

NAADP triggers the fertilization potential in starfish oocytes

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Abstract

In invertebrates oocytes or eggs, the fertilization or activation potential establishes the fast electrical block to polyspermy and, in some species, provides the Ca^{2+} influx which contributes to the following intracellular Ca^{2+} wave. In echinoderms, the molecule triggering the activation potential is still unknown. The aim of this study was to assess whether nicotinic acid-adenine dinucleotide phosphate (NAADP) elicited the fertilization potential in starfish oocytes. The changes in membrane potential induced by the sperm were measured in oocytes held at a low resting potential, so that the Ca^{2+} -action potential was inactivated and only the initial slower depolarization caused by the sperm could be studied. Decreasing extracellular Na^+ concentration did not prevent the onset of the fertilization potential, while removal of external Ca^{2+} abolished it. The pre-incubation with SK&F 96365 and verapamil and the pre-injection of BAPTA inhibited the fertilization potential, while the injection of heparin only reduced its duration. The biophysical and pharmacological properties of the sperm-elicited depolarization were similar to those displayed by the NAADP-activated Ca^{2+} -mediated current recently described in starfish oocytes. Indeed, the desensitization of NAADP-receptors prevented the onset of the fertilization potential. Taken together, these data suggest that NAADP could trigger the fertilization potential in starfish oocytes.

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1. Introduction

At fertilization, both oocytes and eggs undergo several ionic responses, including a change in the membrane potential (the so-called “fertilization” or “activation potential”) [1], an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [2,3], and a raise in intracellular pH [4]. In invertebrates and amphibians, the activation potential consists in a sperm-induced depolarization, that may be amplified by an action potential driven by voltage-gated Ca^{2+} channels [5–8] and which provides the fast electrical block to polyspermy [9]. Indeed, in the species where such a mechanism is missing, such as mammals [10] and fish [11], fertilization triggers a hyperpolarizing response [12,13]. The shift in membrane potential is accompanied by an increase in $[\text{Ca}^{2+}]_i$, which takes the form of a single Ca^{2+} transient or repetitive Ca^{2+} oscillations, depending on the species [2,3]. For instance, in echinoderms and frog, a single Ca^{2+} wave propagates across the oocyte upon sperm attachment

[14–16], while, in mammals and ascidians, fertilization triggers a series of periodic Ca^{2+} spikes [17,18]. The rise in $[\text{Ca}^{2+}]_i$ is required to activate the oocytes by restarting the cell cycle and promoting the exocytosis of cortical granules, which establishes the slow block to polyspermy [2,3]. The intracellular Ca^{2+} response is mainly sustained by Ca^{2+} release from the inositol 1,4,5-trisphosphate (InsP_3) receptors (InsP_3Rs) of the endoplasmic reticulum (ER) [2,3]. However, the contribution of nicotinic acid-adenine dinucleotide phosphate (NAADP)- and ryanodine (RyRs)-sensitive stores has been recently pointed out [16,19–21]. In echinoderms, such as sea urchin and starfish, the Ca^{2+} wave is usually preceded by a transient ring of brightness at the oocyte surface, the so-called “cortical flash” [14,22], which is due to Ca^{2+} influx during the action potential that accompanies the sperm-induced depolarization [5,6,8], although in the starfish *Astropecten auranciacus* the “cortical ring” may follow the Ca^{2+} sweep [23]. Therefore, beside providing the fast block to polyspermy, the fertilization potential mediates an influx of Ca^{2+} which may participate to the intracellular Ca^{2+} signal. In addition, in molluscs and echinoderms, the Ca^{2+} required for the onset of the Ca^{2+} wave at fertilization enters primarily through the voltage-gated Ca^{2+} channels

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activated during the activation potential [24,25]. The mechanism by which the sperm initiates the depolarization is, however, still uncertain. In sea urchin eggs, prior injection of NAADP to desensitize the NAADP-receptors abolished the fertilization-induced “cortical flash” [26]. In agreement with the role played by NAADP in the activation of sea urchin eggs, a dramatic increase in NAADP concentration occurs during fertilization, that is largely due to production in sperm upon contacting egg jelly [26,27].

Starfish oocytes undergo monospermic fertilization after a maturation process induced by the hormone 1-methyladenine (1-MA), which is revealed by the breakdown of the nucleus (germinal vesicle) and the subsequent intermixing of the nucleoplasm with the cytoplasm [28]. Both in immature and mature oocytes of the two species of starfish that we have studied, i.e. *Asterina pectinifera* and *A. aurantiacus*, a cortical Ca^{2+} flash is produced by NAADP following the influx of extracellular Ca^{2+} [16,23]. Indeed, the response to NAADP is absent or very low upon removal of external Ca^{2+} [29]. The main difference between the two species is that, in the former, the Ca^{2+} influx is propagated inside the cytosol by the activation of InsP_3Rs and RyRs through a mechanism of Ca^{2+} -induced Ca^{2+} release [16,29], while in the latter it remains localized in the cortex [23]. The NAADP-sensitive cortical ring is due to the activation of a Ca^{2+} -mediated membrane current [30]. Such a current is mainly brought by Ca^{2+} , but not Na^+ , is insensitive to the inhibition of InsP_3Rs and RyRs , but is strongly affected by the preincubation with thapsigargin and by the preinjection of the Ca^{2+} chelator, BAPTA [30]. These features suggest that NAADP triggers a highly localized, cortical Ca^{2+} increase from a thapsigargin-sensitive store that is tightly coupled to the NAADP-dependent membrane channels. The activation of the latter would, then, require both the cortical Ca^{2+} pulse and NAADP itself [30]. In a recent report, it has been suggested that the cortical flash observed at fertilization in mature oocytes is due to the activation of NAADP-receptors, while the InsP_3Rs would be responsible for the propagation of the following Ca^{2+} wave [16]. Such a model implies that NAADP could elicit the activation potential in starfish oocytes, but the electrophysiological evidence of this hypothesis is missing.

The aim of the present study was to investigate whether the Ca^{2+} current induced by NAADP was responsible for the fertilization potential in starfish oocytes from *A. aurantiacus* by impaling the cells with a single-electrode and measuring the changes in membrane potential induced by the sperm by employing both Ca^{2+} imaging and electrophysiological techniques, as shown elsewhere [30]. Our results provide the first evidence that the fertilization potential in starfish oocytes can be triggered by NAADP, thus suggesting that NAADP-induced Ca^{2+} influx is necessary to activate starfish oocytes at fertilization and indicating a further physiological role for this recently discovered second messenger.

