Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 167-174

ENZYMATIC SYNTHESIS AND DEGRADATION OF NICOTINATE ADENINE DINUCLEOTIDE PHOSPHATE (NAADP), A Ca^{2+}-RELEASING AGONIST, IN RAT TISSUES

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Received February 20, 1995

We have recently found that nicotinate adenine dinucleotide phosphate (NAADP) is a potent agonist that triggers Ca^{2+} release from intracellular stores of sea urchin eggs, and that its action is distinct from effects of IP_3 and cyclic ADP-ribose (J. Biol. Chem. 270:3216, 1995). Now we report that extracts from rat brain, heart, liver, and spleen but not kidney cortex contain enzymatic activity which catalyzes NAADP synthesis by exchange of nicotinamide for nicotinic acid and which is probably catalyzed by NAD(P)-glycohydrolase. Extracts from these tissues also inactivate NAADP in the rank inverse to their ability for NAADP synthesis. These results suggest that NAADP, a Ca^{2+}-releasing agent, can be generated in mammalian tissues, namely in brain.

Controlled release of ionized calcium (Ca^{2+}) from intracellular stores is an important component of intracellular signaling pathways that regulate biological processes in various cell types (1). The most comprehensively studied is the Ca^{2+} release triggered by inositol-1',4',5'-trisphosphate (IP_3) an agonist (2) which binds onto a specific IP_3-receptor/Ca^{2+}-channel within endoplasmic membranes. More recently, Lee et al (3-5) discovered that cyclic ADP-ribose (cADPR), a nucleotide synthetized from β-NAD, binds on a specific receptor and stimulates Ca^{2+}-induced Ca^{2+}-release system (CICR) through the ryanodine channel, which is also located in endoplasmic membrane. Most recently (6), we found that nicotinate adenine dinucleotide phosphate (NAADP), a deamidated derivative of NADP triggers release of Ca^{2+} in sea urchin egg homogenates by a mechanism which is clearly different from actions of either IP_3 or cADPR (6). Simultaneously, NAADP was identified as product of alkaline treatment of NADP (7). The

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Abbreviations: NADP, nicotinamide adenine dinucleotide phosphate; NAADP, nicotinate adenine dinucleotide phosphate, IP_3 inositol-1,'4,'5'-trisphosphate; cADPR, cyclic ADP-ribose; NAD, nicotinamide adenine dinucleotide.

0006-291X/95 $5.00

distinct nature of the NAADP-stimulated Ca-release system was documented by specific homologous desensitization (6) and the lack of inhibition by established blockers of IP_3 and antagonists of cADPR-stimulated Ca^{2+}-release (6,7). Furthermore, we found that NAADP-triggered release is specifically and dose-dependently blocked by thionicotinamide-NADP (thio-NADP); thio-NADP has no effect upon actions of cADPR or IP_3 (6). NAADP triggers release of Ca^{2+} from sea urchin egg homogenates in low nanomolar (< 20 nM) concentrations, a potency similar to Ca^{2+}-releasing action of cADPR; like cADPR or IP_3, NAADP triggers Ca^{2+} release when microinjected to sea urchin eggs (7,8). Taken together, all these observations strongly suggest that NAADP may function as a distinct regulator of intracellular Ca^{2+} release through a specific, hitherto unknown Ca^{2+} channel, and potentially might function as second messenger akin to IP_3 (2) or cADPR (9).

All previous observations have been made on a system of sea urchin homogenate (6), or later on whole egg (7,8), which both are excellent experimental models for study of intracellular Ca^{2+} fluxes (3,5,6). However, in view of the potential role of NAADP as a second messenger (6), it is of paramount importance to determine whether NAADP can be metabolized, synthetized and degraded, in mammalian tissues. In the present study we investigated and found that biosynthesis and biodegradation of NAADP can be catalyzed by enzyme activities present in several rat tissues which greatly differ in these activities.

METHODS AND MATERIALS

Ca^{2+}-release bioassay (3,6). Homogenate for *Lytechinus pictus* eggs were prepared as described previously (6) and stored at -70°. For each experiment new aliquot of frozen homogenates was first diluted in "intracellular medium" and described in our previous report (6). Calcium was measured by fluo-3 fluorescence in 250 ml cuvette at 17°C thermostated by circulating bath and continuously mixed with a magnetic stirring bar, with 490 nm excitation and 535 nm emission using Hitachi (F-2000) spectrofluorimeter.

