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COMMENTARY

The NAADP receptor: commentary on Billington *et al.*

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NAADP is a recently described calcium-mobilizing messenger. First discovered as a potent calcium-releasing molecule in sea urchin eggs, its actions have now been reported in several mammalian cell types. In the sea urchin egg, NAADP-sensitive calcium release channels appear distinct from inositol trisphosphate or ryanodine receptors, and are mainly localized to acidic compartments. In this study, Billington *et al.* extend the pharmacology of the putative NAADP receptor utilizing molecules unrelated to NAADP itself. This work may provide an important step in developing selective NAADP receptor modulators that will help define the role of NAADP in cell signalling.
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Abbreviations: cADPR, cyclic adenosine diphosphate ribose; NAADP, nicotinic acid adenine diphosphate ribose; RyR, ryanodine receptor; IP_3, inositol 1,4,5 trisphosphate

A report in this issue of the *British Journal of Pharmacology*, by Billington and co-workers, suggest that triazine dyes interact with calcium-mobilizing $NAADP^+$ receptors. This represents the first report of compounds structurally unrelated to $NAADP^+$, which are active at this site. Calcium mobilization by the putative $NAADP^+$ receptor displays several interesting pharmacological curiosities in terms of concentration–response relationships and peculiar inactivation characteristics (Patel *et al*., 2001; Genazzani & Billington, 2002). Since little is known about the mechanisms of calcium release by this most potent of calcium-mobilizing messengers, the work of Billington and colleagues may help in the creation of new selective pharmacological agents to study the physiological roles of $NAADP^+$ and its receptor. Creating chemical tools, ideally those that are cell permeant, is a key challenge for the interrogation of the intriguing aspects of this signalling pathway at the molecular level, yet there are very few reports to date that describe approaches toward this goal.

Nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) (**1**, Figure 1) is a very close structural analogue of the more familiar pyridine nucleotide coenzyme $NADP^+$ in which the nicotinamide moiety is replaced by nicotinic acid (Lee & Aarhus, 1995). This modest change in structure results in profound changes in biological activity with the result that $NAADP^+$ is the most potent calcium-mobilizing molecule described to date. In addition to its calcium-mobilizing effects, it may also stimulate calcium entry into cells (Churchill *et al*., 2003; Masgrau *et al*., 2003; Moccia *et al*., 2003). It was discovered as a contaminant in commercial stocks of $NADP^+$ by Lee and co-workers, while investigating the effects of pyridine nucleotides on calcium release from organelles in sea urchin egg homogenates (Clapper *et al*., 1987). In the same series of experiments, the NAD metabolite, cyclic ADP-ribose (cADPR) was discovered as an additional calcium-mobilizing molecule, and later shown to be an endogenous regulator of ryanodine receptors (RYRs) (Galione *et al*., 1991). Enzymes for the synthesis of cADPR from NAD^+ were first discovered in the sea urchin egg and termed ADP-ribosyl cyclases (Rusinko & Lee, 1989). Two mammalian counterparts, named CD38 and CD157 (BST-1) have been characterized and are multifunctional enzymes (Howard *et al*., 1993). These mammalian enzymes can catalyse the synthesis of $NAADP^+$ from $NADP^+$, through base exchange in the presence of nicotinic acid (Aarhus *et al*., 1995). Although changes in $NAADP^+$ levels in cells in response to cellular stimuli have been measured by a radio-receptor assay (Churchill *et al*., 2003; Masgrau *et al*., 2003), it is not clear whether ADP-ribosyl cyclases or base exchange mechanisms are involved (Lee, 2003).

Sea urchin eggs have long been employed as important experimental systems in cell biology, and in particular for the study of fundamental aspects of calcium signalling. These large cells (around 100 μm in diameter) display prominent regenerative calcium waves at fertilization, which are entirely based on calcium release from intracellular stores. Indeed, the initial description of the action of the first calcium-mobilizing messenger, inositol 1,4,5-trisphosphate (IP_3), in an intact cell was reported in these cells (Whitaker & Irvine, 1984). However, at fertilization, there are pronounced changes in the levels of pyridine nucleotides (Schomer & Epel, 1998) and this led Lee and co-workers to investigate their effects in these cells. The great advantage of sea urchin eggs over other systems for studying calcium-mobilization mechanisms in detail, is that cell-free systems can be readily prepared that pump calcium into organelles in the presence of ATP and retain robust sensitivity to calcium-mobilizing messengers. $NAADP^+$ was found to release calcium by a mechanism that was distinct from the two known calcium-release channels, IP_3 and cADPR/ryanodine receptors (IP_3Rs and RYRs), also present in sea urchin eggs (Lee & Aarhus, 1995). Data such as these allowed the formulation of the important hypothesis that $NAADP^+$ acts on a novel calcium-release channel. This release mechanism in sea urchin eggs has several key properties

