Pharmacological characterization of NAADP-induced Ca\textsuperscript{2+} signals in starfish oocytes

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Abstract

The recently discovered second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) is central to the onset of intracellular Ca\textsuperscript{2+} signals induced by several stimuli, including fertilization. The nature of the Ca\textsuperscript{2+} pool mobilized by NAADP is still controversial. Depending on the cell type, NAADP may target either an acidic compartment with lysosomal properties or ryanodine receptors (RyRs) on endoplasmic reticulum. In addition, NAADP elicits a robust Ca\textsuperscript{2+} influx into starfish oocytes by activating a Ca\textsuperscript{2+}-mediated current across the plasma membrane. In the present study, we employed the single-electrode intracellular recording technique to assess the involvement of either acidic organelles or RyRs in NAADP-elicited Ca\textsuperscript{2+} entry. We found that neither drugs which interfere with acidic compartments nor inhibitors of RyRs affected NAADP-induced depolarization. These data further support the hypothesis that a yet unidentified plasma membrane Ca\textsuperscript{2+} channel is the target of NAADP in starfish oocytes.

Keywords: Calcium signaling; NAADP; Ryanodine receptors; Lysosomes; Starfish oocytes

A large number of cellular responses are regulated by intracellular Ca\textsuperscript{2+} signals whose spatiotemporal properties may differ depending on the nature of the eliciting stimulus [1]. Nicotinic acid adenine dinucleotide phosphate (NAADP), the latest entry to the list of Ca\textsuperscript{2+}-regulating second messengers, has been suggested to play a pivotal role in the onset of Ca\textsuperscript{2+} signals [2]. Unlike inositol-1,4,5-trisphosphate (InsP\textsubscript{3}) receptors (InsP\textsubscript{3}Rs) and ryanodine receptors (RyRs), the NAADP-dependent Ca\textsuperscript{2+} release lacks Ca\textsuperscript{2+} feedback and does not behave as a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) system [3]. This feature renders NAADP an ideal messenger to provide a triggering Ca\textsuperscript{2+} signal which is then propagated via nearby InsP\textsubscript{3}Rs and RyRs by CICR [4]. An increase in intracellular level of NAADP has recently been reported in several cell types in response to stimuli as diverse as fertilization [5,6], hormones [7], and blood glucose [8]. Despite the mounting body of evidence on the involvement of NAADP in cell signaling, the intracellular target of NAADP remains controversial [9]. The NAADP-sensitive Ca\textsuperscript{2+} store is pharmacologically and physically distinct from the pools activated by InsP\textsubscript{3} and cyclic ADP-ribose (cADPr) [10], the putative agonist of RyRs [1]. Accordingly, it has been shown that NAADP receptors are located on lysosomal-related acidic organelles of sea urchin eggs [11], rat pancreatic acinar and β cells [7], rat arterial smooth muscle [12], and rat cortical neurons [13]. Moreover, evidence that NAADP receptors are present on acidic secretory vesicles has been reported in a basophilic cell line [14]. These observations do not concur with the finding that NAADP modulates the activity of RyRs on the endoplasmic reticulum [15,16]. Additional complexity has been added to the scenario by the suggestion that NAADP not only serves as an intracellular second messenger, but also has the potential to act as an autocrine/paracrine signal in some cell types [14]. Accordingly, extracellularly applied NAADP...
may induce rises in intracellular Ca\(^{2+}\) in a basophilic cell line [14] and in cortical astrocytes in rat brain slices [17].

