Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP)-mediated Calcium Signaling

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Hon Cheung Lee
From the Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a metabolite of NADP that was first identified as the most potent Ca\textsuperscript{2+} stores mobilizing molecule in sea urchin egg homogenates more than a decade ago (1, 2). It has since been shown to be effective in a wide variety of cells, from plant to animal, including human (reviewed in Refs. 3–5). Its mechanism of action is distinct since been shown to be effective in a wide variety of cells, from plant to animal, molecule in sea urchin egg homogenates more than a decade ago (1, 2). It has been demonstrated (18). In lieu of a specific antagonist, this desensitization provides convincing evidence that the NAADP-sensitive mechanism is pres-

Structure and Ca\textsuperscript{2+} Release Activity of NAADP

NAADP is a simple derivative of NADP, with the only modification being the conversion of the amide of the nicotinamide group to a carboxyl group (Fig. 1A). The two compounds differ by only one mass unit and have identical group at the adenine ring and the 2'-phosphate are both critical to its biological activity (9) (circled in Fig. 1A). Attaching a caged group to the 2'-phosphate produces an inactive analog (10) that can regenerate NAADP and elicit large changes upon photolysis in many cells (e.g. sea urchin eggs, Fig. 1B), providing convincing evidence that the NAADP-sensitive mechanism is present and functional in live cells (8, 11). Removal of the 2'-phosphate by phosphatases or hydrolysis by nucleotide phosphodiesterase also inactivates its biological activity (10). The general presence of these enzymes in cells assures rapid removal of NAADP after its signaling function is completed.

That NAADP is effective in mobilizing Ca\textsuperscript{2+} stores was first demonstrated in sea urchin egg homogenates, a simple and stable system for readily accessing Ca\textsuperscript{2+} stores of the cells (1, 2). Results indicated that the extent of the Ca\textsuperscript{2+} release by NAADP was similar to those induced by cADPR or IP\textsubscript{3}, the two Ca\textsuperscript{2+} stores that are closely associated with the plasma membrane, or whether it is particularly intriguing is its regional distribution seen in the brain as revealed by autoradiography, which shows concentration of the receptor in the thalamus, hippocampus, midbrain, and the medullar region (23).

Ca\textsuperscript{2+} Stores Targeted by NAADP

Density gradient fractionation of sea urchin egg homogenates first showed that the NAADP-sensitive stores had a broader distribution than the endoplasmic reticulum (ER) stores sensitive to cADPR and IP\textsubscript{3}, and were particularly concentrated in the denser fractions, suggesting separate stores were targeted by NAADP (2, 24). Stratifying cellular organelles by centrifugation in live sea urchin eggs confirmed that the NAADP-sensitive stores (Fig. 1C) were separable and could be moved to a pole distal to the nucleus, where the ER and the cADPR- and IP\textsubscript{3}-sensitive stores (Fig. 1D and E) were located (25).

Marker enzyme analyses identified the NAADP-sensitive fractions in the sea urchin egg homogenates as lysosome-like organelles that possessed a thapsigargin-insensitive Ca\textsuperscript{2+} transport system distinct from that of the ER (26, 27). Instead of utilizing a Ca\textsuperscript{2+}-ATPase, this system employed a combination of a bafilomycin-sensitive V-H\textsuperscript{-}ATPase and a Ca\textsuperscript{2+}/H\textsuperscript{+}-exchanger for the sequestration of Ca\textsuperscript{2+} via a vesicular proton gradient (27). Thus treatments with bafilomycin as well as with ionophores that discharged the vesicular proton gradient depleted the stores and selectively inhibited the NAADP-dependent Ca\textsuperscript{2+} release. Likewise, lysosomotropic agents that disrupted lysosomes, such as glycylphenylalanine 2-naphthylamide (GPN), could induce Ca\textsuperscript{2+} increase in sea urchin eggs and selectively eliminated the NAADP-sensitive stores without affecting the ER Ca\textsuperscript{2+} stores that were sensitive to cADPR and IP\textsubscript{3} (27). Similar selective effects of lysosomotrophic agents on the NAADP-sensitive Ca\textsuperscript{2+} mobilization were seen also in mammalian cells, such as pancreas acinar and B-cells (MIN6) as well as in smooth muscle cells (29).

The NAADP mechanism, however, is not exclusively associated with lysosomes. In MIN6 cells, store-targeting expression of the Ca\textsuperscript{2+}-sensor protein, aequorin, identified insulin containing secretory granules as NAADP-sensitive Ca\textsuperscript{2+} stores as well (30). These granules, however, were also acidic organelles like lysosomes. Fractionation studies in cauliflower homogenates showed colocalization of the NAADP-sensitive stores with ER markers and not with the bafilomycin-sensitive ATPase, a vacuolar marker (16). The nuclear envelope had also been found to possess the NAADP mechanism, which is a functioning Ca\textsuperscript{2+} store possessing both Ca\textsuperscript{2+} release and thapsigargin-sensitive sequestration systems (31).