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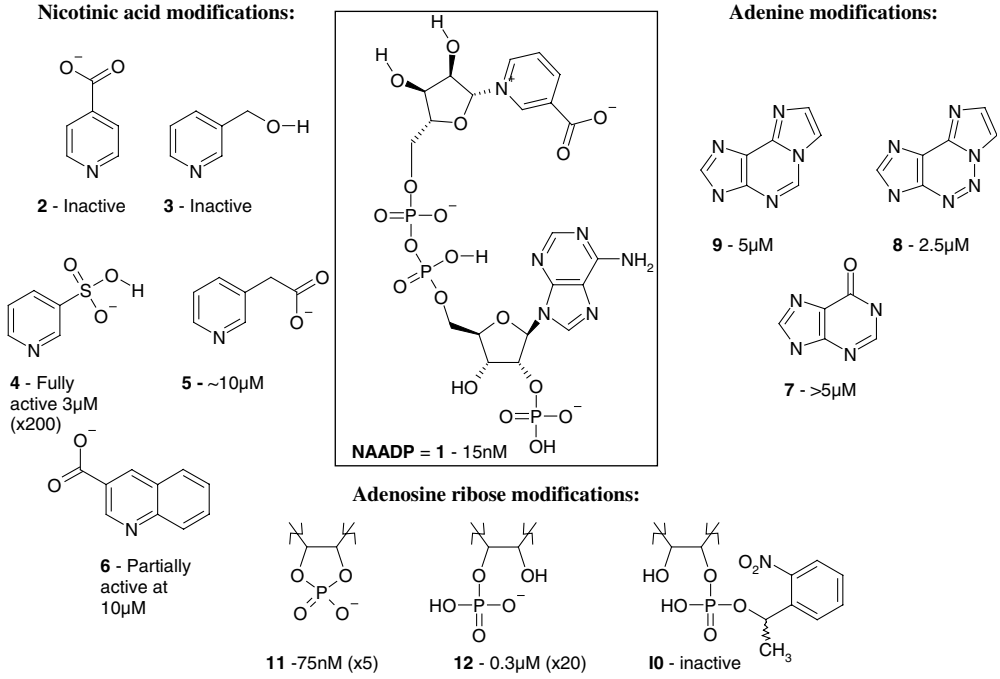

Figure 1 Structure–activity relationships for NAADP and structural analogues. Concentrations refer to approximate EC_{50}s for calcium release in sea urchin egg homogenates.

that distinguish it from IP_3Rs and RYRs. The first is that it is insensitive to antagonism by heparin or by ruthenium red and ryanodine, which block IP_3Rs and RyRs, respectively. Secondly, unlike IP_3Rs or RYRs, $NAADP^+$-induced calcium release is not modulated appreciably by calcium or other divalent cations. Thirdly, $NAADP^+$ receptors seem to reside largely on a store, which is distinct, and can be separated, from those sensitive to either IP_3 or cADPR (Lee & Aarhus, 2000). Cell fractionation studies, egg stratification and pharmacological analysis in intact eggs have shown that the $NAADP^+$-sensitive calcium store is distinct from the endoplasmic reticulum, usually associated with messenger-mediated mobilization, and likely acidic in nature (Churchill *et al*., 2002). Fourthly, the $NAADP^+$ calcium-release mechanism exhibits rather extreme inactivation properties in that concentrations of $NAADP^+$ ($IC_{50} \sim 100$ pM) that are below threshold to cause measurable calcium release ($EC_{50} \sim 20$ nM), nevertheless, can fully inactivate the $NAADP^+$ receptor (Aarhus *et al*., 1996; Genazzani *et al*., 1996). This is apparently a time- and concentration-dependent effect. The mechanism of inactivation is unknown, but a two-site model, with a high-affinity site leading to slow inactivation and a lower-affinity site leading to fast activation, has been proposed (Patel, 2004), although other scenarios, such as partial occupancy of a multimeric receptor complex leading to slow inactivation, could be considered. Finally, high-affinity binding sites (termed $NAADP^+$ receptors here) for $[^{32}P]NAADP^+$ have been reported for membranes from sea urchin eggs and mammalian cells (Billington & Genazzani, 2000; Patel *et al*., 2000). However, in the case of sea urchin egg membranes, the interaction of $NAADP^+$ with its receptor is essentially irreversible in the presence of physiological potassium concentrations (Dickinson & Patel, 2003).