In addition to mobilizing intracellular Ca\(^{2+}\), NAADP may stimulate Ca\(^{2+}\) entry from the extracellular space. In starfish oocytes, NAADP elicits a localized Ca\(^{2+}\) increase beneath the plasma membrane, the so-called cortical flash, upon the activation of a plasma membrane Ca\(^{2+}\) current [18,19]. The NAADP-induced current displays an inwardly rectifying I–V relationship, reverts at potentials more positive than +50 mV, and is mainly brought by Ca\(^{2+}\), but not Na\(^{+}\) [19]. In agreement with the electrophysiological data, Ca\(^{2+}\) imaging experiments revealed that no response to NAADP is discernible in the absence of extracellular Ca\(^{2+}\) in starfish oocytes. Moreover, the NAADP-induced current is reduced by verapamil and SK&F 96365, two known blockers of Ca\(^{2+}\)-permeable membrane channels, but is insensitive to the inhibition of InsP\(_3\)Rs and cADPr receptors [18,19]. All these data indicate that a plasma membrane Ca\(^{2+}\) channel mediates the increase in intracellular Ca\(^{2+}\) level induced by NAADP in starfish oocytes [9]. However, it has not yet been determined whether either a lysosomal-related acidic compartment or RyRs contribute to the bioelectrical response to NAADP.

In the present study, we employed the single-electrode intracellular recording technique to measure the positive shift in membrane potential induced by photolysis of caged NAADP in starfish oocytes. Our main goal was to assess the involvement of either a lysosomal-like acidic store or RyRs in NAADP signaling. We showed that neither drugs which interfere with Ca\(^{2+}\) storage in acidic organelles nor agents which block RyRs affected NAADP-evoked depolarization. These results provide a further evidence that NAADP stimulates starfish oocytes by a mechanism different from that described in other cell types, i.e., by activating a plasma membrane Ca\(^{2+}\) channel.

Materials and methods

Preparation of oocytes. Starfish (*Asterina pectinifera*) were collected during the breeding season in the Mutsu Bay (Aomori, Japan) and maintained in running natural sea water (16 °C). Fully grown immature oocytes were harvested from the ovaries and washed in filtered artificial sea water (ASW: 500 mM NaCl, 8 mM KCl, 10 mM CaCl\(_2\), 12 mM MgCl\(_2\), and 2.5 mM NaHCO\(_3\), pH 8.0) for 40–50 min before use. Maturation was promoted by adding 10 \(\mu\)M 1-methyladenine (1-MA) (Sigma Chemicals Co., St. Louis, MO), the hormone which induces the maturation process in starfish immature oocytes, to the recording chamber containing an immature oocyte impaled with a single microelectrode (see below).

Synthesis of caged NAADP. Caged NAADP was synthesized according to the protocols of Lee and Morgan [20,21]. Briefly, 2-nitroacetophenone hydrazone was converted to 1(2-nitrophenyl)diazoethane (NPE; 1.4 mmol) by stirring with 1 g MnO\(_2\) in chloroform for 10 min [20]. The resulting product was filtered and 4 ml was added to 10 mg NAADP in 4 ml H\(_2\)O, pH 1.3. This reaction mixture was stirred in the dark and the organic phase was removed and replaced with fresh NPE when the color had changed from orange to yellow. When the reaction was complete as

Fig. 1. NAADP depolarizes immature starfish oocytes. (A) Ca\(^{2+}\)-action potential elicited by a brief depolarizing current pulse in an immature starfish oocyte. (B) Membrane depolarization induced by the photoliberation of caged NAADP (100 \(\mu\)M in the injecting pipette) injected 10 min before the exposition to UV light. (C) Depolarizing response to photolysis of caged NAADP (100 \(\mu\)M in the injecting pipette) measured in another oocytes collected on the same day from the same animal. In this oocyte, it is evident the slow foot preceding the fast upstroke of the Ca\(^{2+}\)-action potential. Note that the first membrane depolarization is followed by six additional Ca\(^{2+}\)-action potentials. (D) No response was visible when NAADP was photoliberated in the absence of extracellular Ca\(^{2+}\). In each panel, the UV flash was given at the time indicated by the arrow. In (D) the UV flash lasted 7 min.
monitored by HPLC (Mono-Q eluted with TEAB) [20], the product was purified using the same HPLC protocol. The peak corresponding to the caged form was aliquoted, dried by evaporation, and stored as a solid at −80 °C until use. Exposure of the caged NAADP to UV light for 10 min led to the uncaging of >90% of the caged compound as assessed by HPLC.

