Unique kinetics of nicotinic acid–adenine dinucleotide phosphate (NAADP) binding enhance the sensitivity of NAADP receptors for their ligand

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Unique kinetics of nicotinic acid–adenine dinucleotide phosphate (NAADP) binding enhance the sensitivity of NAADP receptors for their ligand

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Nicotinic acid–adenine dinucleotide phosphate (NAADP) is a novel and potent Ca^{2+}-mobilizing agent in sea urchin eggs and other cell types. Little is known, however, concerning the properties of the putative intracellular NAADP receptor. In the present study we have characterized NAADP binding sites in sea urchin egg homogenates. [^{32}P]NAADP bound to a single class of high-affinity sites that were reversibly inhibited by NaCl but insensitive to pH and Ca^{2+}. Binding of [^{32}P]NAADP was lost in preparations that did not mobilize Ca^{2+} in response to NAADP, indicating that [^{32}P]NAADP probably binds to a receptor mediating Ca^{2+} mobilization. Addition of excess unlabelled NAADP, at various times after initiation of [^{32}P]NAADP binding, did not result in displacement of bound [^{32}P]NAADP. These data show that NAADP becomes irreversibly bound to its receptor immediately upon association. Accordingly, incubation of homogenates with low concentrations of NAADP resulted in maximal labelling of NAADP binding sites. This unique property renders NAADP receptors exquisitely sensitive to their ligand, thereby allowing detection of minute changes in NAADP levels.

Key words: calcium, channel, sea urchin egg, signal transduction.

INTRODUCTION

The multitude and diversity of cellular processes regulated by the cytosolic Ca^{2+} concentration highlights the importance of Ca^{2+} in physiology [1]. In many cells, increases in cytosolic Ca^{2+} derive primarily from intracellular Ca^{2+} stores via activation of intracellular Ca^{2+} channels. Receptors for the intracellular messenger inositol 1,4,5-trisphosphate (IP_3) [2] are ubiquitous, and are well established in mediating agonist-dependent Ca^{2+} signals in a variety of excitable and non-excitable cells [3]. The structurally and functionally related ryanodine receptors [4], initially characterized in muscle cells, are also widespread and are gated by cADP-ribose (cADPR) [5,6]. Both IP_3 and ryanodine receptors are regulated by Ca^{2+} [7], a property that probably underlies the complex spatial and temporal nature of cytosolic Ca^{2+} signals typically observed in response to physiological stimuli [8].

Nicotinic acid–adenine dinucleotide phosphate (NAADP) is a novel Ca^{2+}-mobilizing messenger that can elicit Ca^{2+} release from sea urchin homogenates desensitized to both IP_3 and cADPR [9]. That NAADP mediates its effects independently of IP_3 or ryanodine receptor activation is supported by its unique pharmacology [10], the ability to separate NAADP-sensitive Ca^{2+} stores from those responsive to IP_3 and cADPR [9,11], and the lack of effect of cytosolic Ca^{2+} on NAADP-induced Ca^{2+} mobilization [12]. Perhaps the most remarkable feature of NAADP-induced Ca^{2+} mobilization is its susceptibility to inactivation. In contrast to IP_3- and cADPR-induced Ca^{2+} release, subthreshold (non-releasing) concentrations of NAADP completely desensitize homogenates to subsequent challenge with normally maximal concentrations of NAADP [10,13]. Thus NAADP, at least in the sea urchin egg, may provide a ‘one shot’ Ca^{2+} signal that may be important in cellular events such as fertilization [10].

Although the effects of NAADP have been most extensively characterized in sea urchin eggs, recent studies have extended the actions of NAADP to ascidian [14] and starfish [15] oocytes, mouse pancreatic acinar cells [16,17], rat brain microsomes [18], and plant preparations [19]. These studies strongly support a general role for NAADP-mediated Ca^{2+} signalling. A binding site for NAADP has previously been identified in sea urchin egg microsomes [13], however, little is known concerning the molecular identity of the putative NAADP receptor.

In the present study we demonstrate that NAADP binds to a site clearly distinct from those for IP_3 and cADPR in sea urchin egg homogenates. From analysis of the kinetics of NAADP binding, we show that NAADP receptors are acutely tuned to detect small changes in NAADP concentration.

