Metabolism of the novel Ca^{2+} -mobilizing messenger nicotinic acid–adenine dinucleotide phosphate via a 2'-specific Ca^{2+} -dependent phosphatase

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Nicotinic acid–adenine dinucleotide phosphate (NAADP) is a newly described Ca^{2+} -mobilizing nucleotide that appears to target intracellular Ca^{2+} -release channels distinct from those sensitive to inositol trisphosphate or ryanodine/cyclic ADP-ribose. Little, however, is known concerning the regulation of cellular NAADP levels. In the present study, we have characterized the metabolism of NAADP by brain membranes. From HPLC and MS analyses we show that loss of NAADP was associated with the appearance of a major product that is likely to be nicotinic acid–adenine dinucleotide (NAAD), the dephosphorylated form of NAADP. Dephosphorylation of NAADP, but not 3'-NAADP, was dramatically attenuated by Ca^{2+} chelators and stimulated by Ca^{2+} over a physiological range in a calmodulin-insensitive manner. In

contrast, NADP was metabolized predominantly to ADP-ribose phosphate via glycohydrolase activity, although slower Ca^{2+} -dependent dephosphorylation of both NADP and 2'-AMP could also be demonstrated. This is the first report describing a Ca^{2+} -regulated 2'-specific phosphatase which is probably the major pathway for the inactivation of NAADP in brain. Our data provide a potential feedback mechanism for limiting NAADP-induced Ca^{2+} release within cells through stimulation of NAADP metabolism by Ca^{2+} and strongly support a signalling role for this novel nucleotide in the brain.

Key words: calcium feedback, calcium oscillations, cyclic ADP-ribose, inositol trisphosphate.

INTRODUCTION

Release of Ca^{2+} from intracellular stores plays a pivotal role in the generation of cytosolic Ca^{2+} signals that in turn are intimately involved in the control of a whole host of cellular functions in both excitable and non-excitable cells [1]. Inositol trisphosphate (IP_3) is a ubiquitous and well characterized messenger which mediates Ca^{2+} increases in many cells through gating IP_3 receptors located primarily on the endoplasmic reticulum [2,3]. A second pathway involves activation of ryanodine receptors [4], a family of related intracellular Ca^{2+} -release channels modulated by cyclic ADP-ribose (cADPR) [5,6]. Central to the generation of complex Ca^{2+} signals, which typically result upon cell stimulation [7], is the regulation of both IP_3 and ryanodine receptors by Ca^{2+} itself [8]. Feedback effects of Ca^{2+} can be both stimulatory and inhibitory, allowing tight control of channel activity resulting in intricate spatial and temporal patterns of Ca^{2+} release [9].

Recently, a third pathway for intracellular Ca^{2+} release has been characterized activated by nicotinic acid–adenine dinucleotide phosphate (NAADP) [10,11], an endogenous NADP metabolite [12,13]. ADP-ribosyl cyclases (ARCs), in addition to catalysing the cyclization of NAD to cADPR, can also catalyse the synthesis of NAADP from NADP in the presence of nicotinic acid (NA). The latter base-exchange reaction is favoured at acidic pH [14,15]. That NAADP-induced Ca^{2+} mobilization in broken-cell preparations is unaffected by inhibitors of IP_3 and ryanodine/cADPR receptors suggests that NAADP targets a novel intracellular Ca^{2+} -release channel [15,16]. This notion is supported by radioligand binding studies [17–21]. Indeed, in contrast with IP_3 and cADPR, NAADP-induced Ca^{2+} mobilization is entirely unaffected by changes in cytosolic Ca^{2+} [13,22–24]. Clearly then, Ca^{2+} mobilized in response to NAADP is unlikely