The calcium-mobilizing actions of $NAADP^+$ are not restricted to sea urchin eggs. Microinjection studies using either $NAADP^+$ or a photo-activatable derivative have shown that $NAADP^+$ is a potent calcium-mobilizing agent in a variety of mammalian cells. A remarkable characteristic of $NAADP^+$-induced calcium release in mammalian cells is that the concentration–response relationship is bell-shaped (Cancela *et al*., 1999; Berg *et al*., 2000; Masgrau *et al*., 2003). That is low concentrations of $NAADP^+$, usually in the nanomolar range, effectively releases calcium, but higher concentrations (in the micromolar range) may cause no release at all. Combined experiments with micromolar $NAADP^+$ injected into cells and photolysis of caged $NAADP^+$ have indicated that micromolar $NAADP^+$ fully inactivates $NAADP^+$ receptors (Cancela *et al*., 1999). In the absence of selective inhibitors of $NAADP^+$ receptors, these higher concentrations of $NAADP^+$ have been used as a method of selectively inhibiting $NAADP^+$-sensitive calcium-release mechanisms. The concentration-dependent inactivation profile of $NAADP^+$ receptors in mammalian cells is therefore different from that in sea urchin eggs and has not been observed in isolated stores such as sarcoplasmic reticular vesicles from rat heart (Bak *et al*., 2001). This raises the possibility that inactivation may rely on processes only present in intact cells and may, for example, rely on the interplay of $NAADP^+$ with other $NAADP^+$ metabolites, such as NAADPH (Billington *et al*., 2004). Indeed, $NAADP^+$ may be appreciably converted to NAADPH in the reducing cellular environments of mammalian cells in contrast to the oxidizing cytoplasm of unfertilized sea urchin eggs.

Experiments employing cellular applications of high $NAADP^+$ concentrations have indicated an important role for $NAADP^+$ in mediating stimulus-specific calcium signalling. For example, cholecystokinin but not bombesin- or cholinergic-mediated calcium signalling in pancreatic acinar cells (Burdakov & Galione, 2000; Cancela *et al*., 2000), and glucose- but not acetylcholine-mediated calcium signals in

pancreatic beta cells (Yamasaki *et al*., 2004), involve $NAADP^+$ as an intracellular messenger. From studies in both mammalian eggs and sea urchin eggs, a model for $NAADP^+$-mediated calcium signalling has been proposed in which $NAADP^+$ triggers local calcium release, which may be amplified by the recruitment of IP_3Rs and RYRs as CICR channels (Cancela *et al*., 1999; Churchill & Galione, 2000). The interactions between the three types of calcium-release channels are complex and both cell and stimulus specific (Galione & Churchill, 2002). For example, in pancreatic acinar cells, $NAADP^+$-induced calcium signals are wholly dependent on activation of IP_3Rs and RYRs, so that calcium signalling patterns depend on which messengers are generated (Cancela *et al*., 2002), while in pancreatic beta cells they are not dependent on other calcium-release channels (Yamasaki *et al*., 2004). In T-cell lines, functional $NAADP^+$ receptors appear to be required for both IP_3 and cADPR evoked calcium signals (Berg *et al*., 2000). In vascular smooth muscle cells, $NAADP^+$ evokes local calcium signals that may then be greatly amplified by RYRs (Boittin *et al*., 2002). Importantly, a number of studies in mammalian cells have shown that, as in sea urchin eggs, the $NAADP^+$-sensitive calcium store is distinct from the endoplasmic reticulum where IP_3Rs and RYRs are predominantly located (Mitchell *et al*., 2003; Yamasaki *et al*., 2004). Pharmacological studies and specific organelle fluorescence markers have indicated that this store is likely to be an acidic compartment with characteristics of lysosome-related organelles. However, until the $NAADP^+$ receptor is characterized at the molecular level, and more selective tools are generated, it is difficult to unravel the complex interactions between the various calcium-release channels with certainty. Indeed, an alternative scenario in which $NAADP^+$ interacts directly with RYRs has also been proposed (Hohenegger *et al*., 2002; Gerasimenko *et al*., 2003).

