr inhibited the Ca$^{2+}$ release specifically induced by Ins-P$_3$ or cADPr in the oocytes (not shown). The injection of heparin or 8-NH$_2$cADPr had a further effect on NAADP$^+$-induced Ca$^{2+}$ release. In the presence of either of the two antagonists, the decay of the Ca$^{2+}$ trace after the initial peak was arrested, i.e. the rise in intracellular Ca$^{2+}$ was prolonged over time, lasting for the duration of the recording (20 min). At the end of the experiments, intracellular Ca$^{2+}$ was still significantly higher than in the noninjected controls. Possibly, the inhibition of either the Ins-P$_3$ or cADPr routes would increase the effectiveness of the coupling of the other to the NAADP$^+$ route.

When one of the two conventional routes is inhibited, the other becomes more effectively coupled, leading to the gradual increase of Ca$^{2+}$. The possibility that the Ins-P$_3$ and cADPr routes influenced each other independently of NAADP$^+$ was ruled out by experiments in which the injection of heparin had no effect on the Ca$^{2+}$ release induced by cADPr, and that of 8NH$_2$cADPr failed to influence the response to Ins-P$_3$ (not shown). Taken together these findings show also that in starfish oocytes NAADP$^+$-induced Ca$^{2+}$ release represents a pathway for mobilizing internal Ca$^{2+}$ distinct from those sensitive to Ins-P$_3$ and cADPr although it is functionally linked to them. This is compellingly supported by the findings in panel d of Fig. 1. Co-injection of both heparin and 8-NH$_2$cADPr together with caged NAADP$^+$ into the cytoplasm inhibited the response induced by NAADP$^+$ by about 50% (n = 4; 0.29 ± 0.1), suggesting that NAADP$^+$-induced Ca$^{2+}$ release triggers or recruits the other two mechanisms.

The pharmacological properties of NAADP$^+$-induced Ca$^{2+}$ release had been previously characterized in sea urchin egg homogenates (8), and it had been shown that the Ca$^{2+}$ response mediated by the NAADP$^+$ channels was antagonized by L-type Ca$^{2+}$ channel blockers. At variance with this, Fig. 1e shows the relatively minor inhibition of the Ca$^{2+}$ response in an immature oocyte injected with NAADP$^+$ and transferred to seawater containing verapamil at a concentration of 250 μM prior to irradiation (n = 3; 0.38 ± 0.1). The other L-type Ca$^{2+}$ channel blocker nifedipine at a concentration of 10 μM also failed to inhibit the transient (data not shown). To add weight to the suggestion that in immature oocytes NAADP$^+$ released Ca$^{2+}$ from intracellular stores, experiments were then performed in Ca$^{2+}$-free seawater containing 2 mM EGTA. In this case also, NAADP$^+$ released Ca$^{2+}$ (n = 3; 0.33 ± 0.1) although the transient had a lower amplitude than in experiments in Ca$^{2+}$-containing seawater.

**Mature Oocytes**—Calcium release following the uncaging of NAADP$^+$ was also observed after the oocytes had been challenged with the hormone 1-MA which induces re-initiation of...
FIG. 2. Ca\(^{2+}\) mobilization induced by the uncaging of NAADP\(^{+}\) in mature oocytes. a, Ca\(^{2+}\)-release triggered by the photolysis of caged NAADP\(^{+}\) injected into the cytoplasm of a mature oocyte; b, uncaging of NAADP\(^{+}\) in an oocyte injected with heparin; c, uncaging of injected NAADP\(^{+}\) in a mature oocyte injected with 8-NH\(_2\)-cADPr; d, uncaging of NAADP\(^{+}\) in a mature oocyte previously injected with heparin and 8-NH\(_2\)-cADPr; e, uncaging of caged NAADP\(^{+}\) in an oocyte in seawater containing 250 \(\mu\)M verapamil; f, uncaging of caged NAADP\(^{+}\) in an oocyte transferred to Ca\(^{2+}\)-free seawater containing 2 mM EGTA. Additional details are given under "Materials and Methods."
NAADP$^+$ and Starfish Oocytes