2. Materials and methods

2.1. Preparation of oocytes

Starfish (*A. aurantiacus*) were collected during the breeding season in February–June in the gulf of Naples and kept in running natural sea water (16°C). Immature oocytes (containing the germinal vesicle, nucleus) were dissected from the ovaries and kept in artificial sea water (ASW) containing in mM: 500 NaCl, 8 KCl, 10 CaCl_2 , 12 MgCl_2 , 2.5 NaHCO_3 , pH 8.0 titrated with NaOH, washed several times with it, and kept for 40–50 min before use. Oocytes in which the breakdown of the germinal vesicle occurred spontaneously were discarded.

2.2. Microinjections and Ca^{2+} imaging

The calcium fluorescent dye Oregon Green 488 BAPTA-1 coupled to a 10 kDa dextran (OGBD, Molecular Probes, Eugene, OR, USA) was injected by pressure, using an Eppendorf Transjector 5246, at a concentration of 5 mg/ml in the injection buffer (IB; 450 mM potassium chloride, 10 mM HEPES, pH 7.4) into the cytoplasm of immature oocytes. As the volume of the injected substances corresponded to 1–2% of the oocyte volume, the final concentration of OGBD was 50–100 $\mu\text{g/ml}$. Cytosolic Ca^{2+} changes were measured by using a cooled CCD camera (MicroMax, Princeton Instruments Inc., Trenton, NJ) mounted on a Zeiss Axiovert 200 microscope with a Plan-Neofluar 20 \times /0.50 objective. Fluorescence images were processed with a MetaMorph Imaging System software (Universal Imaging Corporation, West Chester, PA). To exclude variations of fluorescence intensity, the signals were corrected for variations in dye concentration by normalizing the fluorescence against baseline fluorescence and the images were displayed in terms of the relative fluorescence $F_{\text{rel}} = [(f_t - f_0)/f_0]$, where f_t is the recorded fluorescence and f_0 is the resting fluorescence before sperm addition. All the experiments were carried out at room temperature (20–23 $^\circ\text{C}$).

2.3. Electrophysiological measurements

Most of the records were obtained with a single electrode which was inserted into an immature oocytes as, just after the germinal vesicle breakdown, the membrane is so elastic that a good impalement is difficult [6,31,32]. Therefore, 1-MA was applied to an impaled immature oocyte and inseminated 50–60 min later, when the germinal vesicle had disappeared. Nevertheless, to study the effect of heparin and BAPTA (Sigma, St. Louis, MO, USA) on the onset of the fertilization potential, the oocytes were impaled at the end of GVBD. The rate of successful impalements was, however, very low and obtaining the measurements from a significant number of cells (no less than five for each condition) required a lot of efforts. Heparin and BAPTA

were injected at the doses of 50 mg/ml and 200 mM, respectively, the final concentrations into the oocytes being 500 μ g/ml and 2 mM. In order to measure the membrane potential, micro-electrodes were pulled from borosilicate glass (filament type, 1.2 mm o.d.; Clark Electromedical, UK), filled with 450 mM KCl and had a resistance of 15–20 M Ω . Membrane potentials were recorded through a headstage connected to an Axoclamp-2B amplifier (Axon Instruments Inc., Union City, CA) in bridge mode [33]. For the measurement of the voltage-dependent currents, electrodes with a lower resistance (2–3 M Ω) were pulled, while the AxoClamp-2B amplifier was used in the discontinuous single-electrode voltage-clamp mode and operated at a sampling rate of 0.8–2 kHz after optimization for the RC characteristics of the cells [30,34]. The current–voltage (I – V) relationship was measured by applying voltage-steps of 1 s to potentials ranging between –100 to 30 mV with an increment of 1 s from an holding potential of –70 mV. Experiments were carried out at room temperature (20–23 °C). Pooled data are given as mean \pm S.E. and the significance of differences between the averages was evaluated by Student's t -test for paired or unpaired observations. $P < 0.05$ was considered significant. When reported, ΔV is the difference between the resting value of V_m and the value of the membrane potential measured at the threshold of activation or at the peak of the fertilization potential.

2.4. Insemination

The oocytes were inseminated by carefully dropping 10 μ l of sperm suspension near the cells. In most of the experiments, the resting potential of mature oocytes was depolarized and resulted in a slow electrophysiological response to the sperm which could favor polyspermy (see Section 3). As the probability of sperm entry depends on the sperm concentration [6], the sperm were diluted to a concentration (10⁵/ml) which was shown to prevent polyspermy. On the other hand, when resting V_m was more positive than –5 mV, the strong membrane depolarization could prevent sperm attachment [6]. Therefore, we carefully increased the sperm concentration until a monospermic response could be measured.

2.5. Solutions

The oocytes were always bathed in ASW, whose composition is given above. To investigate the Na⁺ permeability of the fertilization potential, 495 mM NaCl was replaced by an equimolar amount of choline chloride (Low-NaSW). For experiments in absence of external Ca²⁺, the oocytes were transferred to a solution containing in mM (CaFSW): 500 NaCl, 8 KCl, 12 MgCl₂, 2.5 NaHCO₃, 2 EGTA, pH 8.0 titrated with NaOH. All the chemicals were of analytical grade and obtained from Sigma Chemical Co.

