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

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

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Luigia Santella‡, Keiichiro Kyozuka§, Armando A. Genazzani¶, Laura De Riso‡, and Ernesto Carafoli‖**

From the ‡Laboratory of Cell Biology Stazione Zoologica "A. Dohrn" Villa Comunale, I-80121, Napoli, Italy, the §Asamushi Marine Biological Station, Asamushi, Aomori 039-3501, Japan, the ¶Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1QJ United Kingdom, and the ‖Department of Biochemistry, University of Padova, 35121 Padova, Italy

An intracellular mechanism activated by nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) contributes to intracellular Ca^{2+} release alongside inositol 1,4,5-trisphosphate (Ins-P_3) and ryanodine receptors. The $NAADP^+$-sensitive mechanism has been shown to be operative in sea urchin eggs, ascidian eggs, and pancreatic acinar cells. Furthermore, most mammalian cell types can synthesize $NAADP^+$, with nicotinic acid and $NADP^+$ as precursors. In this contribution, $NAADP^+$-induced Ca^{2+} release has been investigated in starfish oocytes. Uncaging of injected $NAADP^+$ 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 $NAADP^+$-induced Ca^{2+} mobilization, which was minor in immature oocytes, was instead essential in mature oocytes. Thus, the $NAADP^+$-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 $NAADP^+$-induced Ca^{2+} mobilization in both immature and mature oocytes. The data also suggest that $NAADP^+$-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 ($NAADP^+$), also releases Ca^{2+} from intracellular stores (4–6). $NAADP^+$ 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 $NAADP^+$-induced Ca^{2+} release (4); (ii) desensitization of Ins-P_3 and ryanodine receptors does not influence $NAADP^+$-induced Ca^{2+} release (4, 5); and, (iii) $NAADP^+$-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 $NAADP^+$ released Ca^{2+} 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 $NAADP^+$ (7). Fractionation experiments have supported these observations because membranes containing Ins-P_3- and cADPr-sensitive pools migrated differently from those containing the $NAADP^+$-sensitive pool in Percoll gradients (4). Furthermore, $NAADP^+$-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 $NAADP^+$, which would be *per se* unable to induce Ca^{2+} release, blocked further Ca^{2+} release by supra-maximal concentrations of $NAADP^+$ (9, 10).

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

To increase the understanding of the process of Ca^{2+} release by $NAADP^+$, 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-methyaldenine (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

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** To whom correspondence should be addressed: Dept. of Biochemistry, University of Padova, Viale G. Colombo 3, 35121, Padova, Italy. Tel.: 0039-049-8276137; Fax: 0039-049-8276125.

[1] The abbreviations used are: Ins-P_3, inositol 1,4,5-trisphosphate; 1-MA, 1-methyladenine; $NAADP^+$, nicotinic acid adenine dinucleotide phosphate; cADPr, cyclic ADPribose; GVBD, germinal vesicle breakdown.