Microinjections and photolysis of caged NAADP. Caged NAADP was injected into the cytoplasm of immature oocytes by pressure, using an Eppendorf Transjector 5246, at a concentration of 100 μM in the injection buffer (450 mM potassium chloride, 10 mM HEPES, pH 7.4). Ryanodine and ruthenium red were injected into immature oocytes at the concentrations of 2 mM and 100 μM in the injection buffer, respectively. As the volume of the injected substances corresponded to 1–2% of the oocyte volume, their final concentration inside the cells was estimated 50–100 times lower than in the micropipette.

After injection, the oocytes were transferred into a recording chamber constituted by the lid of a Petri dish, 60 × 10 mm, containing 2 ml of ASW and mounted on the mechanical stage of a Zeiss Axiovert 100 microscope. Caged NAADP was photolyzed by irradiating the oocyte with ultraviolet (UV) light (330 nm) emitted by a 75 W xenon lamp, passed through a 290–390 band-pass filter, and focused onto the oocyte through a Plan-Neofluar 20×/0.50 objective. The excitation filter-wheel was driven by a computer-controlled shutter (Lambda 10–2, Sutter Instruments, Co., Novato, CA, USA). The UV flash was extinguished shortly after the onset of membrane depolarization [22].

Electrophysiological measurements. Intracellular recordings were performed by impaling immature oocytes with sharp glass electrodes (filament type, 1.5 mm o.d.; Harvard Apparatus, UK), which were filled with 450 mM KCl and had a resistance of 40–50 MΩ. The microelectrodes were pulled by using a Sutter Instrument P-87 pipette puller. The impalement was obtained by applying short hyperpolarizing current pulses through the electrode until the membrane potential became steady at the negative value. Only oocytes whose resting membrane potential remained stable within ±1 mV at least 10 min after the impalement were exposed to UV light. A good impalement of mature oocytes was prevented by the high membrane elasticity at the end of the maturation process [23]. Unlike Astrepercen auranticus oocytes [19], when 1-MA was added to impaled oocytes, the electrode tended to come out of the cell after the germinal vesicle (i.e., the nucleus) breakdown, the most remarkable morphological change occurring during maturation. Therefore, we could record the bioelectrical response to NAADP only in a few mature oocytes.

The membrane potential was recorded through a headstage connected to an Axoclamp-2B amplifier (Axon Instruments Inc., Union City, CA, USA) in bridge mode [24]. The changes in membrane potential induced by uncaged NAADP were sampled at 1 kHz and digitized with a Digidata 1200B data acquisition system (Axon Instruments Inc.). The membrane input resistance was measured by injecting hyperpolarizing current pulses to induce steady-state voltage responses between 3 and 5 mV [24]. The oocytes were always bathed in ASW. For experiments in absence of external Ca^{2+}, the oocytes were transferred to a solution containing (CaFSW): 500 mM NaCl, 8 mM KCl, 12 mM MgCl₂, 2.5 mM NaHCO₃, and 2 mM EGTA. Experiments were carried out at room temperature (20–23 °C).

Statistics. Pooled data were given as means ± standard error (SE) and the significance of differences between the averages was evaluated by Student’s t test for unpaired observations. p < 0.05 was considered significant.

Reagents. All the chemicals were of analytical grade and obtained from Sigma Chemical Co.