3. Results

3.1. Fertilization-induced Ca²⁺ wave in starfish oocytes

The increase in [Ca²⁺]_i induced by the sperm has been recorded with a cooled CCD camera in oocytes pre-injected with OGBD. In 20 out of 37 oocytes, the first detectable Ca²⁺ signal was the cortical flash, which mirrored the activation of voltage-gated Ca²⁺ channels [22,35] and occurred 35.6 \pm 6.9 s ($n = 17$) after sperm addition to the bath (Fig. 1A and B). In these cells, a point-source wave of Ca²⁺ started 2.3 \pm 0.2 s ($n = 20$) after the cortical flash (Fig. 1A and B) from the site of sperm interaction and travelled to the antipode of the oocyte within 1–2 min, reaching a peak amplitude of 0.81 \pm 0.02 ($n = 16$) (Fig. 1C and D). The duration of the Ca²⁺ elevation was of about 12 min (Fig. 1C), although in several oocytes the Ca²⁺ signal did not completely decay to the baseline during the period under observation (up to 20 min; data not shown). In 11 oocytes, the Ca²⁺ wave preceded the cortical flash by 3.3 \pm 0.5 s ($n = 11$; not shown) and initiated 46.0 \pm 9.3 s after sperm addition ($n = 13$; not shown), a value which is not significantly different from the latency of the signal starting with the cortical flash ($P = 0.37$). Also the height and the kinetics of the Ca²⁺ response were similar to those displayed by the oocytes where the Ca²⁺ sweep followed the Ca²⁺ ring (not shown). Although unusual, such a pattern of increase in [Ca²⁺]_i has already been observed in oocytes from *A. aurantiacus* [23]. Finally, no cortical flash was detectable in the remaining six cells, although both the peak fluorescence and the time course of the Ca²⁺ wave did not differ from those of the oocytes which displayed the cortical flash (not shown). In all the oocytes, the fertilization membrane appeared approximately 2–5 min after the onset of the wave and was fully elevated after 12–15 min (Fig. 1A).

3.2. Changes in the voltage-dependent K⁺ and Ca²⁺ currents during hormone-induced maturation of starfish oocytes

The fertilization potential was measured by impaling immature oocytes with a single microelectrode and recording the electrophysiological response to the sperm 50–60 min later, when the germinal vesicle had disappeared. The mean membrane potential (V_m) of immature oocytes was –69.4 \pm 0.9 mV ($n = 50$), which is not different from the value reported in a previous paper [30], whereas the specific membrane resistance was equal to 0.545 \pm 0.025 M Ω cm^{–2} ($n = 5$). The I – V relationship of the immature oocytes was measured by applying a step-like protocol from –100 to +30 mV to cells held at –70 mV. The resting I – V showed a clear inward rectification at potentials lower than –70 mV, a transient inward Ca²⁺ current between –50 and –20 mV, and a transient outward K⁺ current at positive potentials (Fig. 2A and C). All of these currents have been extensively studied in starfish oocytes and we did not proceed in their

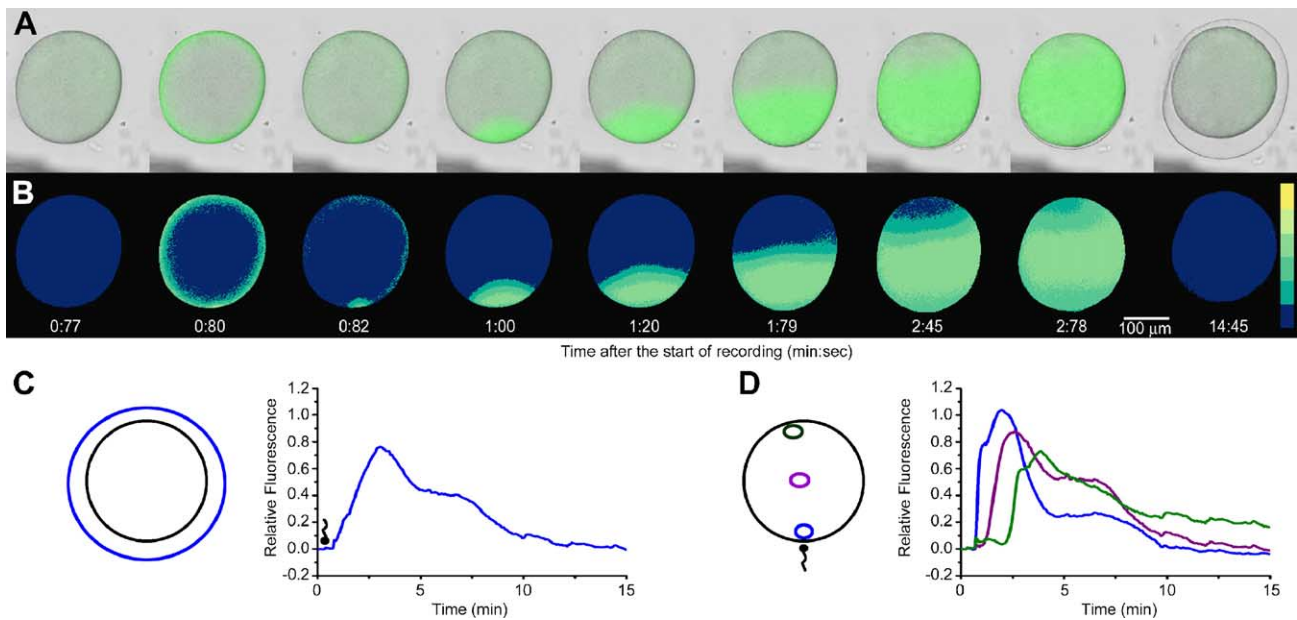


Fig. 1. Ca^{2+} wave observed at fertilization in starfish oocytes. Sequence of the fluorescence images showing the onset and the propagation of the Ca^{2+} sweep induced by the sperm in starfish oocytes (A and B). In order to better illustrate the spreading of the Ca^{2+} signal from the point of sperm–oocyte contact to the antipode of the cell, in Panel A, the relative fluorescent images have been overlapped with the corresponding light transmitted images. The fertilization membrane is first visible 2.45 min after the start of the recording and fully elevated after 14.45 min. (C) Graph of the relative fluorescence of the Ca^{2+} indicator, measured from a region of interest (ROI) around the oocyte (as indicated by the blue circle drawn around the oocyte), which offers a numerical equivalent of the pseudocolors shown in Panel B as function of the time. (D) The ROIs were positioned inside the oocyte to graphically illustrate the pattern of propagation of the Ca^{2+} wave: in accordance with the relative fluorescent images in Panels A and B, the Ca^{2+} sweep propagated from the entry point of the sperm (blue trace) to the centre of the oocyte (purple trace) and the antipode (dark green trace). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

