Structural Determinants of Nicotinic Acid Adenine Dinucleotide Phosphate Important for Its Calcium-mobilizing Activity*

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Hon Cheung Lee and Robert Aarhus

From the Department of Physiology, University of Minnesota, Minneapolis, Minnesota 55455

Nicotinic acid adenine dinucleotide phosphate (NAADP) mobilizes Ca\(^{2+}\) through a mechanism totally independent of cyclic ADP-ribose or inositol trisphosphate. The structural determinants important for its Ca\(^{2+}\) release activity were investigated using a series of analogs. It is shown that changing the 3-carboxyl group of the nicotinic acid (NA) moiety in NAADP to either an uncharged carbinol or from the 3-position to the 4-position of the pyridine ring totally eliminates the Ca\(^{2+}\) release activity. Conversion of the 3-carboxyl to other negatively charged groups, either 3-sulfonate, 3-acetate, or 3-quinoline carboxylate, retains the Ca\(^{2+}\) release activity, although their half-maximal effective concentrations (EC\(_{50}\)) are 100-200-fold higher. Changing the 6-amino group of the adenine to a hydroxyl group results in more than a 1000-fold decrease in the Ca\(^{2+}\) release activity. Conversion of the 2-phosphate to 2',3'-cyclic phosphate or 3'-phosphate likewise increases the EC\(_{50}\) by about 5- and 20-fold, respectively. Similar to NAADP, all of the active analogs can also desensitize the Ca\(^{2+}\) release mechanism at subthreshold concentrations, suggesting that this novel property is intrinsic to the release mechanism. The series of analogs used was produced by using ADP-ribosyl cyclase to catalyze the exchange of the nicotinamide group of various analogs of NADP with various analogs of NA. An important determinant in NA that is crucial to the base exchange reaction was shown to be the 2-position of the pyridine ring. Neither pyridine-2-carboxylate nor 2-methyl-NA support the exchange reaction. The negative charge and the position of the 3-carboxyl group are nonessential since both pyridine-3-carbinol and pyridine-4-carboxylate support the base exchange reaction. In addition to the information on the structure-activity relationships of NAADP and NA, this study also demonstrates the utility of the base exchange reaction as a general approach for synthesizing NAADP analogs.

A novel mechanism for mobilizing intracellular Ca\(^{2+}\) stores has recently been characterized in sea urchin eggs, which is activated by nicotinic acid adenine dinucleotide phosphate (NAADP), a metabolite of NADP (1, 2). The mechanism is independent of those activated by either inositol trisphosphate (IP\(_3\)) or cyclic ADP-ribose (cADPR), since it is insensitive to inhibition by their respective antagonists, heparin and 8-amino-cADPR (2). The Ca\(^{2+}\) stores that are sensitive to NAADP can be separated by Fercoll gradient density centrifugation from those sensitive to IP\(_3\) and cADPR (2), and these stores are also insensitive to thapsigargin, suggesting the presence of a novel Ca\(^{2+}\) pump (3). That the action of NAADP is likely to be mediated by a receptor is shown by specific binding of \([3^{25}P\)]NAADP to egg microsomes, which can be competitively inhibited by NAADP but not by cADPR (4). Another unique feature that distinguishes this mechanism is that the agonist, NAADP, at subthreshold concentrations in the range of nanomolar, can totally inactivate the release mechanism (4, 5). This novel desensitization is shown to be occurring at the receptor level (4). A caged analog of NAADP has been synthesized (6), and releasing NAADP in live sea urchin eggs by photolyzing the analog produces not only a Ca\(^{2+}\) transient but long term Ca\(^{2+}\) oscillations lasting for more than 30 min (4), indicating that the Ca\(^{2+}\) release mechanism is present and operative in live cells.

