Determination of cellular nicotinic acid–adenine dinucleotide phosphate (NAADP) levels

Dev CHURAMANI*, Elizabeth A. CARREY†¹, George D. DICKINSON* and Sandip PATEL*²

*Department of Physiology, University College London, The Old Squash Courts, London WC1E 6BT, U.K., and †Purine Research Unit, Guy's Hospital, Thomas Guy House, London SE1 9RT, U.K.

Nicotinic acid–adenine dinucleotide phosphate (NAADP) is fast emerging as a new intracellular Ca^{2+} -mobilizing messenger. In sea urchin egg homogenates, binding of NAADP to its receptor is not readily reversible; hence, prior incubation with low concentrations of NAADP is more effective in inhibiting subsequent binding of radiolabelled NAADP than incubating the preparation with the two ligands simultaneously [Patel, Churchill and Galione (2000) *Biochem. J.* **352**, 725–729]. We extend this finding to show that NAADP is more effective still in inhibiting the subsequent radioligand binding at lower homogenate concentrations, an effect again quite probably due to the non-reversible nature of the

receptor–ligand interaction. Enhanced sensitivity of the preparation to NAADP afforded by simple manipulation of the experimental conditions has been applied to determine low levels of NAADP in acid extracts from human red blood cells, rat hepatocytes and *Escherichia coli* without interference from NADP breakdown. Our improved method for the quantification of NAADP should prove useful in the further assessment of its signalling role within cells.

Key words: calcium, nicotinic acid–adenine dinucleotide phosphate (NAADP), radioreceptor assay, sea urchin egg.

INTRODUCTION

Mobilization of intracellular Ca^{2+} stores by Ca^{2+} -mobilizing messengers is a ubiquitous pathway for the generation of cytosolic Ca^{2+} signals [1]. Tight regulation by Ca^{2+} itself of receptor– Ca^{2+} channel complexes, activated by, for example, IP_3 (inositol trisphosphate) [2,3], contributes to the remarkably complex Ca^{2+} signals that often result [1,4]. Indeed, subtle differences in the way Ca^{2+} signals are organized in terms of both time (frequency-modulated Ca^{2+} oscillations) and/or space (local versus global Ca^{2+} increases) are probably decoded by the cell, thus providing appropriate specificity to allow a single ion to control such a vast array of cellular processes [5].

Of late, much interest has been devoted to the Ca^{2+} -mobilizing properties of NAADP (nicotinic acid–adenine dinucleotide phosphate) [6–11]. In the sea urchin egg, where the actions of this NADP analogue were first described [12–14], the pharmacology of NAADP-induced Ca^{2+} release is consistent with the existence of a novel intracellular Ca^{2+} channel [15]. This channel undergoes an unusual form of desensitization [16,17] and is not regulated by changes in cytosolic Ca^{2+} [18,19]. Radioligand binding studies and biochemical analysis are also consistent with the activation by NAADP of a novel target protein [20]. For example, binding of NAADP to its receptor in sea urchin eggs is not readily reversible [16,21,22], at least in the presence of physiological concentrations of K^+ [23]. Moreover, recent studies in several cell types suggest that these receptors may reside on Ca^{2+} stores distinct from the endoplasmic reticulum [18,24–27], an established Ca^{2+} store where IP_3 and ryanodine receptors reside. Thus several features of NAADP-mediated Ca^{2+} release clearly distinguish it from more established Ca^{2+} mobilization mechanisms regulated by IP_3 and cyclic ADP-ribose.