further characterization [31,36–40]. The addition of 1-MA (10 μM) resulted in a decrease in the amplitude of both the K^+ currents and in an increase in the peak of the Ca^{2+} current. This feature is clearly shown in Fig. 2B and C and has been also shown in oocytes from *A. pectinifera* [31] and *Lep-tasterias hexactis* [37]. In agreement with the data reported in the latter studies, the disappearance of the inward rectifier K^+ current raised the specific resistance of the oocytes to $25.4 \pm 8.1 \text{ M}\Omega \text{ cm}^{-2}$ ($n = 5$). It has been shown that, as a consequence of the increase in membrane resistance, any slight change in the inward current during maturation could result in a large potential change [6,31,36]. Indeed, both the leakage current due to electrode penetration and the increase in Na^+ permeability occurring upon 1-MA addition [41] may shift the resting potential of mature oocytes to more depolarized value than those measured at the immature stage [6,31,36]. A similar jump in the membrane potential of the oocytes from *A. aurantiacus* following 1-MA addition is shown in Fig. 2D. A slow depolarization started 10–20 min after 1-MA addition and culminated in one or more spikes due to the activation of the voltage-gated Ca^{2+} channels, which brought V_m to the average value of $-13.8 \pm 1.9 \text{ mV}$ ($n = 49$; ranging from -40 to $+3 \text{ mV}$). In 12 out of 30 oocytes, replacement of external Na^+ with an equimolar amount of choline caused a recovery of V_m to $-70.1 \pm 0.8 \text{ mV}$, suggesting that an increase in Na^+ permeability could occur during maturation and depolarize the mem-

brane in a fraction of cells. In the other 18 oocytes, removal of external Na^+ shifted the resting potential of mature cells to $-27.5 \pm 3.6 \text{ mV}$, which is significantly more negative than the value of V_m measured in ASW ($-11.4 \pm 2.4 \text{ mV}$; $P = 0.002$), but more depolarized than V_m recorded at the immature stage ($-69.5 \pm 2.1 \text{ mV}$; $P = 0.0000081$). It is, therefore, conceivable that the leakage current due to electrode penetration may contribute to the jump in membrane potential measured upon 1-MA addition in the majority of the oocytes, as suggested by Miyazaki et al. [6,31].

3.3. Ca^{2+} entry is responsible for the fertilization potential in starfish oocytes

In starfish oocytes [6], the activation potential lacks the Ca^{2+} -action potential when the resting V_m is too small, due to the inactivation of voltage-gated Ca^{2+} channels [42]. The slow depolarization observed under these conditions represents the first step of the response to the sperm [5,6], which leads to the threshold of activation of the regenerative process when V_m is maintained at more negative values. The initial depolarization is evidently mediated by a sperm–oocyte interaction [43] and provides a valuable tool to assess the trigger of the activation potential in starfish without any contamination by the voltage-sensitive action potential. Therefore, we focussed our attention on the shift in V_m which occurred at fertilization in depolarized oocytes

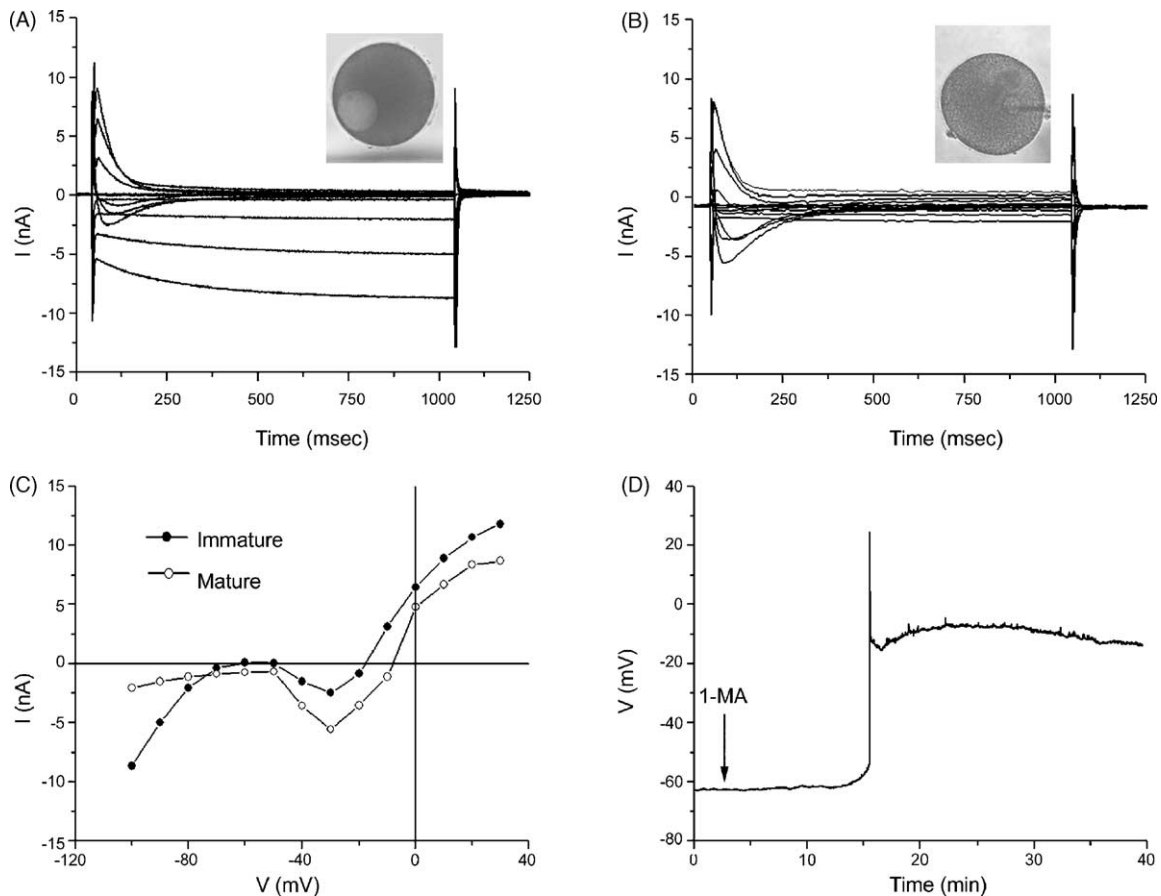


Fig. 2. Effect of 1-MA on the voltage-dependent currents in starfish oocytes. Ionic currents recorded from an immature oocyte not treated with 1-MA (A) and 45 min after (B) the addition of 1-MA ($10 \mu\text{M}$) by applying a voltage-step protocol from -100 to $+30$ mV. The holding potential was -70 mV. Note the disappearance of the inward rectifier K^+ current (i.e., the current activated at potentials lower than -70 mV) in mature oocytes. (C) Current–voltage relation of the cell shown in Panels A (closed circles) and B (white circles). For potentials more negative than -70 mV, steady-state currents are plotted. For more positive potentials, peak currents are shown. The oocyte from which the voltage-dependent currents have been measured is shown in the light transmitted images in the inset of Panels A and B at the immature and mature stage, respectively. Note the lack of the germinal vesicle (i.e., the nucleus) after the completion of the maturation process (inset in Panel B). (D) jump in the resting membrane potential of a different oocyte after the addition of 1-MA ($10 \mu\text{M}$) (see text for further explanations).

and tested the hypothesis that NAADP could be the trigger of the activation potential [16].