to exert direct negative-feedback effects on channel opening, and other mechanisms must exist to limit NAADP-mediated Ca^{2+} increases. The unusual inactivation of this channel is probably one such mechanism. In sea-urchin egg homogenates, where the effects of NAADP have been most extensively studied [25], concentrations of NAADP that do not mediate any detectable Ca^{2+} release can cause full inactivation of the receptor [17,26]. Thus, Ca^{2+} mobilization in response to a normally maximal concentration of NAADP is completely abolished following pretreatment with concentrations of NAADP as low as 1 nM [17,26]. In pancreatic acinar cells [27] and T-lymphocytes [28], the concentration-dependence of NAADP-induced Ca^{2+} increases is again most unusual. In these systems, optimal Ca^{2+} release is observed at ≈ 100 nM, whereas at higher concentrations (1–100 μM), NAADP receptors inactivate without detectable Ca^{2+} release, giving rise to a bell-shaped concentration–response curve [27,28]. These data provide strong evidence that NAADP receptors are a new class of intracellular Ca^{2+} -release channels, which, as a recent elegant study suggests, may be located on a Ca^{2+} store physically separate from those expressing IP_3 and ryanodine receptors [29]. However, in intact cells, NAADP-, IP_3 - and cADPR-induced Ca^{2+} -release pathways interact functionally, a process which is likely to be important in co-ordinating Ca^{2+} signals [30,31]. Although sea-urchin eggs [32,33] and several mammalian tissues [34] have the capacity to both synthesize and metabolize NAADP, little is known concerning the regulation of these activities.

In the present study we have characterized the metabolism of NAADP by brain membranes. We show for the first time that (i) NAADP is metabolized predominantly by dephosphorylation to nicotinic acid–adenine dinucleotide (NAAD) and (ii) that this activity is stimulated by Ca^{2+} . Such regulation by Ca^{2+} may

Abbreviations used: ADPR, ADP-ribose; ADPRP, ADP-ribose phosphate; ARC, ADP-ribosyl cyclase; AP, alkaline phosphatase; BAPTA, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; 2'-P-AMP, 2',5'-adenosine bisphosphate; cADPR, cyclic ADP-ribose; IP_3 , inositol trisphosphate; MALDI-TOF, matrix-assisted laser-desorption-ionization time-of-flight; Nic, nicotinamide; NA, nicotinic acid; NAAD(P), nicotinic acid–adenine dinucleotide (phosphate); NAMN, NA mononucleotide; NMN, Nic mononucleotide.

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provide a feedback mechanism for terminating NAADP-induced Ca^{2+} increases further supporting a signalling role for NAADP in the brain [35].

MATERIALS AND METHODS

Membrane preparation

All procedures were performed at 4 °C. Whole brains from male CD1 mice were homogenized using an Ultra-Turrax homogenizer in a medium composed of 20 mM Hepes, pH 7.2, supplemented with a protease-inhibitor cocktail (Boehringer-Mannheim) either in the absence or presence of EDTA (1 mM) as indicated. A cytosolic fraction was prepared by centrifugation of the crude homogenate at 100 000 *g* for 60 min and collection of the supernatant. Membranes were prepared by centrifugation of the homogenate at 20 000 *g* for 10 min and resuspending the pellet using a Dounce homogenizer (tight-fitting plunger). This procedure was repeated twice more and the final preparation (≈ 10 mg of protein/ml) was divided into portions and stored at -80 °C until use. In some experiments, brain membranes (2.5 mg of protein/ml) were solubilized with 1% Triton X-100 for 60 min. Samples were centrifuged at 100 000 *g* for 60 min, the resulting pellets washed twice by centrifugation (10 min, 20 000 *g*) and the detergent-insoluble membranes resuspended in their original volume.

Metabolism assays and HPLC analysis

Membranes (≈ 5 mg of protein/ml) were incubated with the indicated nucleotide at 37 °C in a buffer containing 20 mM Hepes, with or without Ca^{2+} chelators as appropriate and, at various times, the reaction was quenched by the addition of an equal volume of ice-cold ethanol. Particulate material was removed by centrifugation (12 000 *g*, 1 min) and supernatants analysed for nucleotide content by anion-exchange HPLC. Samples (125 pmol–125 nmol) were injected on to a 3 mm $\times$ 150 mm column packed with the ion-exchange support material AGMP1 (Bio-Rad). Elution was performed at a flow rate of 4 ml/min using a gradient of trifluoroacetic acid that increased linearly from 0 to 2% over the first 1.5 min, to 4% at 3 min, to 8% at 4.5 min, to 16% at 6 min, to 32% at 7.5 min, to 64% at 9 min and to 100% (150 mM trifluoroacetic acid) at 10.5 min. Absorbance was monitored at 254 nm and area measurements determined using Breeze Software (Waters). Initial rates were measured as disappearance of substrate over time points where metabolism of the nucleotide was approximately linear (typically 20–30% metabolism over 5 min–5 h, depending on substrate concentration). For purification of the metabolites, the flow rate was reduced to 1 ml/min as described in [19,20], and the collected fractions dried by vacuum concentration, washed twice in methanol to remove trifluoroacetic acid and resuspended in water.