Although NAADP-induced Ca^{2+} release has been described in many systems, including plants, echinoderms, amphibians and

mammals [6–11], scant information is available regarding cellular levels of NAADP. Indeed, it is only very recently, using a radioreceptor assay based on the binding of [^{32}P]NAADP to sea urchin egg homogenates [16,21,22], that NAADP levels have been quantified within cells. Sea urchin spermatozoa appear to contain high (μM) levels of NAADP [28,29]. Importantly, NAADP levels increase in the spermatozoa on contact with egg jelly, raising the exciting possibility that the spermatozoa may provide a bolus of NAADP to the egg during fertilization [29]. Increases in NAADP levels have also been reported in pancreatic β cells stimulated with glucose [30]. Therefore, at least in these two cellular systems, NAADP levels are controlled by physiologically relevant stimuli. Taken together with the finding that metabolism of NAADP, via dephosphorylation, is increased by Ca^{2+} and, thus, regulated [31], these new studies provide strong evidence that NAADP is a bona fide intracellular Ca^{2+} -mobilizing messenger [32–34]. However, molecular details of how NAADP is generated and degraded are lacking. Although ADP-ribosyl cyclases, a family of multifunctional enzymes that include the mammalian homologue CD38 [35], are capable of synthesizing NAADP from NADP and nicotinic acid *in vitro* [36], and this base-exchange activity is demonstrable in various tissues from wild-type mice, but not in mice in which the gene for CD38 has been genetically deleted [37], whether NAADP is derived from this enzymic route *in vivo* is not known.

In general, NAADP mediates Ca^{2+} release over the nM range [6–11]. With the exception of sea urchin spermatozoa [28,29], cellular levels of this nucleotide are therefore probably maintained well below this value. Sensitive methods to detect NAADP are therefore vital to quantify accurately cellular levels, particularly under basal (non-stimulated) conditions. In the present study, we have utilized the non-dissociating nature of NAADP binding to its receptor in sea urchin eggs [16,21,22] to improve the sensitivity of this preparation as a radioreceptor assay for the

Abbreviations used: IP_3 , inositol trisphosphate; NAADP, nicotinic acid–adenine dinucleotide phosphate; NAD(P)ase, NAD glycohydrolase.

¹ Present address: Institute of Child Health, 30 Guilford Street, London WC1N 1EH, U.K.

² To whom correspondence should be addressed (e-mail patel.s@ucl.ac.uk).

determination of NAADP levels. We have applied this method to quantify low levels of NAADP in several cell types. Our methods should prove useful in studies examining the signalling role of this novel nucleotide.

MATERIALS AND METHODS

Preparation of human red blood cell extracts

Red blood cells were isolated from human blood obtained from volunteers by the method described in [38]. Cells were pelleted by centrifugation and extracted with 2 packed cell volumes of trichloroacetic acid (10 %, w/v) for 1–240 min (as indicated) at room temperature (22 °C). The precipitated protein was removed by centrifugation (12 000 *g*, 2 min) and trichloroacetic acid was back-extracted from the supernatant with water-saturated diethyl ether (5 × 1 ml). Extracts not analysed immediately were frozen at –20 °C or freeze-dried. Packed red blood cells typically had haematocrit values of 96–98 %, measured by microcentrifugation in capillary tubes (Hawksley, Lancing, U.K.), 33 % of which were assumed to be cell membranes. Thus, in a typical extraction [100 µl of packed cells (1.1 × 10⁸ cells, 25 mg of protein) + 200 µl of trichloroacetic acid and 97 % haematocrit], the cytoplasmic volume was estimated to be 65 µl (0.97 × 0.67 × 100 µl) and the total volume was 268 µl (65 µl of cytoplasm + 3 µl of dead volume + 200 µl of trichloroacetic acid). Cytoplasmic constituents were therefore diluted by approx. 4-fold (268:65) after extraction. The efficiency of nucleotide recovery (88 ± 5 %, *n* = 3) was determined by including [³²P]NAADP (approx. 20 000 c.p.m.; see below) with the cells and acid during extraction, followed by scintillation counting of the recovered sample.

Red blood cells were also extracted using HClO₄ as described in [30]. In these experiments, cells were extracted with 1 packed cell volume of HClO₄ (1.5 M) for 60 min on ice. The samples were then centrifuged for 1 min at 13 000 *g* and the supernatant samples were diluted with an equal volume of KHCO₃ (2 M). After a further 60 min incubation on ice, the insoluble potassium perchlorate was removed by centrifugation. The final pH of the supernatant was 8–8.5. Recovery of NAADP by this method was 60 ± 3 % (*n* = 3).