Additionally, NAADP has been observed to be capable of stimulating Ca\textsuperscript{2+} influx in both invertebrate eggs as well as in mammalian T-cells (32–34). Whether the influx is an indirect consequence of mobilization of the Ca\textsuperscript{2+} stores that are closely associated with the plasma membrane, or whether it is due to direct activation of membrane Ca\textsuperscript{2+} channels by NAADP, remain to be settled. It thus appears that the NAADP mechanism is quite widely distributed among cellular organelles.

**Messenger Functions of NAADP**

That NAADP is a naturally occurring molecule in cells was first shown in sea urchin sperm (35). The endogenous concentration measured was unexpectedly high in this terminally differentiated cell that lacks most of the normal organelles. This has led to the proposal that the sperm may serve as vehicles for delivery of NAADP to the egg at fertilization. Consistently, contact of the sperm with the jelly components surrounding the eggs greatly increases the endogenous NAADP levels (33). Measurements of the NAADP level in eggs immediately after fertilization showed large increases that appeared to correlate with the flash of Ca\textsuperscript{2+} increase in the egg cortex seen immediately after sperm-egg fusion. Photolysis release of only NAADP, but not Ca\textsuperscript{2+}, cADPR, or
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PI$_p$ from their respective caged analogs preloaded into the eggs induced cortical flashes similar to that observed at fertilization. Prior desensitization of the NAADP stores blocked the fertilization-induced cortical flash, indicating it was specifically mediated by the mobilization of the NAADP stores, perhaps those localized in the egg cortex (33).

Photorelease of NAADP in the eggs not only produced a large Ca$^{2+}$ increase (cf. Fig. 1B) but could also induce prolonged Ca$^{2+}$ oscillation, which neither PI$_p$ nor cADPR could do (10, 11, 36). Unexpectedly, blockage of the PI$_p$ and the cADPR mechanisms inhibited the NAADP-induced oscillation. Also, measurements showed that the Ca$^{2+}$ content of the ER stores increased after mobilization of the NAADP stores (36). These results indicate a two-pool mechanism is involved, whereby Ca$^{2+}$ released from the NAADP stores is sequestered by the ER stores, overloading the latter and triggering spontaneous release via the cADPR and PI$_p$ mechanisms (3, 10, 11, 36). It is thus clear that Ca$^{2+}$ stores can be separate and have distinct mechanisms for mobilization, and yet they can functionally interact to produce Ca$^{2+}$ oscillation.

Ascidian oocytes also possess all three Ca$^{2+}$-mobilizing mechanisms. Injection of NAADP produced rapid modulation of membrane currents similar to that observed at fertilization (37). On the other hand, cADPR could effectively induce vesicular fusion, which NAADP could not. Neither cADPR nor NAADP could elevate cytoplasmic Ca$^{2+}$, which was mainly activated by IP$_3$ (37). These results are consistent with the NAADP and the cADPR stores being localized in the cell cortex, allowing them to effectively modulate membrane events, while the IP$_3$-sensitive ER stores are mainly distributed in the cytoplasm. Selective localization of Ca$^{2+}$ stores can thus allow different messenger pathways to subserve distinct cellular functions.

The first mammalian cells shown to be responsive to NAADP are the pancreatic acinar cells (8). Cholecystokinin (CCK) is an important secretagogue in these cells, which also elicits local Ca$^{2+}$ spiking and global Ca$^{2+}$ waves (38). Physiological concentrations of CCK stimulated immediate cellular production of NAADP that peaked in seconds (39) and triggered localized Ca$^{2+}$ spiking in the secretory pole of the cells (28, 39). Infusion of NAADP into the cells activated similar spiking. Prior desensitization of the NAADP receptor and lysosomotrophic agents, bafilomycin and GPN, all blocked the CCK signaling (8, 28). These results establish that NAADP satisfies all the criteria of being a second messenger for mediating the signaling function of CCK.

Additionally, CCK also stimulated the cellular production of cADPR, although with a time course lagging behind the NAADP elevation (39). Infusion of cADPR into the cells also triggered Ca$^{2+}$ spiking but could not be blocked by prior desensitization of the NAADP receptor (8). These results are consistent with NAADP being the trigger, whose signal is then amplified by mobilizing the cADPR stores, perhaps through Ca$^{2+}$-induced Ca$^{2+}$ release (8, 38). Indeed, 8-amino-cADPR, a specific antagonist of cADPR (40), blocked Ca$^{2+}$ spiking induced by CCK, indicating the mobilization by the cADPR stores was critical to the observed spiking (8, 19, 38). That NAADP was the messenger specifically for the CCK signaling was shown by the fact that acetylecholine, another secretagogue in the acinar cells, even at supramaximal concentrations, could only stimulate the production of cellular cADPR but not NAADP. The former reached peak values in about a minute, similar to the CCK-induced cADPR production (39). These results provide a clear example of the specificity of NAADP and illustrate the selective coupling of an agonist (CCK) to a second messenger pathway.