EXPERIMENTAL

Synthesis of [^{32}P]NAADP

[^{32}P]NAADP synthesis described here is a variation of the procedure reported previously [13]. [^{32}P]NADP was synthesized from [^{32}P]NAD (2 μM; specific activity 1000 Ci/mmol; Amersham Pharmacia Biotech) by incubation with 50 units/ml NAD kinase (Sigma) and 10 mM MgATP for 2 h at 37 °C in a buffer containing 5 mM Hepes (pH 7.5). The remaining ATP and ADP was converted into AMP by incubation with apyrase (2 units/ml; Sigma) for 1 h at 37 °C. The reaction was then diluted 5-fold into a medium containing 10 mM Mes (pH 5.0), 19 mM nicotinic acid and 2 μg/ml ADP-ribosyl cyclase (Sigma), and was incubated for a further 2 h in order to convert [^{32}P]NADP into [^{32}P]NAADP by base-exchange. The final mixture was separated by anion-exchange HPLC on a 3 mm × 150 mm column packed with AGMP1 (Bio-Rad). Elution was performed

Abbreviations used: cADPR, cADP ribose; IP_3, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid–adenine dinucleotide phosphate; TFA, trifluoroacetic acid.

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at a flow rate of 1 ml/min using a gradient of trifluoroacetic acid (TFA) that increased linearly from 0–2 % over the first 6 min, to 4 % at 11 min, to 8 % at 16 min, to 16 % at 21 min, to 32 % at 26 min and to 100 % (150 mM TFA) at 26.1 min. Fractions were collected every minute, were neutralized by the addition of Tris base (final concentration 75 mM) and their radioactivities were determined by Cerenkov counting. A major peak was observed at 25.6 ± 0.4 min ($n = 3$) that corresponded to authentic NAADP (determined by monitoring the absorbance at 254 nm) run on a parallel AGMP1 column (elution time 25.1 ± 0.2 min).

NAADP binding assays

Binding of [^{32}P]NAADP (0.1–0.5 nM) to sea urchin homogenates [0.25–0.5 % (v/v)] was determined at either 4 °C or 20 °C (as indicated) in a medium containing 20 mM Hepes (pH 7.2), 250 mM potassium gluconate, 250 mM *N*-methyl D-glucamine and 1 mM $MgCl_2$ (final volume of 250 μl). Bound and free radioligand were separated by rapid filtration. The effects of NaCl (Figure 2A) were performed at pH 7.2 in a medium containing 20 mM Hepes and 1 mM $MgCl_2$. In experiments examining the pH dependence of [^{32}P]NAADP binding (Figure 2B), medium containing 20 mM Mes (to obtain pH values of 6 and 6.5), Hepes (to obtain pH values of 7 and 7.5) or Tris (to obtain pH values of 8 and 8.5) was used. Total and non-specific binding (determined in the presence of 100 nM NAADP) were typically 2000 and 100 c.p.m. respectively. Collection of sea urchin (*Lytechinus pictus*) eggs and preparation of homogenates were performed as described previously [10].

Ca^{2+} release assay

The Ca^{2+} concentration of the medium was determined fluorimetrically using 2.5 % (v/v) homogenates at 17 °C in binding medium supplemented with 1 mM MgATP, 10 mM creatine phosphate, 10 units/ml creatine phosphokinase and 3 μM fluo-3, essentially as described previously [10].

Data analysis

Curves were fitted to the following equation (using the least-squares method):

$$S = S_0/[1 + ([\mathrm{C}]/\mathrm{IC}_{50})^h]$$

where IC_{50} is the concentration of competitor (C) causing half-maximal inhibition of specific [^{32}P]NAADP binding (S), S_0 is specific binding in the absence of competitor and h is the Hill coefficient. All data are presented as means ± S.E.M.