MS

Matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) MS was performed on a Voyager Elite XL mass spectrometer (Applied Biosystems, Framingham, MA, U.S.A.) in the reflector mode using delayed extraction. A 0.5 μ l aliquot of an aq. 5–10 μ M analyte solution was mixed with 1 μ l of a saturated aq. 2,5-dihydroxybenzoic acid solution directly on the target plate and subsequently dried under a warm stream of air. The mass spectrum recorded was externally calibrated using the calibration mixture 1 of the Sequazyme™ peptide mass standard kit (Applied Biosystems).

Enzyme assays

In experiments, examining the effects of nucleotide pyrophosphatase (1.75 unit/ml) and NADase (0.25 unit/ml), incubations were performed at 37 °C in a buffer containing 20 mM Hepes, pH 7.2. Alkaline phosphatase (AP; 1 unit/ml) and ARC (1 unit/ml) treatments were performed in buffers containing 20 mM Tris, pH 8.8, and 20 mM NNA/20 mM Mes, pH 5, respectively. All enzymes were from Sigma Chemical Co.

Other methods

3'-NAADP was synthesized as described previously [36]. Free Ca^{2+} concentrations in the medium were determined with a Ca^{2+} electrode [37]. Protein concentrations were determined using bicinchoninic acid ('BCA'; Sigma Chemical Co.). Data are presented as means $\pm$ S.E.M. from at least two different membrane preparations.

RESULTS AND DISCUSSION

Metabolism of NAADP by brain membranes

Crude brain membranes (prepared in the absence of EDTA) were incubated with NAADP and, at times thereafter, NAADP content quantified by HPLC. Following a 120 min incubation with 100 μ M NAADP, $68 \pm 9\%$ ($n = 3$) of the adenine nucleotide had been metabolized (Figure 1A). The initial rate of NAADP metabolism increased with increasing NAADP concentration over the range 1–100 μ M and was half-maximal at a substrate concentration of ≈ 7 μ M (Figure 1B). This value is within the concentration range over which NAADP mediates Ca^{2+} mobilization from brain microsomes ($\text{EC}_{50} \approx 1$ μ M) [24] and K_m values for metabolism of the established messengers, IP_3 (25 μ M for IP_3 phosphomonoesterase) and cADPR (160 μ M for cADPR hydrolase) [38,39]. Although the initial rate of NAADP metabolism was maximal at NAADP concentrations of 100 μ M (163 ± 6 pmol/min per mg, $n = 9$) and 300 μ M (163 ± 23 pmol/min per mg, $n = 5$), the rate was somewhat reduced at a substrate concentration of 1 mM (105 ± 15 pmol/min per mg, $n = 6$; Figure 1B). This apparent decrease in the rate may be due to inactivation of the enzyme during the extended incubations (> 3 h) required in order to accurately measure metabolism of NAADP at this substrate concentration. Metabolism of NAADP (100 μ M) by cytosolic fractions was minimal (5 ± 2 pmol/min per mg, $n = 3$). Previous studies have demonstrated NAADP metabolism by brain homogenates using a sea-urchin-egg bioassay to determine NAADP concentration by its ability to mobilize intracellular Ca^{2+} stores [34]. Our data confirm more directly that NAADP is metabolized by brain and that this activity is membrane-bound.

NAADP is dephosphorylated to NAAD

NAADP could be metabolized in a number of ways, including dephosphorylation to NAAD, and amidation to NADP. Both of these potential metabolites are inactive in Ca^{2+} -release and [^{32}P]NAADP-binding assays [15–17,19]. Analysis of chromatograms indicated that loss of NAADP (retention time 6.7 ± 0.03 min, $n = 10$) in the presence of brain membranes was associated with the appearance of a prominent product with a retention time of 4.2 ± 0.02 min ($n = 22$) (Figure 1A). The retention time of this primary metabolite was similar to that of NAAD (4.1 ± 0.04 min, $n = 7$); NADP, however, was consistently eluted later (retention time 4.5 ± 0.03 min, $n = 7$).