Preparation of other cell extracts

Hepatocytes from male Sprague–Dawley rats were isolated by EDTA perfusion of the liver, followed by centrifugation on a Percoll gradient as described in [39].

Competent *Escherichia coli* (JM109; Promega) were transformed with a vector conferring ampicillin resistance (pGEM®-3Z) and cultured for 16 h at 37 °C in Terrific Broth, supplemented with 0.4 % (v/v) glycerol and 100 µg/ml ampicillin. J774 murine macrophages were cultured in Dulbecco's modified Eagle's medium, supplemented with 10 % (v/v) foetal bovine serum and antibiotics, and were maintained as suspensions at 37 °C in an atmosphere of 5 % CO₂. All the cells were harvested by centrifugation and washed twice with PBS. Extractions (100 µl packed cell volume + 200 µl of trichloroacetic acid) were performed as described above for red blood cells.

The numbers of cells (and total protein content) used were 1.3 × 10⁷ (10 mg), 7 × 10⁷ (5 mg) and 5 × 10⁷ (12 mg) for hepatocytes, *E. coli* and J774 cells respectively.

Anion-exchange HPLC

Nucleotides were separated either on a Hypersil® NH₂ column (250 mm × 3.2 mm; Phenomenex, Macclesfield, Cheshire, U.K.)

or on a column (3 mm × 150 mm) hand-packed with AG® MP-1 (Bio-Rad Laboratories). For separations using Hypersil®, the samples (10–100 µl) were injected on to the column after equilibration with 5 mM KH₂PO₄ (pH 2.65). The bound material was eluted with a buffer composed of 0.5 M KH₂PO₄ and 1 M KCl (pH 3.8) using a linear gradient (30 min separation; flow rate, 0.5 ml/min). For separations using AG® MP-1, samples (200 µl) were injected on to a 1 ml column equilibrated with water. The bound material was eluted with a concave-up gradient of trifluoroacetic acid, which increased linearly to 2 % at 6 min and to 4, 8, 16, 32, 64 and 100 % (150 mM trifluoroacetic acid) at 12, 18, 24, 30, 36 and 40 min respectively. The flow rate was either 0.5 or 1 ml/min as indicated. Absorbance was measured at 254 nm.

Purification of red blood cell extracts and NADP

Red blood cell extracts or NADP (10 mM; Sigma) were separated using AG® MP-1 as described above at a flow rate of 1 ml/min. Fractions (1 ml) were collected, evaporated to dryness under vacuum, washed three times with methanol (1 ml) and resuspended in 200 µl of water.

Treatment of red blood cell extracts with hydrolytic enzymes

Apyrase (low ATP/ADPase activity ratio; Sigma) and NAD(P)ase (NAD glycohydrolase; Sigma) were reconstituted with water (25 units/ml). Alkaline phosphatase conjugated with agarose (Sigma) was washed twice by centrifugation and resuspended in a buffer containing 100 mM Tris (pH 9; approx. 700 units/ml). Cell extracts (24 µl) were treated with the appropriate enzyme or the corresponding vehicle (6 µl) and incubated at room temperature for 1 h. The final concentrations of enzymes in the incubations were 5 units/ml [for apyrase and NAD(P)ase] and approx. 140 units/ml (for alkaline phosphatase–agarose). Alkaline phosphatase was removed by centrifugation at 12 000 *g* for 5 min before radioligand binding.