HPLC purification of nucleotides was performed by anion exchange chromotography using AG MP-1 (Bio-Rad) (1 x 10 cm): eluted with gradient of trifluoroacetic acid, as described previously (6). NAADP was biosynthetized from β-NADP by base exchange reaction catalyzed by NAD(P)-glycohydrolase according to C. Bernofsky (10) and described in principle in our previous study (6). cADPR was biosynthetized using ADP-ribose cyclase contained in homogenized *Aplysia californica* ovotestis and incubated with 5mM β-NAD (11). After preparative procedures NAADP and cADPR (6) were evaporated to dryness in Speed Vac concentrator. Both NAADP and cADPR used in our experiments were at least 95% pure, as determined by HPLC.

Tissues from adult male Sprague-Dawley rats (200-250 g b wt) sacrificed under anesthesia namely brain, spleen, heart, liver and renal cortex were quickly dissected, chilled and minced in a solution containing 0.25 M sucrose, 20 mM triethanolamine-acetic acid; 20 μg/ml soybean trypsin inhibitor, 20 μg/ml leupeptin 20 μg/ml aprotinin and adjusted to pH=7.2. Tissues were homogenized (1:4 ; wt/vol) in a Dounce homogenizer using 4-5 strokes and centrifuged at 4000 x rpm for 10 min at 4° C. The supernatants were collected and used for enzymatic NAADP synthesis and hydrolysis. The protein content was measured by method of Lowry et al (12). In present study we used only freshly prepared tissue homogenates.

Sea urchins *Lytechinus pictus* and *Aplysia californica* were purchased from Mariners Inc., Long Beach, CA. Fluoro-3 was purchased from Molecular Probes, Eugene, OR; Inositol-1',4',5'-trisphosphate (IP_3), ryanodine, oligomycin and antimycin were from Calbiochem, Los Angeles, CA. All other reagents , all of the highest purity grade available, were supplied from Sigma Co., St. Louis, MO. Each experiment was repeated at least three times.

RESULTS

When β-NADP was coincubated with nicotinic acid and rat brain homogenate under the conditions outlined in legend to Fig. 1, the incubation mixture accumulated Ca^{2+}-releasing activity with properties indicating its identity as NAADP (Fig. 1). At the zero incubation time no Ca^{2+} release activity was detected indicating absence of any intrinsic Ca^{2+}-releasing compound in the tissue extract or in the assay medium containing NADP and nicotinate. During incubation at 37°C the Ca^{2+} releasing activity gradually appeared, as determined by Fluo-3 fluorescence, that increased with time and reached near-plateau between 40 and 60 minutes of incubation (Fig. 1, inset). Incubation of β-NADP with heat-inactivated extract produced no Ca^{2+}-releasing activity. Also, when the homogenate was incubated with α-NADP, no Ca^{2+}-releasing activity was produced (not shown). In the following the product of NADP incubation with nicotinate and tissue extract will be referred to as "incubate" or "assay media". In all following experiments the standard incubation was 60 minutes (Fig. 1).

The Ca^{2+}-release activity triggered by accumulated incubate was blocked neither by heparin, the blocker of the IP3 receptor, nor by ruthenium red, well-known inhibitor of cADPR activation of CICR (Fig. 2, inset); on the other hand, thio-NADP a selective inhibitor of NAADP (6) completely blocked Ca^{2+} release (Fig. 2). In the sea urchin homogenate bioassay, Ca^{2+} release triggered by the addition of the incubate rendered the system insensitive to the effect of subsequently added NAADP; however, addition of cADPR readily stimulated Ca $^{2+}$ release comparable to that elicited by incubate and also similar Ca^{2+} release was achieved by added IP3 (Fig. 2). Finally, when the incubate was deproteinized and subjected to HPLC analysis (6) an UV-absorbing peak, that contained Ca^{2+}-release activity, eluted exactly coincident with NAADP standard (Fig.3).