NAADP, although structurally and functionally distinct from cADPR, can be synthesized enzymatically by ADP-ribosyl cyclase (1), the same enzyme that cyclizes NAD to produce cADPR (7). The cyclase can also use NADP as substrate and exchange the nicotinamide group of NADP with nicotinic acid (NA) at acidic pH to form NAADP (1). A soluble form of the cyclase has been purified and crystallized, and its crystal structure solved at 2.4 Å (8–10). The enzyme crystallizes as a dimer formed by two monomers in a head-to-head fashion, enveloping a central cavity (9). In this study, the structural determinants in NA critical for the base exchange reaction were assessed by testing a series of analogs of NA for their ability to support the reaction catalyzed by the cyclase. The products, analogs of NAADP, were assayed for Ca\(^{2+}\) release activity to determine the structure-function relationship of NAADP and its Ca\(^{2+}\)-mobilizing activity.

EXPERIMENTAL PROCEDURES

Synthesis—NAADP (1 mM) or its analog was incubated with NA or its analog (20–30 mM) at pH 4.5 with the Aplysia ADP-ribosyl cyclase (0.2 μg/mL) for 1–2 h at 20–25 °C, and the products were purified by HPLC using either an AG MP-1 column as described previously (1) or the column described below. All substrates were prepurified by the same HPLC method. An alternative HPLC procedure used a Poros 20 HQ column on a BioCad HPLC system (PerSeptive Biosystems) with a flow

triphosphate; NA, nicotinic acid; 3-PSA, 3-pyridinesulfonic acid; 3-PSA-ADP, 3-PSA adenine dinucleotide phosphate; 3-PAA, 3-pyridyl-acetic acid; 3-PAA-ADP, 3-PAA adenine dinucleotide phosphate; 3-QCA, 3-quinoline carboxylic acid; 3-QCA-ADP, 3-QCA adenine dinucleotide phosphate; 3-PC, 3-pyridylcarbinol; 3-PC-ADP, 3-PC adenine dinucleotide phosphate; 2-methyl-NA, 2-methylnicotinic acid; P-4-COOH, pyridine-4-carboxylic acid; 4-NAADP, pyridine-4-carboxylic acid NAADP; P-2-COOH, pyridine-2-carboxylic acid; HPLC, high pressure liquid chromatography.

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rate of 10 ml/min. A nonlinear gradient of trifluoroacetic acid was used: 0–1.33 min, 100% A (water); 1.33–1.83 min, linearly to 4% B (B was 150 mM trifluoroacetic acid); 1.83–2.66 min, linearly to 8% B; 2.66–3.49 min, linearly to 10% B; 3.49–5.15 min, linearly to 32% B; 5.15–6.81 min, linearly to 100% B; at 6.81 min stepped down to 0% B and maintained until 7.68 min.

Ca2+ Release in Sea Urchin Egg Homogenates—Homogenates of sea urchin egg (Strongylocentrotus purpuratus) were prepared as described previously (6). Frozen egg homogenates (25%) were thawed at 17 °C and sequentially diluted to 1.25% with a medium containing 250 mM N-methylglucamine, 250 mM potassium gluconate, 20 mM Hepes, 1 mM MgCl2, 2 units/ml creatine kinase, 8 mM phosphocreatine, 0.5 mM ATP, and 2 μM fluo 3 (Molecular Probes, Inc.), pH 7.2, adjusted with acetic acid (6). Ca2+ release was measured spectrofluorimetrically with an excitation wavelength of 485 nm and emission wavelength of 535 nm. The measurements were done in a cuvette maintained at 17 °C, and the homogenates were continuously stirred. The volume of homogenate used was 0.2 ml, and additions were usually made in 2-μl volumes.

Materials—ADP-ribosyl cyclase was purified from Aplysia ovotestis as described previously (7, 8). [14C]NADP was synthesized by phosphorylating [14C]NAD (Amersham Corp.) with 10 mM ATP using a NAD kinase partially purified from sea urchin egg extracts as described previously (4). The radioactive product, [14C]NADP, was purified by HPLC. The concentrations of various analogs of NAADP were determined by absorbance using extinction coefficients of 16,000 at 250 nm for deamino-NAADP, 48,000 at 260 nm for 3-QCA-ADP, and 18,000 at 260 nm for the rest of the analogs. The extinction coefficients were determined by absorbance using extinction coefficients of 16,000 at 250 nm for deamino-NAADP, 48,000 at 260 nm for 3-QCA-ADP, and 18,000 at 260 nm for the rest of the analogs.