Radioligand binding

[³²P]NAADP was enzymically prepared from [³²P]NAD (1000 Ci/mmol; Amersham Biosciences) as described previously [21]. Briefly, [³²P]NAD was first phosphorylated (using NAD kinase) and the NADP generated was converted into NAADP by exchange of nicotinamide with nicotinic acid (using ADP-ribosyl cyclase). [³²P]NAADP (0.5 nM) was allowed to bind to sea urchin egg homogenates (0.1–0.5 %, v/v) at room temperature in a binding medium, containing 20 mM Hepes (pH 7.2), 250 mM potassium gluconate, 250 mM *N*-methyl-D-glucamine and 1 mM MgCl₂ [21,23]. Homogenates were incubated with cell extracts (at the dilutions indicated) or increasing concentrations of NAADP added either before or together with the radioligand. The bound and free radioligands were separated by rapid filtration through Whatman GF-B filters using a cell harvester.

Determination of NADP levels

NADP levels in the cell extracts were determined (by comparison with known concentrations of NADP) either by HPLC or using a spectrophotometric enzyme-cycling assay. The latter was based on the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; 500 µM] by NADPH, which was formed from NADP present in the extract after enzymic oxidation of glucose 6-phosphate (5 mM) by glucose-6-phosphate dehydrogenase (2.5 units/ml). Reactions were performed at room temperature in the presence of 2 mM phenazine ethosulphate in a buffer composed of 100 mM Tris (pH 8). Absorbance of the

Figure 1 Optimization of a radioreceptor assay for NAADP determination

(A) A schematic representation showing that incubation of NAADP receptors (large spheres) with a submaximal concentration of unlabelled NAADP ($\circ$) results in the labelling of two receptors. At an arbitrary receptor concentration of 8 (left panel), the subsequent binding of a saturating concentration of [32 P]NAADP ($\bullet$) is decreased by 25 % (six out of eight receptors labelled) relative to the binding in the absence of unlabelled NAADP. At a lower receptor concentration of 4 (right panel), proportionally more receptors are occupied by unlabelled NAADP, such that binding of [32 P]NAADP is inhibited to a greater extent (two out of four receptors labelled; 50 %) at this concentration than at higher receptor concentrations. Thus the non-dissociating nature of NAADP binding to its receptor in sea urchin eggs can be readily exploited to enhance the detection of low concentrations of NAADP. (B) Binding of [32 P]NAADP was determined in the presence of the indicated concentration of unlabelled NAADP added before (open symbols) or together with (closed symbols) the radioligand. Experiments were performed at a sea urchin egg homogenate concentration of 0.1 % (v/v) ($\circ$, $\bullet$) or 0.5 % (v/v) ($\square$, $\blacksquare$). Results are expressed relative to the binding of radioligand in the absence of NAADP after correction of non-specific binding determined in the presence of 1 μ M NAADP.

formed product was monitored at 570 nm using a plate-reader. NAADP levels were similar when determined by HPLC or the cycling assay (results not shown).

RESULTS AND DISCUSSION

Previous studies have demonstrated that binding of NAADP to sea urchin egg homogenates is not readily reversible [16,21,22]. Accordingly, we have shown that low concentrations of unlabelled NAADP are more effective in inhibiting the binding of radiolabelled NAADP to homogenates when added before the radioligand than when homogenates are exposed to the two ligands simultaneously [21]. This property substantially increases sensitivity to the detection of low concentrations of NAADP. We reasoned that, at a fixed submaximal concentration of NAADP, decreasing the receptor concentration should result in increased receptor occupancy; hence, binding of [32 P]NAADP should be inhibited to a greater extent at this concentration than at higher receptor concentrations (Figure 1A). To test this prediction, the binding of [32 P]NAADP at two concentrations of sea urchin egg homogenates was compared. From conventional isotope dilution experiments