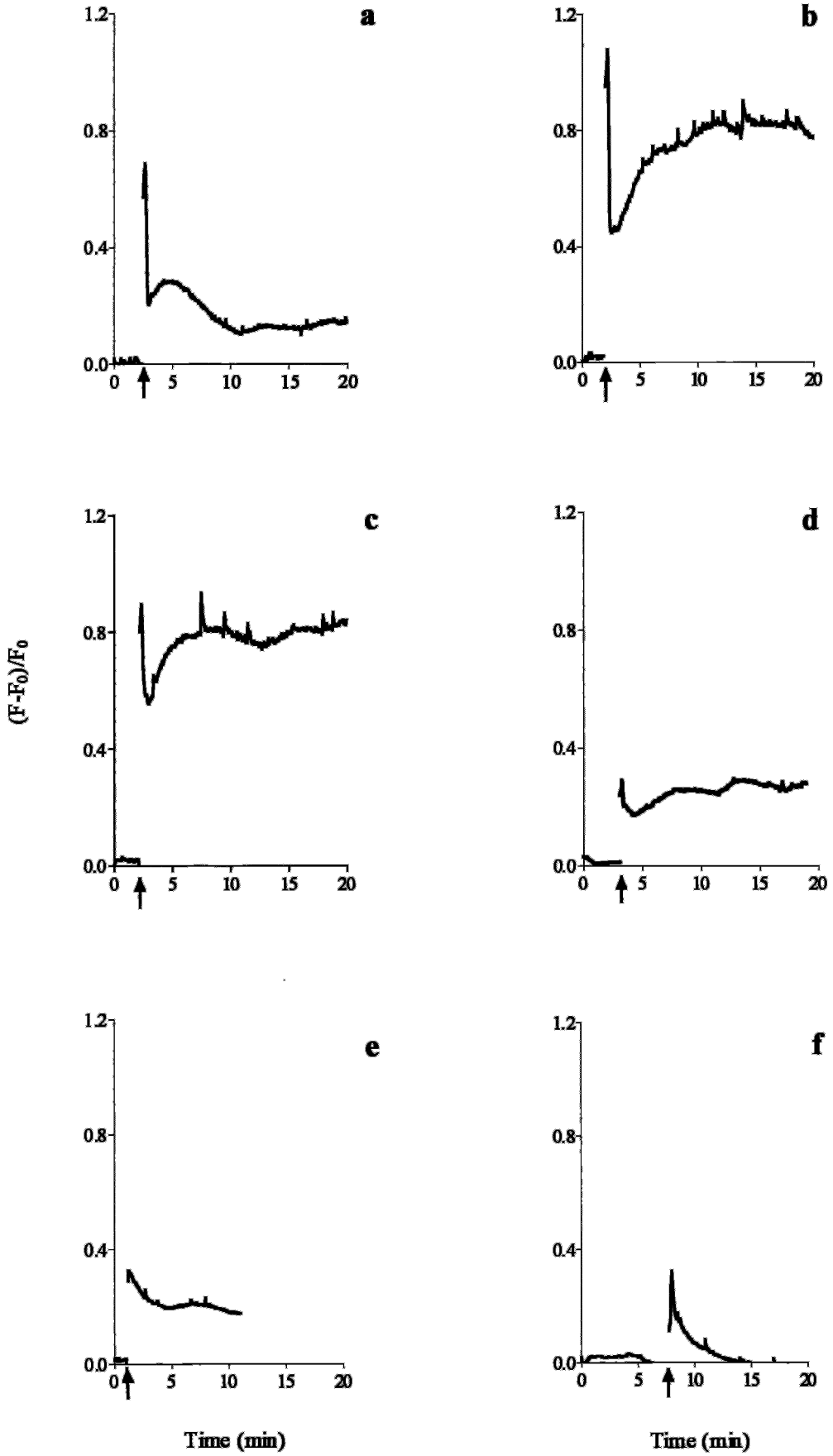

FIG. 1. **Ca^{2+} mobilization induced by the uncaging of NAADP$^+$ in immature oocytes.** *a*, Ca^{2+} release triggered by the photolysis of caged NAADP$^+$ injected into the cytoplasm of an immature oocyte. The *arrow* indicates the beginning of the 5-s uncaging process. *b*, heparin injected prior 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 μM 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.

MATERIALS AND METHODS

Starfish (*Asterina pectinifera)* were collected in the Mutsu Bay (Japan) in September during the breeding season and kept in running seawater (15). Immature oocytes containing the germinal vesicle (nucleus) were dissected from the ovaries in filtered seawater, washed 2–3 times, and kept in it for 30 min before using. Oocytes in which the rupture of the envelope of the germinal vesicle (GVBD) spontaneously occurred during this period were discharged. When mature oocytes were needed, maturation (GVBD) was promoted by the addition of the hormone 1-MA (5 μM). Oocytes at different stages of maturation were used for the microinjection experiments.