In order to further establish its identity, the primary metabolite was purified, subjected to various enzyme treatments and the

Figure 1 Metabolism of NAADP by brain membranes

(A) HPLC analysis of NAADP content (initial concentration 100 μM) following incubation with brain membranes for the times indicated. The primary NAADP metabolite (PM) is marked. Elution of NAAD, NADP (broken line) and NAADP is shown for comparison. (B) Effect of substrate concentration on initial rate of NAADP metabolism. Results are means ± S.E.M. for three to nine experiments.

products generated compared with those following similar treatment of authentic NADP and NAAD as depicted in Figure 2. Alkaline phosphatase converted NADP into a product with retention time of 2.1 ± 0.1 min ($n = 3$) similar to that of NAD (2 ± 0.06 min, $n = 3$) (Figure 3) and consistent with removal of the 2'-phosphate group (Figure 2). In contrast, NAAD (which lacks the 2'-phosphate group; Figure 2) and the primary metabolite were unaffected by similar treatments (Figure 3, $n = 2$). NADP, NAAD and the primary metabolite were also incubated with ARC in the presence of NA. Under acidic conditions (pH 5), this enzyme catalysed the conversion of NADP into a product (retention time 6.7 ± 0.04 min, $n = 3$) that was co-eluted with NAADP (retention time 6.7 ± 0.03 min, $n = 10$; Figure 3). These results confirm base-exchange of nicotinamide (Nic) of NADP with NA [14,15]. In contrast, incubation of NAAD ($n = 3$) or the primary metabolite ($n = 2$) with ARC and NA was without effect on retention time of the substrate, probably reflecting futile exchange of NA (Figure 3). Finally, the effects of nucleotide pyro-

Figure 2 Schematic structure of NADP and NAAD

(A) NAAD (right) differs from NADP (left) in that the amide and phosphate groups (circled) are replaced by hydroxy groups. NAAD is therefore not a substrate for alkaline phosphatase (AP) or ARC/NA and yields distinct products to NADP following nucleotide pyrophosphatase (NP) treatment (B).

Figure 3 NAADP is dephosphorylated to NAAD

HPLC analysis following treatment of 25 μM NADP (top row), NAAD (second row) and purified primary NAADP metabolite (PM, third row) with alkaline phosphatase (AP, left column), ADPCR/NA (middle column) and nucleotide pyrophosphatase (NP, right column). Retention times of the products were compared (shaded boxes) with known standards (5 nmol; bottom row).

phosphatase were compared. Incubation of NADP with this enzyme generated three products with retention times of 0.3 ± 0.03 min, 2.1 ± 0.08 min and 5.4 ± 0.07 min ($n = 3$, Figure 3). The

Figure 4 MS analysis of the NAADP metabolite

MALDI-TOF mass spectra of NADP (**A**), NAAD (**B**) and purified primary NAADP metabolite (**C**). *m/z* values are marked. The theoretical *m/z* values for protonated NADP and NAAD ions are 744.08 and 665.10 respectively. Cleavage of the N-glycosidic bond of NADP and NAAD results in the generation of ADPRP (theoretical *m/z* 622.04, loss of nicotinamide) and ADPR (theoretical *m/z* 542.07, loss of nicotinic acid) respectively. Data are representative of experiments using two different preparations of purified NAADP metabolite.

first product (eluted in the void volume and thus not negatively charged) is likely to be Nic mononucleotide (NMN; retention time 0.4 ± 0.06 min, $n = 3$) and the most highly charged species eluted last is likely to be 2',5'-adenosine bisphosphate (2'-P-AMP), consistent with cleavage of the pyrophosphate linkage. The identity of the second product is unknown at present. In contrast, treatment of NAAD with this enzyme resulted in the generation of two products with retention times of 2.4 ± 0.05 min and 3.2 ± 0.05 min ($n = 3$, Figure 3). The first product was co-eluted with AMP (retention time 2.2 ± 0.09 min, $n = 3$), again confirming hydrolysis of the pyrophosphate bond (Figure 2). Treatment of the primary metabolite with nucleotide pyrophosphatase also yielded two products with similar retention times (2.3 ± 0.02 min and 3.2 ± 0.07 min, $n = 3$) as for NAAD (Figure 3). Clearly then, NAADP is metabolized to a product treatment