Previous studies on the pharmacology of $NAADP^+$ receptors have addressed the structural determinants of the $NAADP^+$ molecule required for receptor activation and inactivation (Lee & Aarhus, 1997). With $NADP^+$ as substrate, the *Aplysia* ADP-ribosyl cyclase [E.C.3.2.2.5] is able to exchange nicotinamide for a range of pyridine analogues bearing substituents in the 3- and 4-position, but base exchange involving 2-position-modified pyridines was not supported. Lee & Aarhus (1997) reported the preparation and evaluation of a small series of analogues from commercially available compounds using this methodology and, thus providing the only current insight into the structural determinants that affect Ca^{2+} release from the $NAADP^+$ store in sea urchin egg homogenates. It was apparent from these studies that a negative charge at the pyridine 3-position is essential for calcium-release activity. $NADP^+$ features a carboxamide at the pyridine 3-position, but is inactive. Moving the carboxylate group to the adjacent 4-position, (Figure 1, structure **2**), or presentation of a methyl alcohol at the 3-position (Figure 1, **3**) both result in completely inactive analogues. Nicotinic acid can be replaced by analogous components that support a negative charge in this position without completely reducing Ca^{2+} release, although these analogues have half-maximal effective concentrations at least 100–200 times higher than $NAADP^+$. Pyridine-3-sulphonate-ADP (**4**) induces calcium release from the $NAADP^+$ store with an EC_{50} of around 3 μM, pyridine-3-acetate-ADP (**5**) required somewhat higher concentrations of about 10 μM, whereas quinoline 3-carboxylate-ADP (**6**) only induced partial release at this concentration. Limited modifications of the adenine base have also been explored. The same paper reported that nicotinic acid hypoxanthine dinucleotide phosphate (deamino-$NAADP^+$), **7**, where the amino group at the 6-position of the adenine ring is replaced with a hydroxyl, showed an approximately thousand fold decrease in activity with calcium release only becoming detectable at concentrations above 5 μM. It is interesting that this compound is reported to desensitize the $NAADP^+$ receptor at much lower concentrations, although less potently than $NAADP^+$ itself. A later paper from the same authors (Lee & Aarhus, 1998) describes the preparation of fluorescent analogues etheno-aza-$NAADP^+$ (**8**, 2.5 μM) and etheno-$NAADP^+$ (**9**, 5 μM) which activate calcium release with half-maximal effective concentrations as shown in brackets (Figure 1) and both desensitize the $NAADP^+$ receptor with IC_{50}s of 60–80 nM. The inactivity of $NAAD^+$ indicates that the presence of a phosphate is important and this is confirmed because installation of a caging group (**10**) at the 2′-phosphate of $NAADP^+$ abolishes calcium-releasing activity (Lee *et al*., 1997). Phosphate regio-isomers of $NAADP^+$, however, induce Ca^{2+} release from the store relatively potently with relative half-maximal concentrations of only about five-fold for 2′,3′-cyclophosphate analogue, **11**, and 20-fold for 3′-phosphate (**12**, EC_{50} 0.3 μM). To summarize, all the active analogues of $NAADP^+$ synthesized to date are less potent than $NAADP^+$ and have similar ratios in terms of concentrations for EC_{50} for calcium release over the IC_{50} for inactivation of receptors. Thus, none of these analogues appear to discriminate between proposed inactivation and activation sites for $NAADP^+$ (Patel, 2004).