Analogs of NA, 3-QCA, 3-PAA, 3-PSA, 3-PC, and 2-methyl-NA were from Aldrich. P-4-COOH, deamino-NAADP, 2′,3′-cyclic PO4, and 3′-NAADP were from Sigma. P-2-COOH was from Fluka.

RESULTS

ADP-ribosyl cyclase cyclizes NAD to produce cADPR (7, 12, 13). The enzyme can also use NAADP as substrate efficiently. Fig. 1 shows the two alternative catalytic pathways of the cyclase using NAADP as substrate. At neutral and alkaline pH, the enzyme cyclizes NAADP by linking position N-1 of the adenine ring with the anomeric carbon of the terminal ribose to produce cADPR phosphate (cADPRP) (1). At acidic pH and in the presence of NA, the cyclase predominantly catalyzes the exchange of the nicotinamide group of NAADP with NA to produce NAADP (1). The structural determinants of NA that are important for the base exchange reaction can be determined by assessing analogs of NA for support of the reaction, and some of them are listed in Fig. 1. The products of the base exchange reaction, analogs of NAADP, can then be used to analyze their structure-function relationships with NAADP and their Ca2+-releasing activity.

Structural Determinants of Nicotinic Acid Important for the Base Exchange Reaction—Fig. 2 shows HPLC chromatograms of the products of the base exchange reaction using NAADP and NA or one of its three analogs as substrate. At acidic pH with NA as substrate, the predominant product was NAADP (Fig. 2A). A small amount of cADPRP was also produced. Similar results were seen with P-4-COOH as substrate (Fig. 2B), which has a carboxyl group at the 4-position of the pyridine ring instead of the 3-position in NA. However, if the carboxyl group is at the 2-position (Fig. 2C, P-2-COOH), no base exchange product was observed; instead, the cyclase cyclized NADP to cADPRP. It appears that it is not just the position of the carboxyl group that is critical; in fact, any substitution at the 2-position may be sufficient to render it incapable of supporting the base exchange reaction. This is shown in Fig. 2D using an NA analog with an extra methyl group at the 2-position (2-methyl-NA). Again, no base exchange product was observed.

The time courses of the reaction with P-4-COOH or P-2-COOH as substrates are shown in Fig. 3. About 20% of the products was cADPRP with P-4-COOH and NAADP as reactants, and the rest was the base exchange product, 4-NAADP (Fig. 3A). On the other hand, with P-2-COOH as substrate, the

FIG. 1. Two catalytic pathways of ADP-ribosyl cyclase. The cyclase produces cADPR by cyclizing NAD. It can also cyclize NAADP by linking the position N-1 of the adenine with the terminal ribose. In the presence of nucleophiles such as NA, the cyclase catalyzes the exchange of the nicotinamide group (Nic) of NAADP with NA producing NAADP. By using various analogs of NA and NAADP, a variety of analogs of NAADP can be synthesized, and some of these used in this study are listed. Modifications include those on the pyridine ring (R and R1), the 6-amino group of the adenine (R4), and the phosphate group at the 2′- (R2) or 3′-position (R3).