Microinjection and Photolysis of Caged NAADP$^+$*, Ins-P*$_3$*, or cADPr*—The injection solution in the pipette for all experiments contained 100 μM caged NAADP$^+$ (19) (Molecular Probes, Inc. Eugene, Oregon), 5 mg/ml calcium green dextran 10,000 (Molecular Probes), 100 mM potassium aspartate, 10 mM Hepes, pH 7.0. The volume injected in all experiments corresponded to 1–2% of the total cell volume. Thus, the final concentration of the injected compounds in the cellular environment was 50–100-fold lower than in the micropipette (15). The UV photolysis and fluorescent measurements were performed using a computer-controlled photomultiplier system as described previously (15). During photolysis, the excitation light was alternated manually (for 5 s) from monitoring (488 nm, band pass filter) to uncaging cassette (Uv-cassette, Olympus, IMT2-DMU). To exclude the variations of the fluorescent intensity by different volumes of injected dye at the beginning, the fluorescence signals were corrected for variations in dye concentration by normalizing fluorescence (F) against base-line fluorescence (F_0), to obtain reliable information about transient Ca^{2+} changes from baseline values. The concentration of heparin (Sigma, M_r ~6000) in the micropipette was 10 mg/ml, that of 8-NH_2-cADPr (Sigma) was 400 μM in the micropipette. The inhibitors were injected into immature or mature oocytes prior to the injection of caged NAADP$^+$ and the dye. For the experiments with the channel inhibitors, immature or mature oocytes were injected with caged NAADP$^+$ and then transferred to seawater containing 250 μM verapamil, or 10 μM nifedipine, or to Ca^{2+}-free seawater containing 2 mM EGTA. For the experiment on the uncaging of Ins-P_3 or cADPr, the microinjection pipette contained 100 μM Ins-P_3 or 400 μM cADPr (Molecular Probes), respectively.

Statistical Analysis—The data in the figures show typical experiments which in all cases have been repeated a number of times. In the text, the results have been analyzed statistically and are expressed as ± S.E.

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. 1*a*).

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. 1*b*) nor 8-NH_2-cADPr (inhibitor of the ryanodine receptor (20); Fig. 1*c*) 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. 1*e* 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) (Fig. 1*f*) 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

of injected caged NAADP$^+$ in an oocyte which had been transferred to Ca^{2+}-free seawater containing 2 mM EGTA. Additional details are given under "Materials and Methods."