The addition of a drop of sperm suspension to the bath depolarized the membrane within 10–90 sec, the mean delay being 41.7 ± 8.3 s ($n = 9$). In accordance with the report by Miyazaki and Hirai [6], the switch in V_m was not accompanied by an action potential and initiated with a slow foot, reminiscent of the “pacemaker potential” of excitable cells, until a threshold of 3.6 ± 2.9 mV ($n = 8$; $\Delta V = 3.2 \pm 0.8$ mV, $n = 8$) was reached (Fig. 3A). The fertilization potential, then, raised to a peak of 17.16 ± 2.33 mV ($n = 8$; $\Delta V = 14.0 \pm 3.3$ mV) with a double exponential kinetics, the values of the fast and the slow time constants being 15.3 ± 2.4 s ($n = 6$) and 4.5 ± 0.5 s ($n = 6$), respectively (Fig. 3A). The membrane potential returned to the baseline with a time course variable from cell to cell and ranging from 10 to 30 min. Several oocytes, however, did not repolarize completely during recordings lasting up to 50 min. This failure may have been due to electrode damage during the

elevation of the fertilization membrane and has already been reported in oocytes from *Mediasteris aequalis* [32]. When the membrane potential of mature oocytes was shifted to negative values (-60 to -70 mV) by injecting a steady-state negative current (around 0.2 nA), the initial depolarization triggered a Ca^{2+} -action potential, which reached a peak of 14.9 ± 4.8 mV ($n = 4$; Fig. 3B). The regenerative process was followed by a slower depolarization which attained an amplitude of 17.9 ± 5.5 mV ($n = 4$) before decaying to the baseline within 30–50 min (not shown). This response is similar to that recorded in *A. pectinifera* maintained at -70 mV by applying a negative dc-current and may be considered as the activation potential triggered by the mature oocytes when they are near to their physiological V_m [6].

In order to investigate the ionic basis of the initial response of the oocytes to the sperm, extracellular Na^+ was first replaced by an equimolar amount of choline. In the cells which were only slightly repolarized by the reduction of extracellular Na^+ , the activation potential did not differ from that

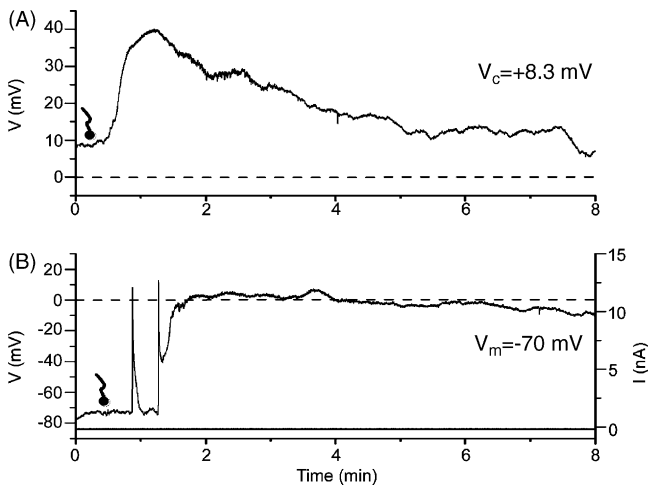


Fig. 3. Effect of the resting V_m on the fertilization potential in starfish oocytes. (A) Fertilization potential triggered in mature oocytes after the depolarization which occurred during the maturation process. The resting V_m of this cell was $+8.6$ mV. The electrophysiological response to the sperm in an oocyte with a low membrane potential lacks the Ca^{2+} -action potential. (B) Fertilization potential recorded in a mature oocyte whose V_m (-2.3 mV) was shifted to ≈ -70 mV by applying a steady-state negative (-0.17 nA) current. In this cell, which is held at a V_m where the voltage-gated Ca^{2+} channels are not inactivated, the electrophysiological response displays the Ca^{2+} -dependent regenerative process. The activation potential is preceded by a transient depolarization, caused by the contact of the oocyte with a spermatozoon which was not able to fertilise the cell. The sperm were added to the bath at the time indicated by the symbol.

measured in ASW in oocytes from the same batch (Fig. 4A and B). Similarly, in the cells whose membrane potential recovered to the value measured at the immature stage upon replacement of Na^+ , the fertilization potential resembled that measured in ASW from oocytes held at $-60/-70$ mV. Indeed, the “pacemaker potential” raised until a threshold of -65.2 ± 1.6 mV ($n = 6$) and was followed by the rapid (291.2 ± 47.2 mV/s, $n = 5$) upstroke of an action potential, which attained a peak of 21.9 ± 4.1 mV ($n = 6$) and decayed to a “well” at -4.5 ± 2.3 mV ($n = 6$). The membrane potential, then, depolarized again, reaching a plateau level of -4.8 ± 2.7 mV ($n = 6$), which was maintained for approximately 60 min before V_m decayed to the pre-stimulation level (Fig. 4C). No difference was observed in the fertilization envelope in Low-NaSW (not shown). It is worth noting that the percentage of polyspermic oocytes significantly increased from 0/24 cells in ASW to 6/12 oocytes when extracellular Na^+ concentration was reduced. The polyspermy was indicated by the occurrence of two to three successive spikes at the beginning of the activation potential (Fig. 4D), whereas each spike was produced by the attachment of a single sperm to the membrane [6,44]. The lack of a significant difference in the electrophysiological response to the sperm measured in Low-NaSW suggested the involvement of Ca^{2+} entry in the sperm-evoked depolarization. The extracellular Ca^{2+} concentration could not be reduced, as starfish sperm do not move in CaFSW. Therefore, Ca^{2+} was removed from