RESULTS AND DISCUSSION

Specific binding of [^{32}P]NAADP to sea urchin egg homogenates was readily detected, constituting 96 ± 0.6 % ($n = 8$) of total binding. Experiments where homogenates were simultaneously incubated with radioligand and various concentrations of unlabelled NAADP (Figure 1A) indicated that the IC_{50} for NAADP was 0.54 ± 0.07 nM ($n = 8$) and the Hill coefficient was 1.07 ± 0.003 ($n = 8$). These data are consistent with labelling of a single high-affinity binding site at this radioligand concentration (0.2 nM). Similar results were obtained with homogenates prepared from *Strongylocentrotus purpuratus* (results not shown). Specific [^{32}P]NAADP binding was not affected by the closely related adenine nucleotides NAD (97 ± 2 %; $n = 3$), NAAD (99 ± 2 %; $n = 3$) or cADPR (98 ± 2; $n = 3$) at concentrations of 10 μM, but appeared to be inhibited by NADP (Figure 1B). The IC_{50} for NADP (639 ± 64 nM; $n = 3$), however, was > 1000

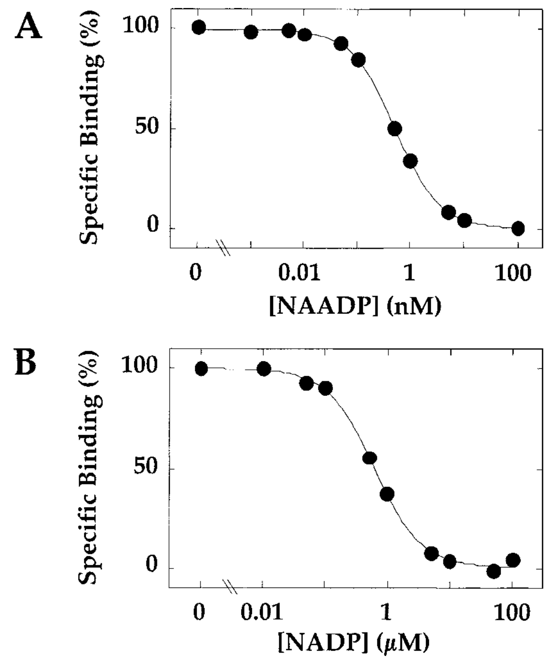

Figure 1 Specific [^{32}P]NAADP binding to sea urchin egg homogenates

(**A**) Binding of 0.2 nM [^{32}P]NAADP to *L. pictus* homogenates in the presence of the indicated concentrations of unlabelled NAADP ($n = 8$). (**B**) Apparent inhibition of [^{32}P]NAADP binding by NADP ($n = 3$). Homogenates were exposed to the specified unlabelled ligand and radioligand simultaneously. All experiments were performed at 20 °C. Error bars were smaller than the symbols.

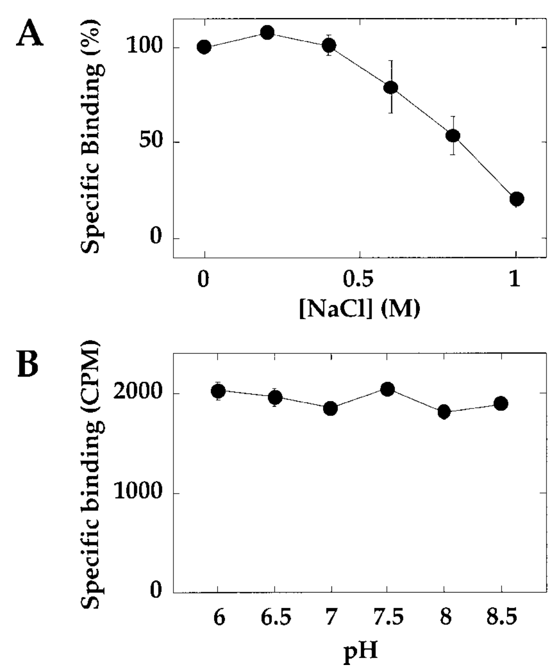

Figure 2 Regulation of [^{32}P]NAADP binding by NaCl and pH

(**A**) Inhibition of [^{32}P]NAADP binding by NaCl. Data ($n = 3$–7) are expressed relative to specific binding in the absence of NaCl. (**B**) Lack of effect of pH on [^{32}P]NAADP binding ($n = 3$). All experiments were performed at 20 °C.