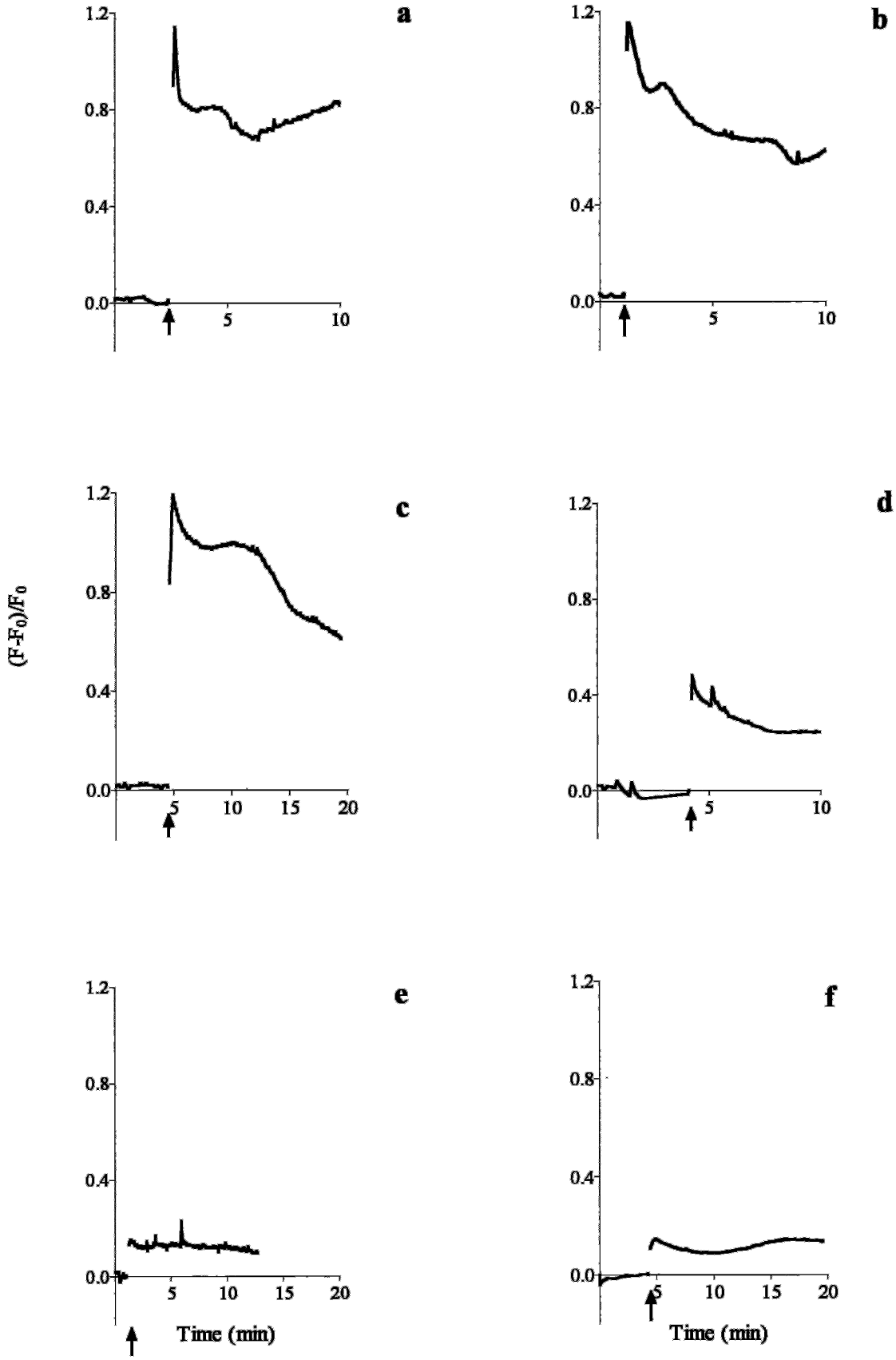

FIG. 2. **Ca^{2+} mobilization induced by the uncaging of $NAADP^+$ in mature oocytes.** *a*, Ca^{2+}-release triggered by the photolysis of caged $NAADP^+$ injected into the cytoplasm of a mature oocyte; *b*, uncaging of $NAADP^+$ in an oocyte injected with heparin; *c*, uncaging of injected $NAADP^+$ 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 μM 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."