The effects of inhibitors of intracellular Ca$^{2+}$ channels and of the absence of Ca$^{2+}$ from the external medium on the response to NAADP$^+$. The values are the average (± S.E.) of three to eleven experiments depending on the various conditions. The fluorescence intensity is expressed as $F^-$/$F^0$ (see “Materials and Methods”).

| Immature oocytes | Mature oocytes |
|------------------|---------------|
| Control          | 0.54 ± 0.1 (100%) | 0.96 ± 0.19 (100%) |
| +Heparin         | 0.92 ± 0.1 (170%) | 1.18 ± 0.12 (122%) |
| +8-NH$_2$-cADPr  | 0.72 ± 0.1 (133%) | 1.15 ± 0.19 (120%) |
| +Heparin + 8-NH$_2$-cADPr | 0.29 ± 0.1 (53%) | 0.46 ± 0.1 (48%) |
| +Verapamil       | 0.38 ± 0.1 (70%) | 0.19 ± 0.1 (20%) |
| No calcium       | 0.33 ± 0.1 (61%) | 0.16 ± 0.1 (17%) |

meiosis (and therefore maturation). 40 min after the addition of 1-MA, the mature oocytes were injected with caged NAADP$^+$. Fig. 2a shows that a 2.5-s photolysis flash produced a rapid elevation of intracellular Ca$^{2+}$, which in this particular experiment reached a height of about 1.2 arbitrary units ($n = 9$; 0.96 ± 0.19). The uncaging of NAADP$^+$ had been previously shown to produce a large Ca$^{2+}$ transient in sea urchin eggs (19) and to activate a massive cortical exocytosis reaction (9, 19, 21). The Ca$^{2+}$ transient induced by NAADP$^+$ triggered the cortical exocytosis reaction also in starfish oocytes (not shown). Occasionally, the uncaging of NAADP$^+$ in mature oocytes elicited Ca$^{2+}$ oscillations, as is the case in sea urchin eggs (19). Up to three Ca$^{2+}$ oscillations were observed over a period of 20 min (not shown). Significant differences with respect to immature oocytes, however, were seen in mature cells: (i) uncaging of NAADP$^+$ induced a significantly greater Ca$^{2+}$-mobilizing response than in immature oocytes; and (ii) after the initial NAADP$^+$-induced Ca$^{2+}$-transient, Ca$^{2+}$ remained elevated, failing to decline to baseline levels.

The effects of the inhibition of the Ins-P$_3$ and cADPr-modulated channels were explored also on mature oocytes. Micoinjection of heparin or 8-NH$_2$-cADPr did not prevent Ca$^{2+}$ release induced by the uncaging of NAADP$^+$ ($n = 4$; 1.18 ± 0.1 and $n = 5$; 1.15 ± 0.19, respectively; Fig. 2, a and c). The concentrations of heparin and 8-NH$_2$-cADPr used, though, were fully effective at blocking release induced by uncaging of Ins-P$_3$ or cADPr (for Ins-P$_3$, see Fig. 3c; for cADPr, data not shown). Interestingly, the post-transient high plateau state observed in control oocytes treated with NAADP$^+$ was not maintained in oocytes co-injected with either heparin or 8NH$_2$-cADPr (Fig. 2, b and c). In accord with the data obtained in immature oocytes, NAADP$^+$-induced Ca$^{2+}$ mobilization was significantly reduced in the presence of both inhibitors ($n = 4$; 0.46 ± 0.1; Fig. 2d)