NADP and analogs

| NAADP: | R=COOH, R2=PO4 |
| 3′-NAADP: | R2=OH, R4=NH2 |
| 2′,3′-cNAADP: | R2=R3=cyclic PO4 |
| deA-NAADP: | R4=O |
| 3PSA-ADP: | R=SO4 |
| 3PAA-ADP: | R=CH3COOH |
| 3PC-ADP: | R=CH3OH |
| 3QCA-ADP: | R1=COOH |

Structural schematic of NAADP and its analogs, with pictorial representation of the exchange reaction. The enzyme can also use NAADP as substrate efficiently.
sum of cADPRP and the residual NADP was conserved throughout the reaction, indicating cADPRP was the only product (Fig. 3B). The base exchange product, 4-NAADP, an analog of NAADP with a carboxyl group at the 4-position instead of the 2-position of the pyridine ring, was tested for its Ca\(^{2+}\) release activity. At concentrations as high as 30 μM, it produced no Ca\(^{2+}\) release in sea urchin egg homogenates (Fig. 3A, inset), whereas NAADP elicited maximal Ca\(^{2+}\) release at 200-fold lower concentrations in the same preparation. Therefore, the position of the carboxyl at the pyridine ring is a critical determinant of the Ca\(^{2+}\) release activity, showing the exquisite specificity of the release mechanism.

To determine if the charge of the carboxyl group is important for the base exchange reaction a neutral analog of NA, 3-PC, with a carbinol group replacing the carboxyl group was tested. The chromatogram of the products after incubation with the cyclase is shown in Fig. 4A. To follow the exchange of the nicotinamide group, [nicotinamide-\(^{14}\)C]NADP was added as tracer. In addition to the 3-PC peak, the absorbance tracing of the products showed a peak with elution time the same as the starting substrate, NADP (Fig. 4B). This peak was the product of the exchange reaction, 3-PC-ADP, and not NADP itself since the peak was devoid of \(^{14}\)C radioactivity (Fig. 4A). All the radioactivity eluted with nicotinamide, which co-migrated with the large 3-PC peak. 3-PC was so effective in forcing the cyclase to the base exchange mode that very little cADPRP, the cyclization product, was detected in its presence (Fig. 4A). In the absence of the NA analog, the cyclase readily cyclized NADP to form cADPRP as shown in Fig. 4B.

The effectiveness of the uncharged analog, 3-PC, in supporting the exchange reaction raises the possibility that the uncharged form of NA may be the reactive species. This could explain the acidic requirement for the base exchange reaction (1). NA has a pK of about 4.8 and about half would be uncharged at the pH of the reaction (pH 4.5). The neutral reactant requirement was tested using an analog of NA, 3-PSA, with a sulfonic acid group at the 3-position instead. The sulfonate is expected to be fully charged at the pH of the reaction. Fig. 5 shows that 3-PSA fully supported the base exchange reaction. After 40 min of reaction, about equal amounts of the exchange product, 3-PSA-ADP, and cADPRP were produced. The proportion of cADPRP was higher with 3-PSA as substrate than when NA was used (cf. Fig. 2), which could be due to the difference in size of the sulfonate group as compared with the
carboxyl group. These results show that either a neutral or a fully charged group at the 3-position of the pyridine ring is capable of supporting the exchange reaction.

Structure-Function Relationship of NAADP and Its Ca\(^{2+}\) Release Activity—Unlike the analog 4-NAADP described above, 3-PSA-ADP elicited full Ca\(^{2+}\) release from egg homogenates as shown in Fig. 6. It also desensitized the homogenates to subsequent challenge with NAADP. Likewise, homogenates desensitized to NAADP would not respond to 3-PSA-ADP. A unique property of the NAADP-sensitive Ca\(^{2+}\) release mechanism is that NAADP can induce complete self-inactivation at subthreshold concentrations (4, 5). Treatment of egg homogenates with non-releasing concentrations of 3-PSA-ADP for 3 min produced such inactivation that saturating NAADP added afterward induced no further Ca\(^{2+}\) release (Fig. 6). Similarly, homogenates inactivated by pretreatment with nanomolar concentrations of NAADP did not respond to maximal concentrations of 3-PSA-ADP. These results indicate that 3-PSA-ADP acts on the same Ca\(^{2+}\) release site as NAADP.