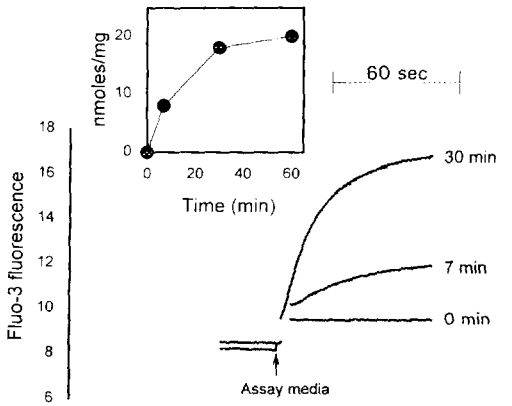

Figure 1. **Synthesis of NAADP incubating β-NADP with rat brain homogenates.** Brain homogenates (1 mg/ml, wet wt/vol), prepared as described in **Methods** and **Materials**, were incubated at 37°C in a medium containing 7 mM nicotinic acid, 0.2 mM β-NADP, and 40 mM triethanolamine-acetic acid buffer adjusted to pH = 7.2 (total volume 500 µl) for 7 min and 30 min. Aliquots (3 µl) of the assay media were tested for Ca^{2+}-release activity using the sea urchin egg homogenate bioassay (6). The inset shows the time-course of NAADP synthesis by rat brain homogenates; NAADP content was determined by calibration of the sea urchin egg homogenates with known concentrations of purified NAADP standards.

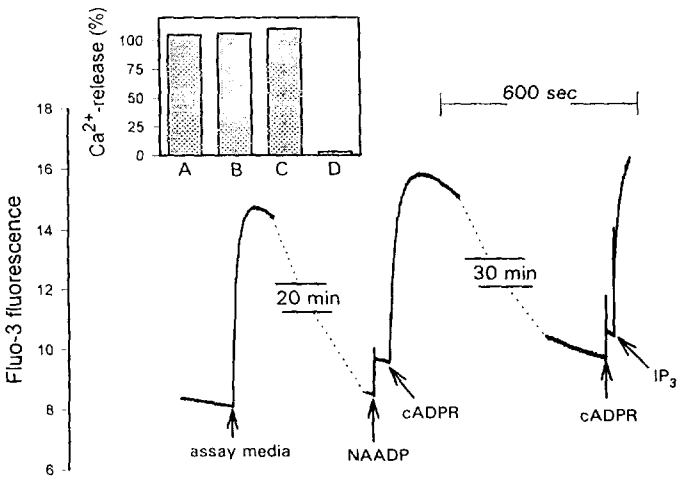

Figure 2. **Functional identification of the metabolite of β-NADP as NAADP.** The properties of Ca^{2+}-releasing activity generated by incubation of β-NADP and nicotinate with the brain homogenate as NAADP were indicated by **a)** homologous desensitization of Ca^{2+}-release system in the sea urchin egg homogenates and **b)** with use of specific inhibitors of Ca^{2+}-releasing agonists. Homologous desensitization of the sea urchin egg homogenate was determined by sequential additions, first of 3 µl of assay media from brain homogenate incubated with nicotinate and β-NADP for 60 min (as described in Fig. 1), then after 20 min of Ca^{2+} reuptake addition of 120 nM NAADP, then 120 nM cADPR, and after 30 min Ca^{2+}-reuptake additions of 120 nM cADPR, and then 4 µM IP_3. The inset shows the effects of specific inhibitors of Ca^{2+}-release systems on the Ca^{2+}-release triggered by addition of 3 µl brain homogenates, incubated as described above with NADP and nicotinate; **(A)** control, no inhibitors added; **(B)** pretreated with 36 µM ruthenium red; **(C)** with 320 µg/ml heparin; **(D)** with 40 µM thio-NADP. The results are expressed as % of the Ca^{2+} release induced under control conditions (**A**, no inhibitors).