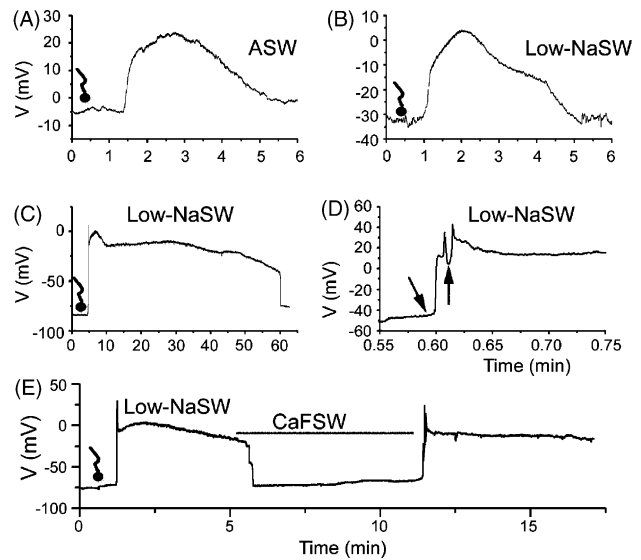


Fig. 4. Ca^{2+} entry is responsible for the onset of the fertilization potential. Electrophysiological response to the sperm measured in ASW (A) and in Low-NaSW (B–E) in oocytes from the same batch. Note that the reduction of external Na^+ repolarized the oocyte to the pre-hormonal level in Panels C–E, but not in Panel B. In Panel E, extracellular Ca^{2+} was removed from the bath during the period indicated by the horizontal bar. The activation potential occurred when extracellular Na^+ was replaced by an equimolar amount of choline, while it was reversibly abolished by removal of external Ca^{2+} . The sperm were added to the bath at the time indicated by the symbol. The high incidence of polyspermy in Low-NaSW is illustrated in Panel D: the onset of each spike activated by the arrival of a new spermatozoon is indicated by the black arrow.

the bath after the peak of the fertilization potential triggered in Low-NaSW in oocytes which completely repolarized upon reduction of Na^+ . Indeed, under these conditions, the long duration of the plateau allows to carefully evaluate the effect of the removal of extracellular Ca^{2+} on the activation potential. As shown in Fig. 4E, the membrane depolarization was reversibly abolished in CaFSW. Taken together, these results demonstrate that Ca^{2+} influx is the main responsible for the onset of the fertilization potential in starfish oocytes.

3.4. SK&F 96365 and verapamil block the fertilization potential

The observation that the depolarization evoked by the sperm was essentially caused by Ca^{2+} influx rather than Na^+ influx suggested that it could be mediated by the NAADP-activated Ca^{2+} current [30]. The latter is strongly affected by SK&F 96365, an inhibitor of receptor-operated Ca^{2+} entry, and by the L-type Ca^{2+} channel blocker, verapamil [30]. The incubation of the oocytes for 20 min in SK&F 96365 ($10 \mu\text{M}$) or in verapamil ($100 \mu\text{M}$) prevented the onset of the fertilization potential in seven and five cells, respectively (Fig. 5). In two out of the five oocytes pre-treated with verapamil, the addition of the drug to the bath elicited a slow depolarization which brought V_m from a resting value of ≈ -10 mV to a plateau level of about 0 mV

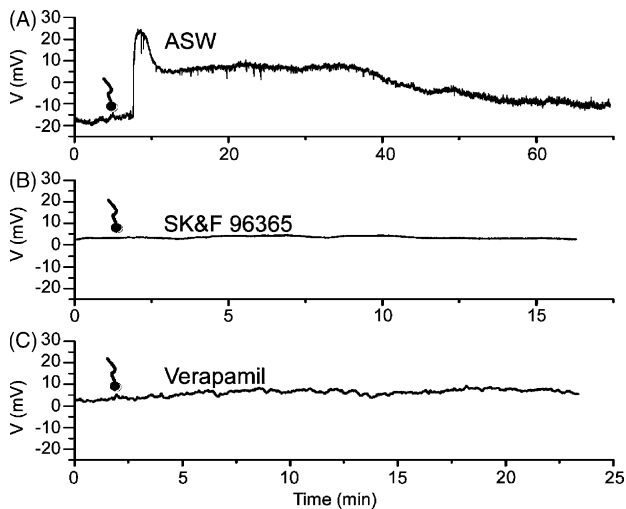


Fig. 5. Effect of SK&F 96365 and verapamil on the fertilization potential. SK&F 96365 (10 μ M; B) and verapamil (100 μ M; C) inhibit the activation potential in starfish oocytes. The cells were pre-incubated for 20 min with both the blockers before the addition of the sperm. In Panel A, a control response, obtained from an oocyte of the same batch, is shown. In each panel, the sperm were added to the bath at the time indicated by the symbol.

(data not shown). When the sperm suspension was added during the plateau of verapamil-induced depolarization, no fertilization potential occurred. As discussed in a previous paper [30], the concentration of verapamil necessary to block the depolarization elicited by the sperm was rather high. However, this dose was in the range of that required to abolish the response to NAADP in homogenates from sea urchin eggs [45] and heart microsomes [46] and in starfish oocytes [30].