Fig. 7 compares the concentration response of four analogs of NAADP with different substitutions on the pyridine ring. At the 3-position, these substitutions are sulfonic acid for 3-PSA-ADP, acetic acid for 3-PAA-ADP, and carbinol for 3-PC-ADP. Also shown is the analog with 3-quinoline carboxylic acid substituting for NA, 3-QCA-ADP. The half-maximal effective concentration (EC\(_{50}\)) of 3-PSA-ADP was about 3 \(\mu\)M, more than 200-fold higher than that of NAADP. The EC\(_{50}\) values for 3-PAA-ADP were even higher at about 10 \(\mu\)M. For 3-QCA-ADP, the largest in size of the series, only a small Ca\(^{2+}\) release was detected at 10 \(\mu\)M. It thus appears that the effectiveness of this series of analogs in releasing Ca\(^{2+}\) decreases roughly with increasing size of the substitution on the pyridine ring. However, the negative charge of the substitution is definitely critical. In the absence of a negative charge, as in the case of 3-PC-ADP, an analog with a carbinol group at the 3-position and the smallest in size of the series, there was no Ca\(^{2+}\) release activity even at 10 \(\mu\)M. This charge requirement is consistent with the previous demonstration that NADP, with an uncharged amide group at the 3-position of the pyridine ring, is likewise inactive in releasing Ca\(^{2+}\) (2, 14).

In addition to the analogs with various substitutions in the pyridine ring described above, structural changes can also be imposed on other parts of the NAADP molecule by using analogs of NADP as substrates for the exchange reaction with NA. Results shown in Fig. 8 indicate that the position of the phosphate group at the 2'-position of the ribose is important but not critical for the Ca\(^{2+}\) release activity. Changing the 2'-phosphate to a cyclic form linking both the 2'- and 3'-positions, 2',3'-cyclic NAADP, decreases its effectiveness but does not eliminate its Ca\(^{2+}\) release activity. A further decrease in effectiveness is seen when the 2'-phosphate is changed to the 3'-position as in 3'-NAADP in which EC\(_{50}\) increases by about 20-fold to 0.3 \(\mu\)M as compared with 15 nM for NAADP. Therefore, as long as a phosphate group is present at the ribose, the Ca\(^{2+}\) release activity is retained. Removal of the phosphate as
in NAAD, however, does eliminate its biological activity as has been shown previously (2).

An unexpected structural determinant in NAADP that is critical for its Ca\(^{2+}\) release activity is the amino group at the 6-position of the adenine ring. Using nicotinamide hypoxanthine dinucleotide phosphate (deamino-NADP) and NA as substrates, deamino-NAADP was synthesized. Fig. 9 shows that the Ca\(^{2+}\) release activity of the analog was only detectable at higher than 5 \(\mu\)M. It did, however, inactivate the release mechanism at non-activating concentrations in a manner similar to NAADP, indicating that it did act on the NAADP-sensitive mechanism. This is in contrast to 3-PC-ADP that did not appear to interact with the NAADP receptor at all since it neither released Ca\(^{2+}\) nor induced desensitization.

### DISCUSSION

The Ca\(^{2+}\)-releasing activity of NAADP is novel in three aspects. First, the release mechanism activated by NAADP is completely independent of the two known mechanisms mediated by the IP\(_3\) and ryanodine receptors, indicating that it is a new and hitherto undescribed pathway (1–5). Second, the NAADP-activated mechanism is the first receptor-mediated mechanism that shows full inactivation by non-stimulating concentrations of the agonist (4, 5). Third, releasing NAADP in live sea urchin eggs by photolyzing its caged analog produces long term Ca\(^{2+}\) oscillations, a function shared by neither IP\(_3\) nor cADPR (4). These three unique and novel features make...
the NAADP-dependent Ca$^{2+}$-signaling mechanism a worthy topic of investigation. This study represents the first to systematically analyze the structure-activity relationship between NAADP and its Ca$^{2+}$-releasing function.