Figure 5 NAADP metabolism is Ca^{2+} -dependent

Membranes prepared either in the absence ($\circ$) or presence ($\bullet$) of EDTA (1 mM) were incubated with $100 \mu\text{M}$ NAADP (**A**) or cADPR (**B**) and nucleotide content determined at various times. (**C**) Ca^{2+} -dependence of NAADP metabolism. Initial rate of metabolism of NAADP ($100 \mu\text{M}$) was determined in a buffer containing 2.5 mM BAPTA and various concentrations of CaCl_2 (0–2.5 mM) to set the free Ca^{2+} concentration as indicated. Data are means $\pm$ S.E.M. for four to six experiments.

of which with three mechanistically distinct enzymes yields a profile identical with that of authentic NAAD.

Finally, we performed MALDI-TOF MS in order to identify the primary NAADP metabolite. In mass spectra recorded from samples of NADP (Figure 4A), the base peak (*m/z* 744.14) corresponded to the theoretical intact protonated molecular ion (*m/z* 744.08) within 100 p.p.m. Similarly, analysis of NAAD, indicated a base peak (*m/z* 665.15) similar to that of its theoretical value (*m/z* = 665.10). In both samples, secondary peaks were also identified with *m/z* values of 622.07 and 542.10 for NADP and NAAD respectively. These ions are probably generated from cleavage of the N-glycosidic bond of NADP to produce ADP-ribose phosphate (ADPRP; theoretical *m/z* 622.04) and NAAD to produce ADP ribose (theoretical *m/z* 542.07) (Figures 4A and 4B). Analysis of the purified NAADP metabolite (Figure 4C) generated a spectrum almost identical with that of NAAD with ion masses of *m/z* 665.16 and 542.10 (Figure 4C). Taken together with the data from Figure 3, dephosphorylation of NAADP at the 2' position to NAAD is likely to be a major metabolic route for inactivation of NAADP in brain tissue.

NAADP metabolism is Ca^{2+} -dependent

Inclusion of the bivalent-cation chelator EDTA (1 mM) during the isolation of brain membranes was found to dramatically attenuate subsequent metabolism of NAADP. Thus the initial rate of metabolism of NAADP ($100 \mu\text{M}$) was reduced from 146 ± 16 pmol/min per mg ($n = 4$) in the absence of EDTA to

Figure 6 Metabolism of 3'-NAADP by brain membranes

(A) HPLC analysis of 3'-NAADP content (initial concentration 100 μ M) before and 60 min after incubation with brain membranes. The primary 3'-NAADP metabolite (PM) is marked. (B) Membranes prepared either in the absence (○) or presence (●) of EDTA (1 mM) were incubated with 100 μ M 3'-NAADP and nucleotide content determined at various times. Results are means $\pm$ S.E.M. for three experiments.

5 $\pm$ 7 pmol/min per mg (n = 6) in its presence (Figure 5A). By contrast, EDTA-treated membranes readily metabolized cADPR (100 μ M) at a rate similar to membranes prepared in the absence of EDTA (Figure 5B). These data uncover a strict requirement for bivalent cations in the metabolism of NAADP. In order to determine whether the effects of EDTA were due to chelation of Ca²⁺, NAADP metabolism by brain membranes was measured in media in which the free Ca²⁺ concentration was buffered with the more Ca²⁺-selective chelator bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid (BAPTA). From these experiments, the initial rate of NAADP metabolism was markedly increased by Ca²⁺, with half-maximal stimulation occurring at a free Ca²⁺ concentration of $\approx$ 40 nM (Figure 5C). This value approximates that of the resting cytosolic Ca²⁺ concentration in intact cells [1]. Clearly then, the phosphatase responsible for NAADP metabolism is well suited to detect physiological changes in cytosolic Ca²⁺ concentration. Preincubation of brain membranes with calmodulin antagonists was without effect on the metabolism of NAADP. Thus the initial rate of NAADP metabolism in the presence of *N*-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide hydrochloride ('W-7'; 100 μ M) and calmidazolium chloride (10 μ M) was 100 $\pm$ 6% (n = 3) and 96% (n = 2) respectively of that measured in the absence of the inhibitors.