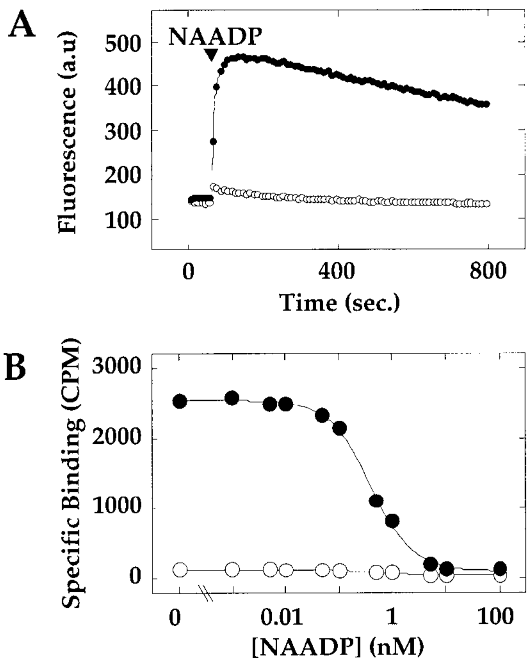

Figure 3 [^{32}P]NAADP binds to putative NAADP receptors linked to Ca^{2+} mobilization

Comparison of NAADP-induced Ca^{2+} mobilization (**A**) and [^{32}P]NAADP binding properties (**B**) in independent sea urchin egg homogenates. Homogenates that responded to NAADP (1 μM) with an increase in the Ca^{2+} concentration of the medium, bound [^{32}P]NAADP (●), whereas homogenates that were insensitive to NAADP in Ca^{2+} release experiments, did not (○). Both preparations mobilized Ca^{2+} in response to 10 μM cADPR and IP_3 (results not shown).

times that of NAADP, probably reflecting contamination of NADP with NAADP as previously reported [9].

[^{32}P]NAADP binding was inhibited by NaCl in a concentration-dependent manner (Figure 2A). The inhibitory effect of high salt was completely reversible. Thus binding of [^{32}P]NAADP, following a 5 min incubation with 1 M NaCl prior to dilution to a final NaCl concentration of 0.4 M, was similar ($103 \pm 4\%$; $n = 3$) to homogenates that were incubated with 0.4 M NaCl throughout. [^{32}P]NAADP binding, however, was unaffected by pH (Figure 2B). [^{32}P]NAADP binding was also found to be insensitive to Ca^{2+}; specific binding of [^{32}P]NAADP in the presence of EGTA (1 mM) or Ca^{2+} (10 mM) was $101 \pm 1\%$ and $99 \pm 1\%$ ($n = 3$), respectively, of that in the control medium (without added Ca^{2+}). These data are consistent with the lack of effect of pH [20] and Ca^{2+} [12] on NAADP-induced Ca^{2+} release from sea urchin egg homogenates, and are in stark contrast to the reported effects of pH and Ca^{2+} on the binding of cADPR [21] and IP_3 [22]. The insensitivity of NAADP binding to Ca^{2+} further indicates that NAADP receptors do not exhibit Ca^{2+}-induced Ca^{2+} release [12], and supports a role for NAADP in providing a 'trigger' Ca^{2+} release that is subsequently amplified by IP_3 and/or cADPR receptors [16]. Preparations that were fully responsive to both IP_3 and cADPR but not NAADP in Ca^{2+} release assays (occasionally encountered; 3 out of 120 preparations) did not bind [^{32}P]NAADP (Figure 3). These data strongly indicate that [^{32}P]NAADP probably binds to a unique site that mediates NAADP-induced Ca^{2+} mobilization.

We subsequently examined the kinetics of [^{32}P]NAADP binding (Figure 4). Binding of [^{32}P]NAADP reached steady state after approx. 20 min at 4 °C (Figure 4A). Consistent with a previous report [13], NAADP binding was irreversible. Thus after a 20 min incubation with [^{32}P]NAADP, the amount of [^{32}P]-NAADP bound following a further 10 min incubation with an excess of unlabelled NAADP (100 nM) was similar ($97 \pm 1\%$; $n = 3$) to that prior to its addition. Longer incubation periods with unlabelled NAADP (up to 12 h) were also without effect on [^{32}P]NAADP binding, as was dilution (11-fold) of the radioligand (results not shown). NAADP was equally ineffective at displacing [^{32}P]NAADP when added at earlier times after the initiation of binding (Figure 4B). These data show that unlabelled NAADP essentially halts further association of [^{32}P]NAADP, but does not displace radioligand already bound, indicating that once [^{32}P]NAADP binds to its receptor, it does so irreversibly. This property probably underlies the inactivation of NAADP-induced Ca^{2+} release by subthreshold concentrations of NAADP [10,13]. Aarhus et al. [13] concluded that NAADP binding is irreversible, but in contrast to our data, that irreversibility developed after a delay. In those experiments, however, the amount of [^{32}P]-NAADP bound following addition of excess unlabelled NAADP was compared with the level of [^{32}P]NAADP bound in control homogenates that had been incubated an additional 10 min. Thus the apparent dissociation of bound [^{32}P]NAADP at early time points may reflect partial labelling of the binding sites due to slow association of the radioligand. High concentrations of NaCl (1 M) that inhibit [^{32}P]NAADP when added simultaneously with the radioligand, did, however, initiate partial dissociation ($57 \pm 5\%$; $n = 3$) of the radioligand once maximal labelling had been attained.