(i.e. binding of radioligand determined in the simultaneous presence of various concentrations of unlabelled NAADP), the concentration of unlabelled NAADP that inhibited [32 P]NAADP binding by 50 % (IC_{50}) was found to be almost the same for sea urchin egg homogenate concentrations of 0.1 % (v/v) (1.2 ± 0.2 nM; Figure 1B, closed circles) and 0.5 % (v/v) (1.4 ± 0.5 nM; closed squares). In contrast, and in accordance with our previous analysis [21], treatment of homogenates with the same concentrations of NAADP before the addition of radioligand resulted in a marked leftward shift in the competition curves (Figure 1B, open symbols). Notably, as predicted, when assayed by this method, sea urchin egg NAADP receptors appeared to be more sensitive to their ligand when more dilute; IC_{50} values were 46 ± 3 and 92 ± 8 pM at homogenate concentrations of 0.1 and 0.5 % (Figure 1B, open circles and open squares respectively). Thus, by simple manipulation of the experimental method, the sensitivity of sea urchin egg homogenates to NAADP can be substantially enhanced, thereby providing an ideal method for detecting low concentrations of NAADP.

Recently, Graeff and Lee [40] have developed an enzymic cycling assay for determining NAADP levels. Although this assay uses less specialized material than a radioreceptor assay and is linear with respect to NAADP concentration, the specificity of this assay is critically dependent on prior removal of certain nucleotide substrates (NAD, NAAD), which would be present in cell extracts. More importantly, the assay described in the present study is approx. 1000-fold more sensitive than that described by Graeff and Lee [40] and is capable of detecting approx. 10 pM NAADP. This is particularly advantageous in the quantification of cellular NAADP, given that NAADP is active over the low nM range (see below), suggesting that basal cellular concentrations of NAADP are probably maintained well below this.

Having established conditions for the detection of low concentrations of NAADP, we analysed extracts from a readily available primary cell type, the human red blood cell, for NAADP content. Binding of [32 P]NAADP to sea urchin egg homogenates was inhibited by red blood cell extracts from six different subjects when homogenates were incubated with radioligand and extract together (Figure 2A, black bars). Given the highly specific nature of the NAADP receptor for its ligand relative to other nucleotides [16,21,29], these results are consistent with the presence of NAADP in the extracts. Importantly, the extent of inhibition was substantially greater when homogenates were incubated with the same concentration of extract before the addition of the radioligand (Figure 2A, open bars). Thus the inhibitory factor(s) present in the extract probably binds sea urchin egg NAADP receptors in an irreversible manner, providing further evidence that the factor is NAADP.

Anion-exchange analysis of the red blood cell extract showed that the major nucleotides present in the sample were ATP, ADP and NADP (Figure 2B). However, these nucleotides are unlikely to contribute to the inhibition of [32 P]NAADP binding by the extract, since the effects of the extract remained unchanged by treatment with apyrase or NAD(P)ase, enzymes which hydrolyse ATP/ADP (to AMP) and NAD(P) to ADP-ribose(-phosphate) respectively (Figure 2C). In contrast, the inhibitory effects of the extract on [32 P]NAADP binding were completely abolished by prior treatment with alkaline phosphatase (Figure 2C). This result indicates the requirement of a free phosphate group(s) and is again consistent with the inhibitory factor being NAADP.

We purified red blood cell extracts by HPLC and analysed individual fractions for their ability to inhibit NAADP binding. As shown in Figure 2(D), the activity of the extract reached a peak in fractions 27–28, corresponding to a retention time of 26.7 ± 0.3 min ($n = 4$). This value is similar to the elution of authentic

Figure 2 NAADP is present in human red blood cells

(A) Binding of [32 P]NAADP to sea urchin eggs was determined in the simultaneous presence of human red blood cell extracts (black bars) at the indicated dilution or to homogenates that had first been incubated with the same concentration of the extract (open bars). Results are shown for extracts from six different subjects. (B) Anion-exchange HPLC analysis (using a Hypersil $^{\text{®}}$ NH $_2$ column) of a typical red blood cell extract. (C) Binding of [32 P]NAADP to sea urchin egg homogenates that had been incubated with extracts (1:200) pretreated with either buffer alone (–), buffer supplemented with apyrase (5 units/ml; + Apy), NAD(P)ase (5 units/ml; + N) or buffer supplemented with alkaline phosphatase (approx. 140 units/ml; + AP) for 60 min at room temperature. (D) Extracts (200 μ l) were separated by anion-exchange HPLC (1 ml/min; AG $^{\text{®}}$ MP-1) and the fractions collected were analysed for their ability to inhibit the binding of [32 P]NAADP to sea urchin egg homogenates (●). Samples were incubated for 60 min at room temperature before the addition of radioligand. The absorbance profile of NAADP (20 nmol; —) separated under identical conditions is shown. Results shown are a representative example from three purifications. Red blood cells were extracted for 1 min in all the experiments.