Significant differences between immature and mature oocytes were also observed with respect to L-type Ca$^{2+}$ channel inhibitors. When the uncaging of NAADP$^+$ was performed in the presence of 250 μM extracellular verapamil, the Ca$^{2+}$-transient induced by the irradiation in mature oocytes was much more significantly inhibited than in immature cells ($n = 4$; 0.19 ± 0.1; Fig. 2e). As mentioned above, blockade of the NAADP$^+$-induced Ca$^{2+}$ release by μM concentrations of verapamil (and of the other L-type Ca$^{2+}$ channel blocker, nifedipine) had been already reported in sea urchin egg homogenates (8). The verapamil finding was corroborated by experiments using nifedipine (10 μM) which also prevented the Ca$^{2+}$ release induced by the uncaging of NAADP$^+$ (data not shown). Furthermore, when experiments were performed in Ca$^{2+}$-free seawater containing 2 mM EGTA, NAADP$^+$ essentially failed to elicit a Ca$^{2+}$-response ($n = 3$; 0.16 ± 0.1; Fig. 2f). These results were at sharp variance with those on immature oocytes and indicate that after the maturation process the NAADP$^+$-sensitive Ca$^{2+}$-release pathway had become functionally different from that of immature oocytes. The pool in mature oocytes

![Graph](image-url)
could still be contained in intracellular membranes (plasma membrane invaginations and/or organelles immediately beneath it) or could be represented by the extracellular space, assuming that the membranes that contained the NAADP$^+$ channel fused with the plasma membrane during the maturation process.

As was the case for immature oocytes, the uncaging of Ins-P$_3$ in seawater triggered a massive and biphasic Ca$^{2+}$ response (Fig. 3a) and the cortical exocytosis reaction (not shown) previously documented by others (22–24). The height of the peak produced by Ins-P$_3$ reached a maximum of 1.2 arbitrary units, the Ca$^{2+}$ increase lasting about 7 min. The magnitude of the response was similar to that produced by uncaging of NAADP$^+$ (Fig. 2a) or of cADPr (data not shown). Unlike the response to NAADP$^+$, however, flash photolysis of caged Ins-P$_3$ in Ca$^{2+}$-free seawater containing 2 mM of EGTA elicited a similar Ca$^{2+}$ spike compared with controls. Interestingly, the shoulder that normally followed the initial peak was in this case barely detectable (Fig. 3b), suggesting that Ca$^{2+}$ influx was responsible for the later phase of the Ins-P$_3$ response. A summary of the effects of the various effectors and inhibitors is offered in Table I.

**DISCUSSION**

As in other cell systems, the properties of the Ca$^{2+}$ release reaction activated by NAADP$^+$ in starfish oocytes clearly indicate an independent signaling pathway. One striking result in this contribution, which deserves to be mentioned at the outset of the “Discussion,” has been the demonstration that the characteristics of the Ca$^{2+}$-release reaction mediated by NAADP$^+$ varied with the maturation stage of the cells. In all cases, the uncaging of the microinjected NAADP$^+$ elicited a Ca$^{2+}$ transient (which in mature oocytes then activated the cortical granule reaction), indicating that the NAADP$^+$ release mechanism, (i.e. the NAADP$^+$ receptors) was present and operative also in this cell type. In both mature and immature cells, the return of the Ca$^{2+}$ spike to the base line was interrupted by a shoulder, after which the decay curve resumed its decline in immature oocytes but failed to do so in the mature ones. In both cell types, the Ca$^{2+}$ spike was (partially) sensitive to the simultaneous release of calcium ions (29)