If NAADP binding is indeed irreversible, then all concentrations of NAADP (provided that they are in excess of receptor concentration; see below) should, with time, lead to maximal occupancy of the binding sites. This was, indeed, found to be the case. Thus the absolute levels of binding attained when homogenates were incubated with 0.2 and 0.4 nM [^{32}P]NAADP (submaximal concentrations relative to apparent affinity) were 126 ± 6 fmol/mg ($n = 3$) and 125 ± 4 fmol/mg ($n = 3$) respectively. These values are therefore B_{max} estimates. Only the rate, and not the extent, of binding, was dependent upon the concentration of radioligand (Figure 4C). This finding is entirely inconsistent with binding to a reversible high-affinity site with a K_d of 0.3 nM (calculated from the IC_{50} and concentration of the radioligand; Figure 1A), where 0.2 and 0.4 nM NAADP should occupy 40 and 60% of binding sites respectively. Furthermore, incubation of sea urchin egg homogenates with submaximal concentrations of unlabelled NAADP inhibited subsequent binding of [^{32}P]NAADP to a greater extent than that predicted for a reversible interaction. Preincubation with 0.125 nM unlabelled NAADP almost completely ($81 \pm 3\%$, $n = 3$) inhibited subsequent binding of 0.2 nM [^{32}P]NAADP; if binding were reversible only 20% of the [^{32}P]NAADP should have been displaced under these conditions. These data also indicate that irreversible binding of [^{32}P]NAADP is not an artefact of the labelling process. Clearly, the irreversible nature of NAADP binding increases the apparent sensitivity of the receptor for its ligand; binding of low concentrations of NAADP will, with time, label more binding sites than a ligand that interacts with its receptor reversibly. NAADP receptors are thus ideally suited for the detection of minute changes in NAADP concentration within an egg.

Finally, we examined the effect of varying the concentration of NAADP during preincubation on subsequent binding of [^{32}P]NAADP (Figure 4D). Incubation with unlabelled NAADP for 30 min prior to [^{32}P]NAADP addition resulted in an approx. 7-fold decrease in the IC_{50} for [^{32}P]NAADP displacement (75 ± 9 pM; $n = 3$) compared with control experiments where labelled and unlabelled NAADP were added simultaneously