Results

NAADP depolarizes starfish oocytes

Immature starfish oocytes were impaled with a single microelectrode 5–15 min after the injection of caged NAADP (100 μM in the injecting pipette) when the inactive messenger had spread to the entire cytoplasm. The sensitivity to NAADP varied from oocyte to oocyte during the breeding season, as previously reported [19]. Therefore, the duration of UV flash could be adjusted depending on appearance of the bioelectrical response (see Materials and methods) [22]. In 38 unstimulated oocytes bathed in ASW, the resting membrane potential and the input resistance averaged −70.5 ± 0.8 mV (n = 38) and 201.2 ± 27.1 MΩ (n = 8), respectively. Starfish oocytes are excitable cells since they are endowed with L-type-like voltage-gated Ca^{2+} channels [24,25]. Accordingly, a brief depolarizing current pulse induced a short (~5 s) lasting Ca^{2+}-action potential (Fig. 1A). The NAADP-dependent current is mainly mediated by extracellular Ca^{2+}, but not Na⁺ [19]. Therefore, it was not surprising that photolysis of caged NAADP depolarized the membrane within 0.2–150 s, the
mean latency being $45.1 \pm 7.2$ s ($n = 36$) (Fig. 1B). The depolarization consisted in an initial slow foot which raised until a threshold of $-62.7 \pm 0.7$ mV ($n = 33$) and was followed by the fast upstroke of a Ca$^{2+}$-action potential which attained a peak of $-11.5 \pm 1.4$ mV ($n = 38$). The action potential was due to the activation of L-type-like voltage-gated Ca$^{2+}$ channels upon NAADP-dependent depolarization. The rapid entry of Ca$^{2+}$ during the upstroke of the action potential, in turn, is responsible for the cortical Ca$^{2+}$ flash observed following photoliberation of NAADP in Ca$^{2+}$ imaging experiments [19,24]. After peak depolarization, the membrane potential decayed to a plateau level of $-23.4 \pm 1.2$ mV ($n = 35$) before rapidly returning to the prestimulation level. In 10 out of 38 oocytes, the first NAADP-induced membrane depolarization was followed by 1–10 additional transient Ca$^{2+}$-action potentials (Fig. 1C). Overall, the duration of the bioelectrical response to NAADP ranged from 3 to 57 min, the average value being 17.0 ± 2.5 min ($n = 36$). In agreement with our previous reports [18,19], removal of extracellular Ca$^{2+}$ abolished NAADP-induced depolarization in 5 out of 5 oocytes (Fig. 1D). As aforementioned, several oocytes bathed in ASW were exposed to a rather long (up to 160 s) UV flash before giving rise to the response. However, irradiation with UV light long up to 6 min did not depolarize either oocytes that had not been preinjected with NAADP (not shown) or oocytes injected with caged NAADP and bathed in CaFSW (Fig. 1D). No evident change in the membrane potential was evoked by the external application of uncaged NAADP (1 mM) in 6 out of 6 oocytes (data not shown), a result which concurs with previous Ca$^{2+}$ imaging experiments [10]. Therefore, NAADP is likely to act only as an intracellular second messenger in starfish oocytes.

The depolarizing response to NAADP was similar in mature oocytes. The resting membrane potential of mature oocytes ranged between $-53$ and $-5$ mV, while the mean input resistance was $601.5 \pm 77.9$ MΩ ($n = 8$), which is significantly higher than the value measured in immature oocytes ($p = 0.000256$). The increase in input resistance upon resumption of the cell cycle and the resulting scattering in the values of resting potential in mature oocytes have been described elsewhere [23,24,26]. Fig. 2 shows that photolysis of NAADP-induced a train of Ca$^{2+}$-action potentials both in immature (Fig. 2A) and mature (Fig. 2B) oocytes collected from the same animal on the same day. Although both the latency and the overall duration of the response were not significantly different between immature (21.6 ± 8.8 s, $n = 5$ and 4.4 ± 0.8 min, $n = 5$) and mature oocytes (23.0 ± 1.9 s, $n = 5$ and 6.5 ± 2.1 min, $n = 5$), in the latter the regenerative process overshot zero by 7.5 ± 2.3 mV ($n = 5$). This feature is due to the higher amplitude of the Ca$^{2+}$-action potential in mature oocytes.

Fig. 3. Inhibition of Ca$^{2+}$ storage in acidic compartments does not affect the response to NAADP. (A) Positive shift in the membrane potential elicited by NAADP (100 μM in the injecting pipette) photoactivated in a control oocyte bathed in ASW. The photolysis of caged NAADP (100 μM in the injecting pipette) evoked a normal depolarization in oocytes incubated in 100 μM GPN (B), 1 μM bafilomycin A1 (C), or 1 μM nigericin (D) [11]. The incubation with each inhibitor ranged from 30 min to 2 h. In each panel, the UV flash was given at the time indicated by the arrow.
In addition, due to their higher input resistance, the slow foot preceding the regenerative process was not evident in mature oocytes [28].