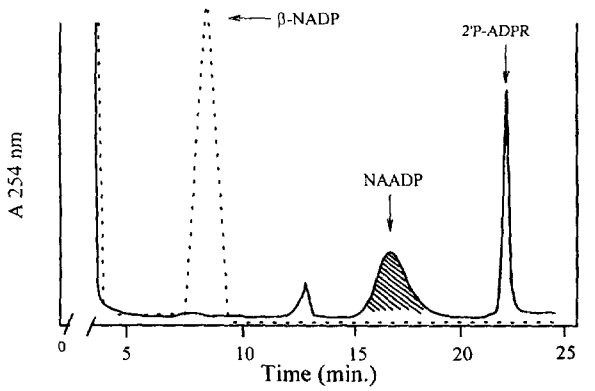

Figure 3. **HPLC analysis of NAADP.** Brain (1 mg/ml) homogenates were incubated with 0.2 mM β-NADP, 7 mM nicotinic acid and in a 40 mM triethanolamine-acetic acid buffer (pH=7.2) at 37°C for 2 hours. The reaction was stopped by addition of equal volume of cold acetone and centrifuged 2,000 x g for 2 min. After acetone evaporation, the supernatant was subjected to anion-exchange HPLC analysis (**Methods and Materials**). The figure shows HPLC analysis of incubate at time 0 min (------), and after 120 min (—) of incubation, (▨) Ca^{2+}-releasing activity.

When extracts from brain, spleen, heart, liver and kidney cortex were incubated with β-NADP and nicotinic acid as substrates under similar conditions, the highest Ca^{2+}-releasing activity was accumulated in brain extract incubate, considerably less Ca^{2+}-releasing activity was found in extracts from spleen, heart and liver and no Ca^{2+}-releasing activity was detected after β-NADP incubation with extract from kidney cortex (Table 1).

The catabolic activity for NAADP in tissue extracts was measured by incubation of 10 μM NAADP with tissue extracts for various time periods and the Ca^{2+}-releasing activity was determined by bioassay (details in legend to Fig. 4). The degradative activities (Fig. 4) in tested tissue extracts were in reverse rank, than the synthetic activities (Table 1). Incubation of NAADP with renal cortical extract resulted in rapid inactivation; the Ca^{2+}-releasing activity disappeared within a few minutes. On the other hand, extracts from liver and spleen were inactivated NAADP at much slower rate. When incubated with brain extract, even after 60 min incubation nearly 50% of the original Ca^{2+}-release activity of NAADP remained active (Table 1, Fig.4).

DISCUSSION

Results presented here show that rat tissues contain considerable enzymatic activities required for biosynthesis of NAADP as well as its enzymatic inactivation. The identity of the Ca^{2+}-releasing activity which resulted from coincubation of β-NADP and nicotinic acid with tissue extracts as authentic NAADP (6) was documented by several lines of evidence: a) selective blockage of the Ca^{2+}- response by thio-NADP, a specific inhibitor of NAADP (6) as compared to inhibitors of IP_3 and cADPR, b) homologous desensitization specific for NAADP and lack of heterologous desensitization as well as c) elution of Ca^{2+}-releasing activity on HPLC system coincident with authentic NAADP standard.

The generation of NAADP was most likely due to an enzymatic reaction catalyzing the exchange of nicotinamide for nicotinic acid in the molecule of NADP, as originally described by Carl Bernofsky (10). This interpretation is also consistent with the findings that omission of

Table 1

Comparison of NAADP synthetic and catabolic activities in homogenates of rat tissues[a]

	NAADP synthesis	NAADP inactivation
brain	++++	+
liver	+++	+++
myocardium	++	N.T.
spleen	++	++
kidney cortex	0	++++

O → + + + + denotes semiquantitative assessment of activity (O = no activity).
N.T. denotes not tested.
Data are based on three independent experiments.
[a]For details see Methods and Materials.

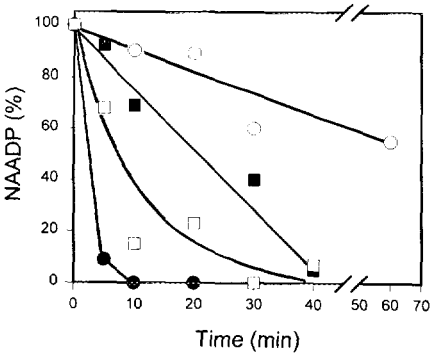

Figure 4. **The time-course of NAADP inactivation due to incubation with rat tissue homogenates.** Tissue homogenates prepared as described in Methods and Materials (1 mg protein/ml) were incubated with 10 μM NAADP in 20 mM Hepes-buffer (pH 7.4) at 37°C. The time course of NAADP inactivation was determined by addition of aliquots (3 μl) of the assay media to the sea urchin egg homogenate Ca^{2+}-release bioassay. Tissue homogenates were prepared from renal cortex (●), liver (□), spleen (■), and brain (○). The results are expressed as % of Ca^{2+}-release activity induced by addition of 3 μl of 10 μM NAADP substrate without incubation (time 0), taken as 100%.