Table 1 Quantification of cellular NAADP and NADP levels

Acid extracts from the indicated cell types were prepared and the NAADP and NADP contents were determined as described in the Materials and methods section. Results are the means $\pm$ S.E.M. for 3–10 independent preparations and are expressed as the concentrations determined within the extract and, for NAADP, after normalization for protein content. NAADP levels were determined by incubating homogenates with extracts before the addition of the radioligand. NAADP was not detected (–) in J774 macrophages.

Cell type	[NAADP]		[NADP]
	Extract (nM)	Cell (fmol/mg)	Extract (μ M)
Human red blood cells	16 $\pm$ 2	142 $\pm$ 6	7.8 $\pm$ 1
Rat hepatocytes	4.5 $\pm$ 0.5	109 $\pm$ 12	20 $\pm$ 11
<i>E. coli</i>	2.5 $\pm$ 0.8	81 $\pm$ 21	4.1 $\pm$ 0.6
J774 macrophages	–	–	3.1 $\pm$ 1.2

NAADP (retention time, 26.8 $\pm$ 0.07 min; n = 3) determined by measuring absorbance. These results provide further evidence that the inhibition of [32 P]NAADP binding by red blood cell extracts is mediated by NAADP.

Compared with inhibition of [32 P]NAADP binding by known concentrations of NAADP, the concentration of NAADP in the extracts was 13 $\pm$ 1 nM (n = 10) when determined by simultaneous incubation with radioligand and was 16 $\pm$ 2 nM after preincubation (n = 7; Table 1). We also determined NAADP levels in red blood cells that were extracted with HClO $_4$, followed by neutralization with KHCO $_3$ as described by Masgrau et al. [30] (see the Materials and methods section). After correction for differential nucleotide recovery, the NAADP content determined

by this method (345 $\pm$ 27 fmol/mg, n = 3) was in reasonable agreement with the values obtained using trichloroacetic acid (156 $\pm$ 18 fmol/mg, n = 3). No significant differences were found in the NAADP content of red blood cell extracts obtained from patients suffering from renal failure (13 $\pm$ 1 nM, five subjects) and healthy volunteers (13 $\pm$ 1 nM, five subjects). Based on the assumptions stated in the Materials and methods section, these values correspond to a cytoplasmic concentration of approx. 60 nM, which is approx. 2–3 orders of magnitude lower than that estimated in sea urchin spermatozoa (4–54 μ M) [28,29] and approx. 5-fold lower than that in sea urchin eggs (290 nM) [29], as reported recently.

We considered the possibility that NAADP detected in our samples is not present in red blood cells, but is instead derived from the breakdown of cellular NADP during the acid extraction procedure. Indeed, the estimated concentration of NADP in the extract (7.8 $\pm$ 1 μ M, n = 10) was approx. 500 times that of NAADP (13–16 nM; see above); hence, even moderate conversion of NADP into NAADP (0.19%) would be sufficient to account for the levels of NAADP we estimated to be present. We therefore quantified NAADP generated from NADP after treatment with trichloroacetic acid (Figure 3). Analysis of commercial preparations of NADP by HPLC (Figure 3A) revealed several contaminants, one of which (retention time, 30 $\pm$ 0.2 min; n = 3) co-eluted with NAADP (retention time, 30 min; n = 2). Since the level of contamination of NADP by NAADP (0.14 $\pm$ 0.01%) may mask any potential NAADP generation during extraction, we first purified NADP as described in the Materials and methods section. The NAADP content after mock extraction (with water) of purified NADP was 0.008 $\pm$ 0.002% (n = 4). Relatively short incubations with trichloroacetic acid did not