3.5. Role of intracellular Ca^{2+} in the onset of the fertilization potential

In order to investigate the role of intracellular Ca^{2+} in the generation of the fertilization potential, mature oocytes were pre-injected with the Ca^{2+} chelator, BAPTA (2 mM), and exposed to the sperm after 10 min. As explained in the Section 2, in this set of experiments, the oocytes were injected with BAPTA and impaled with the recording electrode after the GVBD. The resting V_m measured in control oocytes was equal to -14.2 ± 3.2 mV ($n = 20$), which is not significantly ($P = 0.91$) different from the V_m measured at the end of the maturation process in the oocytes impaled before the exposition to 1-MA. As shown in Fig. 6B, buffering of cytosolic Ca^{2+} with BAPTA prevented the occurrence of the electrophysiological response in four out of four oocytes (see Fig. 6A for a control response recorded in an oocyte from the same batch). The Ca^{2+} wave at fertilization is affected by heparin [47], an inhibitor of $InsP_3Rs$, but not by 8-NH₂-cADPr [23], which prevents the cADPR-dependent Ca^{2+} release from RyRs. However, the pre-injection of heparin (500 μ g/ml) did not affect the onset of the activation

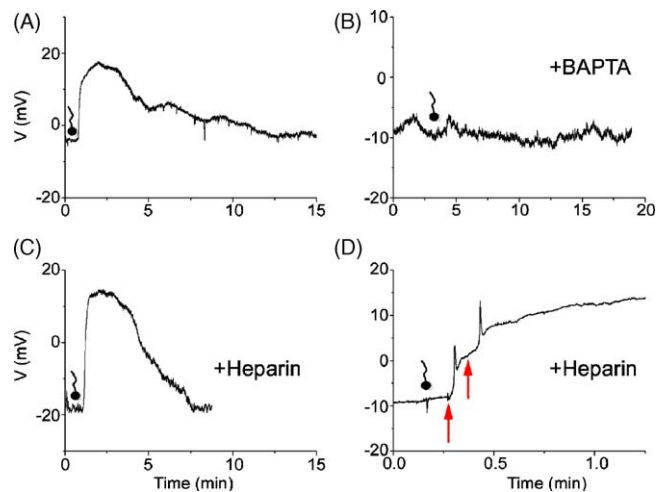


Fig. 6. The injection of BAPTA, but not of heparin, inhibits the onset of the fertilization potential. The pre-injection of BAPTA (2 mM; B) but not of heparin (500 μ g/ml; C and D) abolishes the electrophysiological response to the sperm. Note that the duration of the activation potential in oocytes injected with heparin (B) was shorter than that recorded in control oocytes (A). In Panel D, an oocyte injected with heparin and displaying a polyspermic response is shown. The fertilization potentials triggered by the arrival of a new sperm are illustrated by the red arrow. The sperm were added to the bath at the time indicated by the arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

potential in six out of seven oocytes (Fig. 6C), while the remaining one was polyspermic (Fig. 6D). Nevertheless, the time required by heparin-treated oocytes to repolarize appeared to be longer than that measured in control oocytes (Fig. 6C: compare to the control response displayed in Fig. 6A). The lack of a large intracellular Ca^{2+} mobilization caused an irregular elevation of the fertilization envelope (not shown). It is worth noting that the concentration of heparin we employed has previously been shown to fully block the response to $InsP_3$ in starfish oocytes [29]. The effect of intracellular Ca^{2+} stores depletion on sperm-induced depolarization could not be studied for two reasons. First, the oocytes of *A. aurantiacus* are not responsive to any of the known inhibitors of the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump. Second, in oocytes extracted from species of starfish responsive to thapsigargin (such as *A. pectinifera*), store depletion results in a rise in $[Ca^{2+}]_i$ which causes the elevation of the fertilization membrane (unpublished data from our lab), thus preventing the fertilization process. The sensitivity of the fertilization potential to BAPTA, but not to heparin, is a further indication of the involvement of NAADP in the electrophysiological response to the sperm [30].

3.6. The desensitization of NAADP-receptors may abolish the fertilization potential

In 4 out of 10 oocytes, the injection of NAADP (1 μ M final concentration) induced a membrane depolarization with

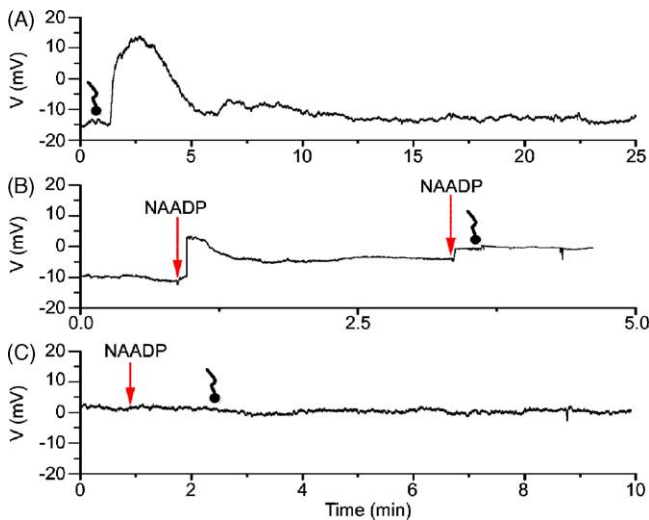


Fig. 7. The desensitization of NAADP-receptors prevents the onset of the fertilization potential. (A) Fertilization potential recorded in a control oocyte from the same batch of cells shown in the following panels. (B) The injection of NAADP (1 μ M; indicated by the red arrow) caused a membrane depolarization, whose amplitude was strongly reduced upon a subsequent injection. The following addition of sperm to the bath did not elicit the activation potential. (C) In an oocyte which did not respond to NAADP (1 μ M), the onset of the fertilization potential was also prevented. The sperm were added to the bath at the time indicated by the arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

a latency of 7.4 ± 5.1 s ($n = 4$), which was significantly faster than the delay of the fertilization potential (Fig. 7B). The electrophysiological response to NAADP exhibited a “pacemaker potential” which attained a threshold of -5.4 ± 1.6 mV ($n = 4$; $\Delta V = 5.1 \pm 2.5$ mV, $n = 4$) before giving rise to a rapid shift in V_m which reached a peak of 14.2 ± 6.2 mV ($n = 4$; $\Delta V = 18.4 \pm 4.7$ mV, $n = 4$) with a slope of 0.030 ± 0.011 mV/ms ($n = 4$). The depolarization induced by NAADP underwent a desensitization process [27,48–50], whereas its amplitude decreased upon repetitive injections of the second messenger (Fig. 7B). Both in the four responsive oocytes (Fig. 7B) and in four out of the six cells which were not depolarized by NAADP (Fig. 7C), the subsequent addition of the sperm did not elicit any fertilization potential. This result further suggests the involvement of a NAADP-sensitive conductance in the onset of the activation potential in starfish oocytes. The lack of a specific and powerful inhibitor of NAADP-receptors imposes a limitation: the desensitization experiments are the best tool to assess the role of NAADP in cell function [48–50]. The inhibition of the sperm-evoked depolarization in oocytes which did not show any detectable response to NAADP is not surprising, as the injection of NAADP could raise its concentration in proximity of the membrane to a level so high to cause the self-inactivation of NAADP-receptors [48,49] and, therefore, to abolish the following response to sperm. It must be observed that the conclusive assessment of the role of NAADP in the onset of the fertilization poten-

tial will, however, require the demonstration that NAADP levels increase in oocytes at fertilization (see Section 4).