**NAADP does not target an intracellular acidic Ca\(^{2+}\) store in starfish oocytes**

Starfish oocytes are endowed with acidic yolk granules which are present within the cortex and represent the functional equivalent of lysosomes [29,30]. In order to assess whether a lysosomal-related acidic pool similar to that described in sea urchin eggs contributes to NAADP signaling in starfish oocytes, we have first disrupted lysosomes by incubating the cells with glycyll-L-phenylalanine 2-naphthylamide (GPN), a substrate of lysosomal cathepsin C whose cleavage results in osmotic lysis of the organelles [11]. A 30-min incubation with GPN (100 \(\mu\)M) did not impair the depolarizing response to NAADP in 6 out of 6 oocytes (Fig. 3B). Subsequently, we used inhibitors which are commonly employed to selectively abrogate Ca\(^{2+}\) storage in acidic compartments, such as bafilomycin A1 and nigericin. Bafilomycin A1 is a blocker of the vacuolar H\(^+\)-ATPase responsible for the proton gradient that drives the Ca\(^{2+}\) uptake into the lysosomes through a H\(^+\)/Ca\(^{2+}\) exchanger [31,32], while nigericin is a protonophore which abolishes the proton gradient itself [32]. In agreement with the result obtained with GPN, either bafilomycin A1 (1 \(\mu\)M) (Fig. 3 C) or nigericin (1 \(\mu\)M) (Fig. 3 D) did not affect NAADP-induced depolarization. The lack of effect of GPN, bafilomycin A1, and nigericin on the response to NAADP is summarized in Fig. 5. These findings indicate that the cortical target of NAADP in starfish oocytes is unlikely to consist in an acidic pool similar to that described in sea urchin eggs [11].

**NAADP does not stimulate RyRs in starfish oocytes**

In our previous work [10,18], we have shown that 8-NH\(_2\)-cADPr, an inhibitor of cADPr-sensitive Ca\(^{2+}\) release from RyRs [33], did not prevent the response to NAADP. However, we have never investigated the effect of the direct block of RyRs on NAADP-induced Ca\(^{2+}\) signals, despite the functional evidence that RyRs are largely present in the cortical region of starfish oocytes [33]. Therefore, we have measured NAADP-elicited membrane depolarization in control oocytes and in oocytes preinjected with ryanodine or ruthenium red, two known inhibitors of RyRs [15]. Accordingly, both agents dampened the Ca\(^{2+}\) oscillations induced in these cells by caffeine (5 mM), a pharmacological agonist of RyRs [24]. However, neither ryanodine (2 mM in the injecting pipette) (Fig. 4B) nor ruthenium red (200 \(\mu\)M in the injecting pipette) (Fig. 4C) altered the response to NAADP. The lack of effect of ryanodine and ruthenium red on NAADP-induced depolarization is summarized in Fig. 5. These results indicate that RyRs are not directly activated by NAADP in starfish oocytes.