Metabolism of 3'-NAADP by brain membranes

We next examined the metabolism of 3'-NAADP, an analogue of NAADP in which the adenine-linked ribose group is phosphorylated at the 3' instead of the 2' position (Figure 6). 3'-NAADP (retention time 6.9 $\pm$ 0.004 min, n = 14) was metabolized predominantly to a product with a retention time of 4.2 $\pm$ 0.01 (n = 11; Figure 6A). This product is similar to the product generated following metabolism of NAADP (retention time 4.2 $\pm$ 0.02 min, n = 22; Figure 1) and is likely to be NAAD (retention time

Figure 7 Metabolism of NADP by brain membranes

(A) HPLC analysis of NADP content (initial concentration 100 μ M) before (broken line) and 5 min after incubation with brain membranes. The primary NADP metabolite (PM) is co-eluted (shaded bar) with ADPR generated following treatment of NADP (100 μ M) with NADase (B). (C) Treatment of the purified NADP metabolite with alkaline phosphatase (AP) converts it to a product that is co-eluted with ADPR (D).

4.1 $\pm$ 0.04 min, n = 7). Thus both 3'-NAADP and NAADP are metabolized via dephosphorylation. However, in contrast with NAADP (Figure 5A), the initial rate of 3'-NAADP metabolism was largely insensitive to EDTA (Figure 6B). These data suggest that the two analogues are dephosphorylated independently and that Ca²⁺-dependent dephosphorylation is specific for the 2' position.

Metabolism of NADP by brain membranes

In order to further assess the specificity of this enzyme, the metabolism of NADP was characterized. NADP, like NAADP, is phosphorylated at the 2' position of the ribose group. Incubation of NADP (100 μ M) with brain membranes resulted in the rapid (14.8 nmol/min per mg, n = 2) conversion of NADP into a product with a retention time of 8.3 $\pm$ 0.1 min (n = 5; Figure 7A). That this product was eluted later than its substrate during anion exchange indicated that it was more negatively charged and thus unlikely to be generated by dephosphorylation (removal of negative charge). Indeed, NAD was eluted much earlier (retention time 2 $\pm$ 0.06 min, n = 3). Two lines of evidence suggest that the primary NADP metabolite is ADPRP. First, this product was co-eluted with the product (retention time 8.3 $\pm$ 0.02 min, n = 3) generated following treatment of NADP with NADase (Figure 7B). This enzyme cleaves the Nic-ribose bond of both NAD and NADP to generate ADPR and ADPRP respectively. Secondly, treatment of the NADP metabolite (following purification) with AP resulted in its conversion into a product (retention time 5.3 $\pm$ 0.02 min, n = 3) that was co-eluted with ADPR (retention time 5.3 $\pm$ 0.06 min, n = 3; Figures 7C and 7D). Taken together, these data suggest that the predominant pathway for metabolism of NADP is through glycohydrolase activity generating ADPRP.

Figure 8 Dephosphorylation of NADP and 2'-AMP

HPLC analysis of NADP (**A** and **B**) and 2'-AMP (**C**) content (initial concentration 100 μ M) following incubation with detergent-treated membranes either in the absence (left) or presence (right) of 2.5 mM BAPTA. NA (10 mM) was included in incubations in (**B**). Reaction times were either 15 min (**A**) or 2 h (**B** and **C**).

Dephosphorylation of NADP and 2'-AMP

As shown previously, metabolism of NAADP by brain membranes was associated with the accumulation of NAAD and not ADPRP, despite incubation times (up to 120 min; Figure 1) well in excess of those required for complete metabolism of NADP to ADPRP (< 5 min). These data indicate clearly that NAADP is not metabolized to an appreciable extent by glycohydrolase activity as is NADP. However, this marked difference in the rate of glycohydrolase activity (against NADP) and phosphatase activity (against NAADP) may have masked possible Ca^{2+} -dependent dephosphorylation of NADP. We therefore sought to separate these two activities. Initial attempts to inhibit glycohydrolase activity with the inhibitor Nic proved unsuccessful. However, we found that treatment of brain membranes with the non-ionic detergent Triton X-100 resulted in significant solubilization of glycohydrolase but not phosphatase activity (results not shown). Accordingly, the rate of metabolism by detergent-treated membranes compared with untreated brain membranes was reduced 1.9-fold for NADP and increased 1.8-fold for NAADP ($n = 2$ independent solubilizations). This treatment therefore resulted in an approx. 3.4-fold enrichment of phosphatase activity relative to glycohydrolase activity. Under these conditions, metabolism of NADP could be effectively inhibited by nicotinamide, the initial rate of NADP metabolism was