**REFERENCES**

1. Galione, A., McDougall, A., Buda, W. B., Willmott, N., Gillot, I., and Whitaker, M. (1993) Science 261, 348–352
2. Lee, H. C., Aarhus, R., and Walseth, T. F. (1995) Science 261, 352–355
3. Lee, H. C. (1998) Cell Biochem. Biophys. 28, 1–17
4. Lee, H. C., and Aarhus, R. (1995) J. Biol. Chem. 270, 2152–2157
5. Chini, E. N., Beers, K. W., and Dousa, T. P. (1995) J. Biol. Chem. 270, 3216–3223
6. Genazzani, A. A., and Galione, A. (1997) Trends Pharmacol. Sci. 18, 108–110
7. Genazzani, A. A., and Galione, A. (1996) Biochem. J. 315, 721–725
8. Genazzani, A. A., Mezina, M., Dickey, D. M., Michelangeli, F., Walseth, T. F., and Galione, A. (1997) Br. J. Pharmacol. 121, 1489–1495
9. Genazzani, A. A., Empson, R. M., and Galione, A. (1996) J. Biol. Chem. 271, 11599–11602
10. Aarhus, R., Dickey, D. M., Graeff, R. M., Lee, K. R., Walseth, T. F., and Lee, H. C. (1996) J. Biol. Chem. 271, 8513–8516
11. Albrieux, M., Lee, H. C., and Villaz, M. (1998) J. Biol. Chem. 273, 14566–14574
12. Aarhus, R., Graeff, R. M., Dickey, D. M., Walseth, T. F., and Lee, H. C. (1995) J. Biol. Chem. 270, 30327–30333
13. Chini, E. N., and Dousa, T. P. (1995) Biochem. Biophys. Res. Commun. 209, 167–174
14. Moreau, M., Guerrier, P., Dörée, M., and Ashley, C. C. (1978) Nature 272, 251–253
15. Santella, L., and Kyozuka, K. (1994) Biochem. Biophys. Res. Commun. 203, 674–680
16. Santella, L., De Riso, L., Gragnaniello, G., and Kyozuka, K. (1998) Biochem. Biophys. Res. Commun. 252, 1–4
17. Kanatani, H., Shirai, H., Nakamichi, K., and Kurokawa, T. (1969) J. Biol. Chem. 244, 4172–4176
18. Aarhus, R., Graeff, R. M., Dickey, D. M., Walseth, T. F., and Lee, H. C. (1995) Biochem. Biophys. Res. Commun. 227, 253–274
19. Longo, F. J. (1997) Fertilization, Chapman and Hall, London
20. Lee, H. C., Aarhus, R., and Kestner, T. (1997) J. Biol. Chem. 272, 4172–4178
21. Lee, H. C., Aarhus, R., and Kestner, T. (1997) J. Biol. Chem. 272, 4172–4178
22. Chihara, K., Kado, R. T., and Jaffe, L. A. (1990) Dev. Biol. 140, 300–306
23. Mohri, T., Kornet, P. L., and Chambers, E. L. (1995) Dev. Biol. 172, 139–157
24. Stricker, S. A. (1995) Dev. Biol. 170, 496–518
25. Cancela, J., Churchill, G., and Galione, A. (1999) Nature 398, 74–76
26. Santella, L., De Riso, L., Gragnaniello, G., and Kyozuka, K. (1999) Exp. Cell. Res. 248, 567–574
27. Miyazaki, S., and Hirai, S. (1979) Dev. Biol. 70, 327–340
28. Schroeder, T. E., and Stricker, S. A. (1988) Dev. Biol. 128, 373–384
29. Creton, R., and Jaffe, L. F. (1995) Dev. Growth Differ. 37, 703–709
Nicotinic Acid Adenine Dinucleotide Phosphate-induced Ca2+ Release: INTERACTIONS AMONG DISTINCT Ca2+ MOBILIZING MECHANISMS IN STARFISH OOCYTES

Luigia Santella, Keiichiro Kyozuka, Armando A. Genazzani, Laura De Riso and Ernesto Carafoli

*J. Biol. Chem. 2000, 275:8301-8306.*
doi: 10.1074/jbc.275.12.8301

Access the most updated version of this article at [http://www.jbc.org/content/275/12/8301](http://www.jbc.org/content/275/12/8301)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 11 of which can be accessed free at [http://www.jbc.org/content/275/12/8301.full.html#ref-list-1](http://www.jbc.org/content/275/12/8301.full.html#ref-list-1)