nicotinic acid from the incubation mixture prevents accumulation of Ca^{2+}-releasing activity. The reaction is apparently stereospecific as is generation of cADPR from β-NAD (4) since α-NADP cannot replace β-NADP as a substrate

The question arises whether NAADP can be generated via this exchange reaction, most likely catalyzed by NAD(P)-glycohydrolase-like enzyme, also in intact cells as well. Under the present conditions, the concentration of cosubstrates, namely nicotinic acid was higher than it may be expected *in vivo*, based on the reports to date (13). However, as in many enzyme reactions, various aspects of the biocatalysis, such as increase of affinity for nicotinate, can be conceivably greatly facilitated by cofactors and activators of different nature present within the intact cells. Since the requirements for optimal exchange reaction catalyzed by NAD(P)-glycohydrolase *in vivo* are virtually unexplored and unknown to date, the NAADP-synthetizing reaction may possibly proceed in intact cells, under physiologic concentrations of cosubstrates and other components.

The inactivation of NAADP by incubation with extract in present experiments is unlikely due to activity of a single specific enzyme. Various phosphomonoesterases and phosphodiesterases, as shown in our previous study (6), may inactivate NAADP. Moreover, since the inosine derivative of NAADP lacks Ca^{2+}-releasing activity (6), deamination of the adenine moiety may also contribute to the loss of activity. Extraordinarily high inactivating activity found in the kidney extract could be due to the very high content and activity of alkaline phosphatase (14) and acid phosphatase (15) in proximal tubules. The rapid inactivation may also account for the observation that incubation of the cosubstrates with kidney extract yielded no detectable Ca^{2+}-releasing activity (Table 1), if nascent NAADP was immediately inactivated by enzymes present in the same incubation mixture. The apparent lack of NAADP generation in kidney cortical extract may be also due to the great diversity of cell populations (16) in this

organ, and may be limited to small portion of renal cortical tissue mass. For example, while we did not find appreciable cADPR formation in homogenates from the whole kidney cortical tissue, rather high activity of cADPR synthesis was found in isolated glomeruli (17), a structure which account for about < 8% of the renal cortical tissue mass (18). The presence of high synthetic capacity and low catabolic activity in the brain might suggest that NAADP-stimulated Ca^{2+}-release system could be very active in neural tissue, a tissue in which numerous other signaling pathways are also abundant and active. However, any speculations as to the functional significance of NAADP-controlled Ca^{2+}-release system ought to be extremely cautious at the present.

Finally, it should be emphasized that not all theoretically possible pathways for NAADP biosynthesis have been yet explored. Conceivably, NAADP can be generated by phosphorylation of NAAD, a compound present in the cells (19), catalyzed by NAD-kinase with ATP as a 2'-P phosphate donor. It remains to be explored whether NAD-kinase (20) accepts also NAAD instead of NAD as a cosubstrate or, alternatively, whether a specific NAAD-kinase may exist in tissues. NAADP can be also theoretically generated from β-NADP by a deamidating enzyme which would act upon nicotinamide moiety of β-NADP. The existence of these two alternative synthetic routes for generation of NAADP ought to be explored in future studies.

In conclusion, our study provides evidence for the existence of enzymatic activities capable of synthesis and degradation of β-NAADP, a novel Ca^{2+}-releasing agonist, in several rat tissues. These observations thus suggest that β-NAADP-regulated Ca^{2+}-releasing system, may not be confined only to sea urchin eggs (6) but potentially may be present and contribute to regulation of intracellular Ca^{2+} fluxes in number of mammalian tissues.

ACKNOWLEDGMENTS

This work was supported by NIH grants DK-30597, DK-16105, Grant-in-Aid from the National Kidney Foundation of the Upper Midwest and by the Mayo Foundation. Ms. Mary E. Bennett provided excellent secretarial assistance.

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