A different type of pharmacological approach was used to study the effects of a range of plasma membrane channel blockers on $NAADP^+$-mediated calcium release in sea urchin egg homogenates, and relative selectively shown by lack of effect on IP_3 and cADPR-mediated calcium release (Genazzani *et al*., 1997). Various blockers of L-type calcium channels, diltiazem and dihydropyridines, were found to selectively block calcium release by $NAADP^+$ without appreciable effects on IP_3Rs or RYRs. Similarly, potassium-channel blockers such as tetrahexylammonium (THA) shared these properties. The effects of these agents were noncompetitive with $NAADP^+$ and did not affect binding of $[^{32}P]NAADP^+$ to egg membranes. Thus, it is likely that they act by blocking the ion conductance pore of the $NAADP^+$ receptor complex. However, the concentrations of these drugs required to block $NAADP^+$ receptors were in excess of those reported to inhibit the plasma membrane channels that they target. Therefore, within the context of the intact cell, these agents are of limited use for studying $NAADP^+$ signalling.

In the current study by Billington and co-workers, the interactions of triazine dyes with $NAADP^+$ receptors were examined based on their ability to interact with nucleotide-binding sites in various proteins. Triazine dyes were found to compete at the $NAADP^+$-binding sites in sea urchin homogenates in a concentration-dependent manner. Reactive red 120 (RR120) was the most potent dye in this respect with an IC_{50} of 1.4 μM. Pretreatment of egg membranes with triazine dyes showed that all the dyes, with the exception of cibacron blue, reversibly bound to the $NAADP^+$ receptor. Perhaps the most intriguing finding was that RR120 at high concentrations (100 μM) was able to mobilize calcium from stores in egg

homogenates. Usually, calcium release from sea urchin homogenates has been measured by fluorometric assays, but the triazine dyes interfere with calcium reporting fluorescent dyes. Instead, they used an indirect assay for calcium release, which involved actively loading the stores with ^{45}Ca and subsequent challenge with releasing agents. A filtration assay determines whether calcium release has occurred by measuring residual calcium retained in stores on the filters. This effect appears to be selective for the $NAADP^+$ receptor since it was insensitive to heparin and ruthenium red, blockers of IP_3Rs and RYRs respectively. Crucially, prior desensitization of $NAADP^+$ receptors with subthreshold concentrations of $NAADP^+$ fully blocked RR120-evoked calcium release. These data, coupled with the finding that $NAADP^+$ and RR120-induced calcium release were nonadditive, strongly suggest that RR120 is an agonist at the $NAADP^+$ receptor. Since RR120 binding to the $NAADP^+$ receptor is reversible, these findings suggest that irreversibility of binding is not a prerequisite for receptor activation. These studies represent the first report of agents that can competitively interact with $NAADP^+$ receptors and activate them, which are not structurally related to $NAADP^+$.

It is clear that the molecular characterization of $NAADP^+$ receptors will greatly enhance our understanding of $NAADP^+$ signalling. Initial purification of solubilized receptors from sea urchin eggs has already been reported, taking advantage of the irreversibility of $[^{32}P]NAADP^+$ binding to tag the receptors during the purification protocols (Berridge *et al*., 2002). Billington *et al*. have extended their studies by using triazine dyes conjugated to agarose and DEAE cellulose beads to further purify the solubilized receptors by affinity chromatography. RG5 beads were found to be useful, since protein interactions with beads were insensitive to NaCl, which could be used to elute the majority of protein, but most of the $NAADP^+$ receptor could be eluted with $BaCl_2$, as assayed by subsequently tagging with $[^{32}P]NAADP^+$. Using RG5 beads a 75-fold enrichment of the receptor was achieved. Thus, the use of triazine dyes may also prove useful in the isolation of the $NAADP^+$ receptor.

The discovery of $NAADP^+$ as a new calcium-mobilizing agent provides an impetus for the design of new agents to modulate the actions of this messenger. That $NAADP^+$ is likely to have crucial roles in pharmacologically important processes such as insulin secretion, lymphocyte activation and smooth muscle contractility, suggests that it may be a useful target for therapeutic intervention in a number of pathophysiologically significant systems. Further, in contrast to the ubiquitous IP_3 system, $NAADP^+$ may be a better target for therapeutics since it may act to modulate the sensitivity of more common calcium-release pathways, may not operate in all cell types, and only then for a subset of receptor-mediated calcium signalling events. The usefulness of triazine dyes in studies in intact cells is obviously precluded by their interactions with a wide variety of cellular proteins, interference with fluorescent reporter dyes and lack of cell permeance. Nevertheless, the study by Billington and co-workers in identifying their actions on the $NAADP^+$ receptors may provide an important step for the discovery of novel therapeutic agents as well as in the identification of the $NAADP^+$ receptor itself.

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