Figure 3 NAADP detected in cell extracts is not derived from NADP

(A) Analysis of NADP (200 nmol) by anion-exchange HPLC (0.5 ml/min; AG[®] MP-1). The area of the trace highlighted by the grey rectangle is enlarged in the inset, revealing contamination of NADP with NAADP (●). (B) Acid breakdown of NADP to NAADP. Purified NADP was incubated either with trichloroacetic acid (for the times indicated) or in control experiments with water (trace marked 0). Acid was removed by ether extraction and samples were analysed by HPLC as in (A). (C) Effect of time of extraction with trichloroacetic acid on the measured NAADP (●) and NADP (○) levels in red blood cells. (D) Lack of correlation between the measured NADP and NAADP levels in extracts from rat hepatocytes.

result in any significant NAADP generation. The NAADP content was $0.01 \pm 0.004\%$ ($n=3$) and $0.017 \pm 0.004\%$ ($n=3$) after extraction with trichloroacetic acid for 1 and 15 min respectively (Figure 3B), values not significantly different from control ($P > 0.05$). In contrast, after longer incubations of NADP with trichloroacetic acid, NAADP was readily detected, accounting for $0.12 \pm 0.01\%$ ($n=3$) of the total nucleotide content at 240 min. Thus the expected concentration of generated NAADP in a solution of $8 \mu\text{M}$ NADP extracted with trichloroacetic acid for 240 min is 9 nM. Since red blood cells were extracted with trichloroacetic acid for only 1 min, acid-mediated generation of NAADP is unlikely to have interfered with the quantification of NAADP reported in the present study. Indeed, increasing the extraction period up to 15 min did not increase the measured NAADP levels in red blood cells (Figure 3C). Only when red blood cells were extracted for longer times did NAADP levels increase significantly (Figure 3C). This increase was not due to a more efficient extraction of the nucleotides with time, since NADP levels in the extract remained unchanged throughout the period (Figure 3C). Rather, acid breakdown of NADP to NAADP is probably the cause for the apparent increase in cellular NAADP levels after extended extractions.

We analysed three other cell types for NAADP content (Table 1). NAADP was detected in acid extracts from hepatocytes and *E. coli*, but only after prior incubation of the homogenate with the extract (Table 1). These results reflect the lower levels of NAADP (relative to red blood cells) in these extracts and further highlight the sensitivity and usefulness of the preincubation method for determining NAADP. The ability of these cell extracts

to inhibit binding of [³²P]NAADP was abolished after prior treatment with alkaline phosphatase (results not shown). NAADP, however, was not detected in J774 murine macrophages by either method. It is worth noting that, of the four cell types examined, the macrophages are the only non-primary cells. Whether this difference contributes to the absence of detectable NAADP in the J774 cells remains to be established, although relatively high NAADP levels (approx. 12 pmol/mg) have been determined in a clonal pancreatic β -cell line [30].

We compared the concentration of NAADP in our extracts with the concentration of NADP. For hepatocytes, although the measured NAADP concentrations in different cell extracts were similar (3.2–5.2 nM), the NADP content was much more variable (7.7–49 μM ; Table 1). Thus there was little correlation between the concentrations of the two nucleotides in the extract (Figure 3D), providing further evidence that NAADP is unlikely to be generated simply by NADP breakdown. Indeed, the NADP concentrations in *E. coli* and macrophage extracts were similar, but NAADP was detected only in the former preparation (Table 1).

In summary, by exploiting the unusual binding characteristics of the target protein for NAADP in sea urchin egg homogenates, we have defined experimental conditions for determining very low concentrations of NAADP, which we have applied to detect NAADP in cells of disparate origin. Our methods may aid future studies in delineating the mechanism of synthesis of NAADP within cells, of which little is known compared with its Ca^{2+} -mobilizing activity.

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