**Discussion**

The pyridine nucleotide NAADP plays a pivotal role in the onset of Ca\(^{2+}\) signals triggered by a variety of extracellular stimuli in a wide range of cells and preparations across the phylogenic spectrum [5–8,12,34,35]. In addition, we recently showed that NAADP plays a key role during fertilization of starfish oocytes by triggering the membrane depolarization which leads to the cortical Ca\(^{2+}\) flash and the ensuing intracellular Ca\(^{2+}\) wave observed upon sperm binding [19,24,26]. What is not clear is the molecular nature of the NAADP-sensitive Ca\(^{2+}\) release mechanism and its sub-cellular localization. Two main models have been proposed to account for the different findings obtained in
different cell types [2,36]. The first model describes a NAADP-sensitive store located on acidic organelles with lysosomal properties, which is responsible for a localized Ca\(^{2+}\) pulse that is amplified by InsP\(_3\)Rs and RyRs on the ER by CICR [6,7,12–14]. The second model assumes that NAADP interacts either directly with RyRs on the ER or via a separate protein that may activate them [15,16]. In an attempt to reconcile this controversy, evidence has been provided that the acidic pool in the secretory region of pancreatic acinar cells contains NAADP-activated RyRs [37]. An alternative model has been put forward by the results obtained in starfish oocytes. Starfish oocytes, which represent a model system to investigate second messenger-induced Ca\(^{2+}\) signals [9,25,38], exhibit a robust Ca\(^{2+}\) influx upon either injection of uncaged NAADP [18] or photolysis of caged NAADP [10,18,19]. NAADP-induced Ca\(^{2+}\) entry, in turn, leads to a centripetally directed intracellular Ca\(^{2+}\) wave by recruiting both InsP\(_3\)Rs and cADPr receptors [10,18]. Removal of extracellular Ca\(^{2+}\), however, prevents the response to NAADP in both Ca\(^{2+}\) imaging and electrophysiological experiments [10,18]. Thus, Ca\(^{2+}\) influx across the plasma membrane plays a pivotal role in NAADP signaling in starfish oocytes. Although these observations indicate that NAADP stimulates starfish oocytes by activating a Ca\(^{2+}\) channel on the plasma membrane, the participation of either lysosomal-related structures or RyRs in the bioelectrical response to Ca\(^{2+}\) entry required to be assessed. Indeed, starfish oocytes are endowed with acidic reserve granules which mediate membrane repair following mechanical wounding [30], a feature which could provide an alternative explanation for NAADP-dependent Ca\(^{2+}\) influx [36]. Moreover, Ca\(^{2+}\) influx through plasma membrane RyRs has been reported in ventricular myocytes and osteoclasts [39,40]. We found that either maneuvers interfering with acidic organelles (GPN, bafilomycin A1, and nigericin) or drugs inhibiting RyRs (ryanodine and ruthenium red) did not significantly affect NAADP-induced membrane depolarization. It is noteworthy that, unlike the closely related sea urchin eggs [11], inhibition of Ca\(^{2+}\) storage into acidic compartments did not impair the response to NAADP in starfish oocytes. However, this result is not surprising when considering the different patterns of NAADP signaling in the two cell types. Accordingly, NAADP activates a modest Ca\(^{2+}\) influx in sea urchin eggs, which is followed by a substantial intracellular mobilization that does not disappear in the absence of extracellular Ca\(^{2+}\) [41]. On the other hand, the lack of effect of RyRs inhibitors on NAADP-induced depolarization is supported by our previous results. Indeed, cADPr-induced current disappears in the absence of extracellular Na\(^+\), but not Ca\(^{2+}\), and displays a rather linear I–V relationship which reverts at a less positive potential (\(\approx +20\) mV) [33]. In conclusion, NAADP activates starfish oocytes by stimulating a plasma membrane Ca\(^{2+}\) channel, which is
unrelated to either acidic lysosomal structures or RyRs. The NAADP-dependent channel presumably belongs to the transient receptor potential (TRP) superfamily of ion channels, which are responsible for the transmembrane flux of monovalent and divalent cations down their electrochemical gradient [42]. The gating and ion permeation properties of TRP channels depend on their assembly in either homo- or hetero-tetrameric [43]. Moreover, TRP properties of TRP channels depend on their assembly in complexes which are closely related molecules to NAADP, may acti-

of monovalent and divalent cations down their electro-

potential of 0 mV and remarkable Na + permeability)

cation gating mechanisms[44]. However, the biophysical properties of TRPM2 (linear I–V relationship, reversal potential of ≈0 mV and remarkable Na + permeability) make it unlikely that TRPM2 channel alone accounts for NAADP-induced current in starfish oocytes. Only the investigation of both the biophysical properties and Ca 2+ -sensitivity of this current will shed light on the member(s) of the TRP superfamily which mediate NAADP signaling in starfish oocytes.

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