This is also the case in arterial smooth muscles, where the NAADP stores were identified as lysosomes that were closely associated with the sarcoplasmic reticulum (29). Edothelin-1, a vasoconstrictor hormone, but not prostaglandin-F$_{2alpha}$, induced NAADP production. Also, bafilomycin selectively inhibited the endothelin-induced Ca$^{2+}$ elevation but not that induced by prostaglandin. On the other hand, thapsigargin blocked the global Ca$^{2+}$ wave induced by endothelin but not the Ca$^{2+}$ spiking that appeared to originate from the NAADP-sensitive lysosomal stores (29). These results again indicate NAADP is functioning as a trigger, which signal is subsequently amplified by mobilization of the ER stores.

A variety of other cellular functions have also been shown to involve NAADP. Pancreatic β-cells (MIN6) are responsive to glucose, which also induces complex Ca$^{2+}$ changes. The cellular NAADP level was found to elevate after glucose (41). Bafilomycin, but not thapsigargin, inhibited the glucose-induced Ca$^{2+}$ changes (28). Binding studies indicated the presence of the NAADP receptor in these cells (41). Photorelease of low concentrations of NAADP elicited similar Ca$^{2+}$ changes in the cells, which could be inhibited by GPN (28). Desensitizing the NAADP receptor with high concentrations of NAADP inhibited the glucose-induced response, indicating the involvement of NAADP in mediating the response (41). Similarly, in human pancreatic islets, insulin induced a complex Ca$^{2+}$ response, which could be mimicked by microinjection of low concentrations of NAADP, while high concentrations desensitized the receptor and blocked the insulin-induced Ca$^{2+}$ changes, again suggesting the involvement of NAADP in the process (42).

NAADP has also been found to mediate neuronal functions. In primary cultures of neurons, delivery of NAADP by liposomal fusion elicited Ca$^{2+}$ changes that could be blocked by bafilomycin but not by thapsigargin. Potentiation of neurite outgrowth was observed in an NAADP concentration-dependent manner after the treatment (43). Similarly, in frog neuromuscular junction, liposomal delivery of NAADP increased transmitter release in a concentration-dependent manner (44). In the buccal ganglion of *Aplysia*, presynaptic injections of NAADP induced Ca$^{2+}$ changes and transiently increased the inhibitory postsynaptic current, indicating increase in neurotransmitter release (45). These results show that not only are the NAADP-responsive cells widespread, so are the functions it mediates.

Enzymatic Synthesis of NAADP

Two enzymes have so far been shown to be capable of synthesizing NAADP (46). The first one is ADP-ribosyl cyclase (cyclase), a soluble protein of about 30 kDa purified from *Aplysia* oocytes (47). It has since been crystallized and its structure solved (48) (Fig. 2A). The second enzyme is its mammalian homolog, CD38, a protein first thought to be a lymphocyte antigen but has since been found to be ubiquitously present intracellularly as well as on the surface of a variety of cells (Ref. 49 and reviewed in Ref. 50). In contrast to the cyclase, CD38 has a single transmembrane segment near its N terminus (49) (Fig. 2C). The crystal structure of its extramembrane domain has recently been solved (51). Both proteins and another homolog, CD157, were first identified as enzymes that can cyclize NAD in a head to tail fashion to produce cADPR with the release of nicotinamide (45, 52, 53). It is now known that both CD38 and the cyclase are multifunctional enzymes capable of catalyzing a base-exchange reaction, exchanging the nicotinamide group of NAD with nicotinic acid and producing NAADP. The exchange reaction is the dominant reaction at acidic pH (46) (Fig. 2C). Additionally, CD38 is the only known enzyme that can hydrolyze cADPR to ADP-ribose (52) (Fig. 2C). Whether CD157 can catalyze either the exchange or the hydrolysis reactions has not been determined. Despite only 25–30% sequence identity among the three proteins, they are...
The extramembrane portion of CD38 is an L-shaped molecule containing 255 residues and has a central cleft dividing the two domains. The N-terminal domain is composed mostly of β-sheets (51). The secondary structures are thus essentially structurally very similar, especially at the enzymatic active sites that are all mainly negative because of the concentration of the critical acidic residues (Fig. 2). The finding that CD38 can catalyze the synthesis of two structurally and functionally distinct Ca²⁺ messengers is truly remarkable and suggests its central role in Ca²⁺ signaling.

**Perspectives**

From the discovery of NAADP as a chemical derivative of NADP to its ascendance to the rank of second messengers, the past decade has seen much progress in the understanding of the Ca²⁺ signaling functions and the enzymatic synthesis of NAADP. Many important issues, however, remain to be resolved. Principal among them is how the external stimulus is coupled to cellular synthesis of NAADP. Intracellular CD38 presumably is responsible, but neither its orientation in internal membranes nor the regulation of its activity is known. Another important issue is the identity of the NAADP receptor. Whether it is a hitherto uncharacterized Ca²⁺-releasing channel or whether it is related to the ryanodine receptor as has been suggested (6, 58, 59) remains to be determined. Nevertheless, the progress on NAADP has strengthened considerably the current view that cells possess not only multiple Ca²⁺ stores but also multiple messengers and signaling pathways for their mobilization. Stimuli can selectively activate certain pathways, and yet the separate Ca²⁺ stores can functionally interact to produce integrated signals. This new found complexity and versatility of Ca²⁺ store mobilization can surely fulfill its expected role as a major signaling mechanism.

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