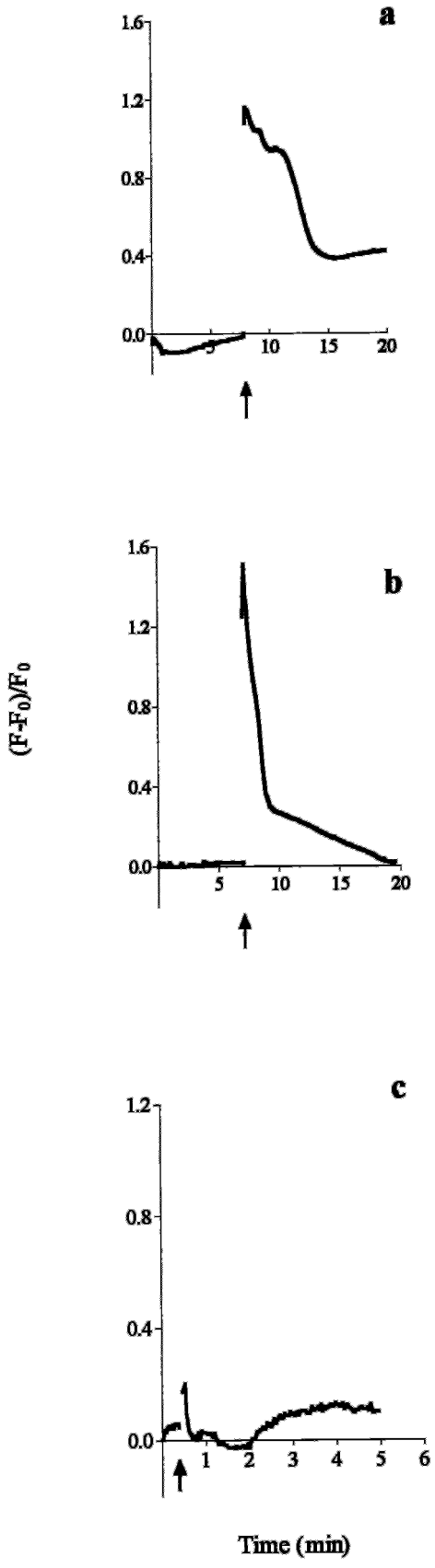

FIG. 3. **Ca^{2+} release elicited by the uncaging of injected Ins-P_3.** *a*, photolysis of injected caged Ins-P_3 in a mature oocyte; *b*, Ca^{2+} transient induced by the uncaging of injected Ins-P_3 in a mature oocyte transferred to Ca^{2+}-free seawater containing 2 mM EGTA; *c*, Ca^{2+} release induced by the uncaging of Ins-P_3 in a mature oocyte injected with heparin. Additional details are given under "Materials and Methods."

TABLE I

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 $F-F_0/F_0$ (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. 2*a* 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 base-line levels.

The effects of the inhibition of the Ins-P_3 and cADPr-modulated channels were explored also on mature oocytes. Microinjection 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, *b* 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. 3*c*; 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. 2*d*)

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. 2*e*). 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. 2*f*). 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

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. 3*a*) 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. 2*a*) 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. 3*b*), 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 injection of both heparin and 8-NH_2-cADPr, supporting the interplay between the routes activated by Ins-P_3/cADPr and that promoted by $NAADP^+$. This is consistent with a recent report on pancreatic acinar cells, where the response elicited by $NAADP^+$ could be blocked by the co-addition of heparin and 8-NH_2-cADPr (25). These two observations could be rationalized as follows: cytosolic increases in $NAADP^+$ induced by extracellular stimuli lead to localized increases in Ca^{2+} that would trigger the activation of Ins-P_3 and ryanodine receptors in the presence of sufficient basal cytosolic amounts of cADPr and Ins-P_3. Physiological support for this model has come from recent experiments in our laboratory: additions of sperm to mature starfish oocytes injected with heparin and 8-NH_2-cADPr still produced small, sub-plasma membrane Ca^{2+} spots, which however, failed to spread to the entire cytoplasm (26). Similarly, a small Ca^{2+} mobilization was observed in sea urchin eggs challenged with sperm after injection of heparin and 8-NH_2-cADPr (1). We propose that in the experiments described here, the uncaging of $NAADP^+$ would produce an initial liberation of Ca^{2+}. This would trigger the opening of the Ins-P_3/cADPr channels, in turn contributing to the initial spike and, especially, to the long lasting elevation of all Ca^{2+} that follows it, including the abortive oscillation. Ca^{2+}-liberated by $NAADP^+$ may thus act as a trigger that activates the channels/pool operated by the other two ligands. A functional interplay between Ins-P_3 and $NAADP^+$ receptors, which supports the proposal, has also been observed in ascidian eggs (11).

Interestingly, the properties of the Ca^{2+} pool mobilized by $NAADP^+$ changed during the maturation process: removal of external Ca^{2+} in mature oocytes inhibited very substantially the Ca^{2+} change induced by the photolysis of caged $NAADP^+$, whereas it affected only marginally the Ca^{2+} response of the immature oocyte. It has already been suggested that the intracellular membranes containing the Ca^{2+} released by $NAADP^+$ in sea-urchin eggs are different from those containing the Ca^{2+} released by cADPr and Ins-P_3 (*i.e.* the endoplasmic reticulum) (4, 7). Evidently, during the maturation process the source of Ca^{2+} mobilized by $NAADP^+$ changed, in part or even completely, from internal stores to the extracellular compartments. This interesting observation may be important to the process of fertilization because the latter is preceded by a number of changes involving the plasma membrane (*i.e.* ionic conductance changes, block to polyspermy, and the elevation of fertilization envelope) (26–28). The finding that the Ca^{2+} response induced by $NAADP^+$ in mature oocytes was affected by external Ca^{2+} adds weight to the suggestion that $NAADP^+$-sensitive Ca^{2+}channels could be the trigger for the initiation of the Ca^{2+} waves at fertilization, which is known to depend upon the influx of calcium ions (29)

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