It is shown that there are at least three critical structural determinants on NAADP. The first one is the negative charge at the 3-position of the pyridine ring. Elimination of the charge or changing it to the 4-position completely inactivates the molecule. The site has some tolerance toward the size of the group containing the negative charge, and the analogs show a decrease in effectiveness as the bulkiness of the group increases. The second important determinant is the 2'-phosphate. The requirement is lenient as long as a phosphate group is attached to the ribose. The molecule retains activity whether the phosphate is on the 2'- or 3'-position or cyclically linked to both positions. Removal of the phosphate as in NAAD, however, eliminates its Ca$^{2+}$ release activity (2). The third important site is the amino group at the 6-position of the adenine ring. Conversion to an -OH group reduces the effectiveness of its Ca$^{2+}$-release activity by more than 1000-fold, even though -OH is just about the same size as -NH$_2$. These results indicate that the NAADP-sensitive Ca$^{2+}$ release mechanism is highly specific and are consistent with its being mediated by a high affinity receptor (4).

This study also provides information on the unique self-inactivation property of the NAADP receptor. All of the analogs tested that are agonists can also induce inactivation at sub-threshold concentrations. The efficacy of the analogs in inducing inactivation directly parallels the efficacy in releasing Ca$^{2+}$. Analogs that have no Ca$^{2+}$-releasing activity also do not cause inactivation. This inextricable linkage between activation and inactivation suggests the inactivation is likely to be intrinsic to the function of the receptor. It is possible that the NAADP receptor contains two binding sites for NAADP. The regulatory site would be of high affinity, and its occupancy results in inactivation. The other site would be of lower affinity and represents the Ca$^{2+}$-releasing site. This two-site mechanism is consistent with the present results.

The series of analogs used in this study was synthesized by taking advantage of the base exchange reaction catalyzed by the Aplysia ADP-ribose cyclase (1, 7, 8). By choosing systematically a set of NA analogs it can be concluded that one of the most critical determinants for the base exchange reaction is the 2-position of the pyridine ring. Any substitution at that position makes the analog incapable of supporting the reaction. It has previously been proposed that the base exchange reaction results from an attack by nicotinic acid on the anemic carbon of an activated ADP-ribose moiety that is formed as an enzyme intermediate during catalysis by the cyclase (15, 16). Substitution at the 2-position apparently reduces the reactivity of the nitrogen of the pyridine ring either by steric hindrance or by reduction of its nucleophilicity. As a result, the chance for the competing intramolecular attack by the N-1 of the adenine of the activated ADP-ribose increases, resulting in the cyclization reaction being the predominant pathway.

The carboxyl group and its negative charge are nonessential for the base exchange reaction. Indeed, the neutral 3-PC was so effective in promoting the base exchange reaction that the cyclization reaction appeared inhibited by its presence. The analog, however, did not really inhibit the cyclase but instead forced the cyclase into the exchange pathway. The apparent inhibition is due to the fact that the product of the exchange has the same HPLC elution time as the substrate NAAD, making it appear that no product was formed. Nicotinamide behaves in much the same manner as 3-PC (data not shown). In the absence of nicotinamide, the cyclase readily cyclizes NA or NADP. The presence of nicotinamide forces the enzyme into the exchange mode, and since the product and the substrate are identical, the enzyme appears inhibited. These results caution against the usual care of nicotinamide and similar compounds as inhibitors of the cyclase without careful determination if the enzyme is forced into an exchange mode instead.

This study demonstrates the usefulness of the base exchange reaction for synthesizing a wide variety of analogs of NAADP. Although only a small number of commercially available substrate analogs was used in this study, the demonstrated feasibility of the approach should set the stage for more thorough investigations into other structural determinants on NAADP or NA that are important for the Ca$^{2+}$-releasing and enzymatic activities, respectively. The large number of pyridine derivatives and analogs of NAADP available make this simple synthesis particularly versatile. In addition to the information on the structure-activity relationships of NAADP and NA, the approach can conceivably lead also to the synthesis of other novel analogs of NAADP, such as fluorescent analogs that may be useful in visualizing the NAADP-sensitive Ca$^{2+}$ stores in cells.

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