4. Discussion

The results presented in this study provide the evidence that a conductance with biophysical and pharmacological properties similar to that activated by NAADP may give rise to the activation potential in starfish oocytes [30]. These data may, therefore, shed further light on the role that this recently discovered second messenger may play in cellular physiology [51].

The following observations suggested that the initial depolarization induced by the sperm was underlain by the NAADP-elicited Ca^{2+} current that we have recently described [30]: (a) the positive shift in V_m at fertilization was mainly due to Ca^{2+} , not Na^+ , influx and was inhibited by SK&F 96365 and verapamil; and (b) the onset of the activation potential was abolished by the pre-injection of BAPTA, but not of heparin. In agreement with this hypothesis, the desensitization of the response to NAADP, a known property of NAADP-receptors [30,48–50], prevented the membrane depolarization upon sperm binding. Furthermore, both the fertilization potential and the NAADP-elicited depolarization started with a pattern reminiscent of the “pacemaker potential” of excitable cells [42]. The longer latency and the slower rise of the response to the sperm were probably due to the time required by the oocyte to synthesize NAADP or to receive it from the sperm (see below). As discussed in a previous study, the sensitivity to BAPTA suggests that, at first, NAADP induces a highly localized Ca^{2+} release from cortical stores that are tightly coupled to Ca^{2+} -permeable membrane channels [30]. Subsequently, both the Ca^{2+} mobilized from the stores and NAADP itself would activate the Ca^{2+} -permeable channel responsible for the electrophysiological response to the sperm [30]. A further indication that the onset of the activation potential does not involve the InsP_3 Rs comes from previous reports showing that the cortical flash at fertilization occurs even in starfish oocytes injected with the SH2 domain of phospholipase $\text{C}\gamma$ [22,35]. The apparent shorter duration of the fertilization potential in oocytes pre-injected with heparin, however, suggests that Ca^{2+} release from InsP_3 Rs may be involved in maintaining the depolarization. Indeed, starfish oocytes express a Ca^{2+} -dependent cationic current [40,52], which could prolong the response to the sperm. In agreement with this hypothesis, in oocytes from *Mediaster aequalis* [32], the sperm may induce a non-selective increase in cation permeability.

In oocytes held at -60 to -70 mV by the injection of a dc-current, the activation potential displayed a Ca^{2+} -action potential [present study], which is responsible for the cortical flash which accompanies the Ca^{2+} wave [22,23]. The finding that NAADP triggers the fertilization potential, therefore, is the first experimental evidence that NAADP may play a pivotal role in the onset of the Ca^{2+} signal at

fertilization in starfish oocytes, as suggested in a previous report [16]. The long duration of the activation potential further suggests the involvement of Ca^{2+} entry in sustaining the intracellular Ca^{2+} sweep. In molluscs and echinurans, the Ca^{2+} response to the sperm consists in an initial cortical flash which spreads inwardly to the center and primarily depends on Ca^{2+} influx through the voltage-gated Ca^{2+} channels [24,25]. This pattern is clearly different from the point-source Ca^{2+} wave occurring in starfish oocytes, whose propagation certainly rely on InsP_3Rs [16,22], but highlights the role that extracellular Ca^{2+} may play at fertilization. The interaction between NAADP-receptors and InsP_3Rs in shaping the sperm-induced Ca^{2+} signal has also been found in ascidian oocytes [19] and sea urchin eggs [26,53]. In the latter, the desensitization of NAADP-receptors prevents the initial cortical Ca^{2+} flash and shortens the duration of the following Ca wave [26,53]. As pointed out above, in sea urchin, the fertilization potential consists in a depolarization which brings the membrane to the threshold of activation of the Ca^{2+} -action potential [5]. The nature of the stimulus causing the initial depolarization is still unknown [54]. As NAADP elicits the Ca^{2+} ring [26], it is conceivable that the NAADP-dependent current may be involved in the onset of the fertilization potential also in sea urchin eggs. The current immediately activated by the sperm–egg contact in sea urchin, however, reverts polarity at about +20 mV [55], a potential which is significantly less positive than +50 mV, the reversal potential of the NAADP-stimulated current [30].

The biophysical properties of the NAADP-activated current are quite different from those exhibited by the fertilization currents recorded in other invertebrates. Indeed, the activation potential is mediated by a non-selective cation current gated by ADP-ribose in ascidian oocytes (ADPr) [56] and by a Cl^- current activated by Ca^{2+} released from the InsP_3Rs in frog oocytes [7]. Furthermore, in molluscs and echinurans, where the Ca^{2+} influx during the activation potential sustains the intracellular Ca^{2+} wave [24,25], the initial depolarization is due to the opening of sperm-gated membrane channels which are mainly permeable to Na^+ [57,58]. The source of NAADP for the onset of the fertilization potential in starfish oocytes remains to be elucidated. NAADP could be synthesized by the oocytes after the binding of sperm or could be injected into the cells by the sperm itself. In accordance with the latter hypothesis, NAADP has been shown to increase in sperm upon contact with the jelly layer and to be, then, released into the eggs in sea urchin [26,59].

In conclusion, this report challenged for the first time the possibility that NAADP may be involved in the onset of the activation potential in starfish oocytes. The results shown indicate the initiation of the fertilization potential requires the activation of an ionic conductance with biophysical and pharmacological properties similar to that activated by NAADP in these cells [30]. In order to provide the clear-cut evidence that the fertilization potential is elicited by NAADP, however, it will be necessary to investigate whether it goes up in sperm and eggs at fertilization

[26]. In a previous study, it has been suggested that the initiation of the Ca^{2+} response to the sperm in starfish oocytes depends on the stimulation of NAADP-receptors, while the propagation of the Ca^{2+} wave throughout the cytoplasm relies on the InsP_3Rs [16]. The future aim of our work will be to assess this hypothesis by the simultaneous measurement of the changes in the membrane potential and in the Ca^{2+} signal at fertilization.

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