reduced from 8.1 ± 0.4 nmol/min per mg ($n = 3$) to 0.48 ± 0.1 nmol/min per mg ($n = 3$) in the presence of 10 mM nicotinamide. Analysis of chromatograms following brief incubations (15 min) of NADP in the absence of nicotinamide (Figure 8A) indicated that the predominant NADP metabolite had a retention time of 8.3 ± 0.009 min ($n = 6$). This product is similar to that following metabolism of NADP by untreated brain membranes and authentic NADase (Figure 7) and thus consistent with metabolism via glycohydrolase activity. However, in the presence of Nic (Figure 8B) and following more prolonged incubations (2 h), the major NADP metabolite produced by detergent-treated membranes had a retention time (1.9 ± 0.1 min, $n = 4$) similar to that of NAD (2 ± 0.06 min, $n = 3$). These data are consistent with slower dephosphorylation of NADP to NAD^+ , revealed only following blockade of glycohydrolase activity. In order to determine whether metabolism of NADP by detergent-treated membranes was Ca^{2+} -dependent, the effect of BAPTA was examined. As shown in Figure 8(A), the initial rate of NADP metabolism was reduced only $21 \pm 4\%$ ($n = 3$) by BAPTA when measured in the absence of Nic. However, in the presence of Nic (Figure 8B), the rate of NADP metabolism (probably via dephosphorylation) was completely inhibited by BAPTA ($n = 3$).

Finally, the metabolism of 2'-AMP was examined (Figure 8C). This analogue (retention time 2.4 ± 0.08 min, $n = 5$), also phosphorylated at the 2' position of ribose, was metabolized to (a) product(s) that did not interact with the anion-exchange medium (retention time 0.28 ± 0 min, $n = 3$). These data are consistent with its conversion into adenosine via dephosphorylation. Furthermore, metabolism of 2'-AMP was Ca^{2+} -sensitive, as it was completely inhibited by BAPTA (Figure 8C; $n = 3$). From competition experiments, both NADP (in the presence of 10 mM Nic) and 2'-AMP reduced the initial rate of NAADP metabolism by $52 \pm 7\%$ ($n = 3$) and $63 \pm 5\%$ ($n = 3$), respectively (substrate concentration 100 μ M). Taken together, these data provide evidence that all three nucleotides are metabolized via the same 2'-specific, Ca^{2+} -dependent phosphatase.

Conclusions

In the present study we have, for the first time, identified a pathway for NAADP metabolism through removal of the 2' phosphate and shown that this newly described activity is regulated by Ca^{2+} over the physiological range. We demonstrate that NAADP is not metabolized through glycohydrolase activity – the major route for metabolism of NADP – although both NADP and 2'-AMP are dephosphorylated in a Ca^{2+} -dependent fashion. Since NAADP activates Ca^{2+} release in a number of varied cell types and preparations, including rat brain microsomes [24] and nerve terminals of the frog neuromuscular junction [40], the ensuing increases in Ca^{2+} are likely to limit further Ca^{2+} increases through accelerating NAADP metabolism. Such regulation may provide a feedback mechanism for regulating NAADP responses. Indeed, that Ca^{2+} release via NAADP receptors is *not* modulated by Ca^{2+} [13,22–24] is consistent with the shaping of NAADP-induced Ca^{2+} signals primarily through regulation of NAADP levels. In this respect, feedback effects of Ca^{2+} on NAADP metabolism could support oscillatory Ca^{2+} signals. The Ca^{2+} sensitivity of NAADP metabolism is analogous with the stimulatory effects of Ca^{2+} on metabolism of IP_3 , through IP_3 3-kinase [41] and cyclic AMP/GMP, through Ca^{2+} -sensitive phosphodiesterases [42]. Tight control of NAADP levels by Ca^{2+} supports further a messenger role for NAADP during Ca^{2+} signalling [35].

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