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Refinement of a radioreceptor binding assay for nicotinic acid adenine dinucleotide phosphate

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Abstract

The measurement of changes in nicotinic acid adenine dinucleotide phosphate (NAADP) levels in cells has been, and remains, key to the investigation of the functions of NAADP as a Ca^{2+}-releasing second messenger. Here we provide details of how to isolate NAADP from cells by extraction with perchloric acid and then measure the NAADP using a radioreceptor assay. We demonstrate that NAADP is neither generated nor broken down during sample processing conditions and that radioreceptor assay is highly selective for the detection of NAADP under cell extract conditions. Furthermore, a number of improvements, such as solid-state detection of the radioactivity, are incorporated to enhance the safety of the procedure. Finally, we have developed a new method to prevent the endogenous metabolism of NAADP by chelating Ca^{2+} with bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA), thereby reducing the difficulty of catching a small transient rise in NAADP levels. In summary, we have refined and improved a method for measuring NAADP levels and presented it in a manner accessible to a wide range of laboratories. It is expected that this will enhance research in the NAADP field.

Keywords: NAADP; Mammalian; Second messenger; Radioreceptor

The cytosolic concentration of Ca^{2+} is very closely regulated in cells; the resting concentration usually is maintained at approximately 10^{-7} M. Changes in Ca^{2+} concentration play an important role in a wide range of processes—from fertilization, to the regulation of cell function throughout life, to cell death [1]. This resting concentration may be increased by means of influx of Ca^{2+} from the extracellular matrix through a channel in the plasma membrane or release of Ca^{2+} from intracellular stores. Release of Ca^{2+} from intracellular stores usually is a result of the binding of a primary messenger, such as a hormone or neurotransmitter, to a receptor in the plasma membrane. This in turn results in the generation of a second messenger inside the cell that is able to release Ca^{2+} from an intracellular store. The best-characterized Ca^{2+}-releasing second messenger is d-myo-inositol 1,4,5-trisphosphate (IP_3).[6] It is synthesized

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[6] *Abbreviations used:* IP_3, D-myo-inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; cADPR, cyclic ADP ribose; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; NADPH, reduced-form NADP; ASW, artificial sea water; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis (acetoxymethyl) ester; CCK, cholecystokinin; NADH, reduced-form NAD; NAAD, nicotinic acid adenine dinucleotide.

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following the activation of a receptor that is coupled to G_q, which in turn activates phospholipase C to cleave IP_3 from phosphatidylinositol 4,5-bisphosphate. IP_3 binds to the IP_3 receptor in the endoplasmic reticulum membrane and releases Ca^{2+} from the endoplasmic reticulum [2].

Nicotinic acid adenine dinucleotide phosphate (NAADP) was first reported as a Ca^{2+}-releasing agent in 1995 [3]. However, in contrast to IP_3, very little is known about the physiological roles of NAADP. The site of action of NAADP remains controversial; there is evidence to suggest that NAADP releases from an acidic lysosome-like store [4,5], although there are also reports of it affecting ryanodine receptors on the endoplasmic reticulum [6]. NAADP has now been categorized as a second messenger, according to the conditions laid down by Sutherland and coworkers [7], in both the sea urchin [8] and model mammalian [9] systems. The ability to measure changes in NAADP levels in cells has been key to this categorization, providing evidence that NAADP is synthesized in response to the binding of an agonist to its cell surface receptor. This remains very important in characterizing NAADP in a wider range of cell types, enabling a more comprehensive picture of the functions of NAADP as a second messenger to be built up. Basal NAADP levels have been measured in a variety of sea urchin [10], plant [11], and mammalian [12,13] cell types. Only recently, however, have agonist-induced changes in NAADP levels been reported in sea urchin sperm [8], pancreatic beta cells [9], smooth muscle cells [14], pancreatic acinar cells [15], and T cells [13]. The majority of these measurements have been made using a radioreceptor binding assay [12], although a cycling assay [13,16,17] has also been described.

In theory, it is possible to use a bioassay in sea urchin egg homogenate, similar to that used for cyclic ADP ribose (cADPR) [18], to detect NAADP. However, there are no reports of NAADP measurements using such a technique. Three variations on a cycling assay have been reported [13,16,17]. These assays offer sensitive detection of NAADP, with 50-fmol quantities being detectable with one method [13], and have the benefit that they do not require the preparation of a radioligand. However, the processing of samples is technically demanding and time-consuming. Cell extracts require purification by anion exchange chromatography and subsequent enzyme treatment prior to determination of the NAADP concentration, which is actually determined indirectly from the resulting concentration of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). The radioreceptor binding assay that we report here is capable of directly detecting 100-amol quantities of NAADP without the risk of interference from other compounds. Furthermore, it is possible to quickly process a large number of samples prepared using a straightforward acid extraction technique without the need for further purification.

Previously expressed concerns encompassing the generation or degradation of NAADP, as well as the selectivity of the assay, and the recovery of NAADP in a cell extract are addressed in detail. Importantly, we report a new method where NAADP increases are measured as NAADP accumulation over time. In summary, we have refined and validated a powerful method for measuring NAADP levels, and we present it in an accessible manner that will enable even inexperienced researchers to measure NAADP levels. The importance of these measurements at this stage in our understanding of the functions of NAADP cannot be overlooked. It is expected that this will enhance research in the NAADP field.

Materials and methods

Materials

All chemicals were purchased from Sigma (UK) except where otherwise indicated below.

High-performance liquid chromatography

All high-performance liquid chromatography (HPLC) was carried out on an anion exchange resin (AGMP1, Bio-Rad, USA), packed in 150 × 2.5-mm Omnifit columns, using a concave upward gradient of trifluoroacetic acid (TFA), delivered by a Waters 600e pump, as described previously [19]. Detection was performed by a UV detector (Waters 2487, UK) at 254 nm.

Stability of nucleotides under different pH conditions

NADP, NAADP, or NADPH (reduced-form NADP) (1 mM) was incubated in the following buffers for 6 h: 50 mM Hepes (pH 7.2), 0.75 M $HClO_4$ (pH 0.88), 1 M $KHCO_3$ (pH 9.1), 0.75 M $HClO_4$/1 M $KHCO_3$ (pH 8.6), or 1 M K_2CO_3 (pH 11). Samples were then neutralized with their base equivalence of either Tris base (for acidic solutions) or Hepes acid (for basic solutions) as applicable. Samples were analyzed by HPLC as described above, with elution times being determined from standard runs with known compounds.

Acid extraction of NAADP

Cells may be prepared in any suitable manner; gametes, isolated cells, chopped tissue, and cells in culture all have been used successfully. Cells were then exposed to agonist, and at the appropriate time points the reaction in cells was stopped by the addition of 0.75 M ice-cold $HClO_4$ added as an equal volume of 1.5 M solution. Sonication (Jencons Vibracell at amplitude 60) was carried out to disrupt the cells, which were then placed on ice for 15 min. The denatured protein was pelleted by centrifugation at 9000*g* for 10 min and stored at –80 °C for later analysis. Supernatant was neutralized with 1 M $KHCO_3$, added as an equal volume of 2 M solution, and vortexed. The resulting $KClO_4$