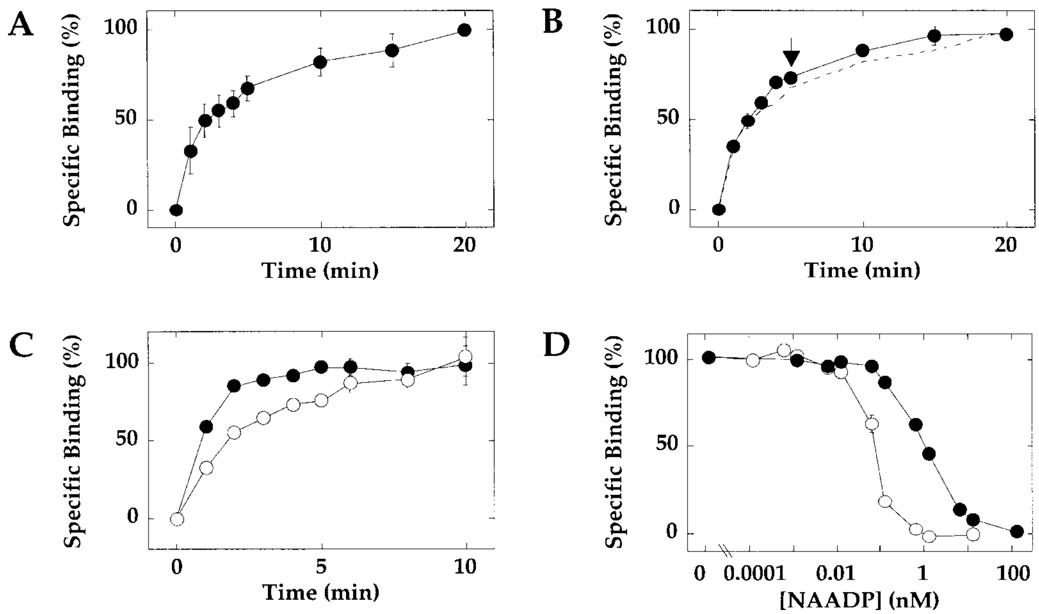

Figure 4 Irreversible [^{32}P]NAADP binding

(**A**) Association kinetics of [^{32}P]NAADP binding. Homogenates were incubated for various times with 0.2 nM [^{32}P]NAADP prior to determination of bound radioligand. (**B**) Lack of effect of excess unlabelled NAADP on binding of 0.2 nM [^{32}P]NAADP. Homogenates were incubated for the indicated times with [^{32}P]NAADP as in (**A**) followed by 100 nM unlabelled NAADP. Bound radioligand was determined 30 min following initiation of the reaction. Thus for the 5 min time point (arrow), homogenates were incubated with [^{32}P]NAADP for 5 min followed by NAADP for 25 min. Despite prolonged incubation with excess unlabelled NAADP, the amount of [^{32}P]NAADP bound was similar to that in association experiments where homogenates were incubated with [^{32}P]NAADP only [dotted line; data taken from (**A**)]. (**C**) Association of 0.2 nM (○) and 0.4 nM (●) [^{32}P]NAADP to sea urchin homogenates. Both submaximal concentrations of radioligand resulted in similar levels of binding (see main text; $n = 3$). (**D**) Effect of incubating homogenates (30 min) with the indicated concentrations of unlabelled NAADP (○) prior to 0.2 nM [^{32}P]NAADP (30 min; $n =$ 3). Data from incubations where labelled and unlabelled NAADP were added simultaneously (●) are shown for comparison. Experiments in (**A**) and (**B**) were performed at 4 °C, whereas those in (**C**) and (**D**) were performed at 20 °C. The IC_{50} values for NAADP binding at 4 °C (0.7 ± 0.2 nM; $n = 4$) and 20 °C (0.54 ± 0.07 nM; $n = 8$) were similar.

(538 ± 66 pM, $n = 8$) [the apparent increase in the slope of the curve in experiments where homogenates were preincubated with NAADP (Figure 4D, open symbols) probably reflects incomplete association of the lower concentrations of NAADP within the time period]. This property can be exploited to form the basis of an extremely sensitive radioreceptor assay for measurement of NAADP levels. Very low concentrations of unlabelled NAADP (< 0.1 nM) only partially inhibited subsequent binding of [^{32}P]-NAADP (Figure 4D), even after incubation periods of up to 12 h (results not shown). This occurred despite the fact that NAADP binding is irreversible and thus should proceed until all binding sites are occupied. Consequently, an intriguing possibility is that the concentration of NAADP receptors may actually be in excess of its ligand at these submaximal inhibitory NAADP concentrations. Thus, only a fraction of receptors would become occupied with NAADP under these conditions. This may also explain why inactivation of Ca^{2+} release by NAADP shows clear concentration dependence [10,13]. Such long-lasting 'quantal' inactivation of occupied receptors [23] would then determine the magnitude of subsequent NAADP responses, an ideal basis for a simple form of memory.

In the present study we have characterized, in detail, the binding properties of NAADP receptors in sea urchin egg homogenates. Factors known to modulate the binding of IP_3 and cADPR to their respective receptors were demonstrated to be without effect on NAADP binding, strongly suggesting that NAADP-induced Ca^{2+} release is mediated via a novel intracellular receptor. Indeed, the kinetics of NAADP binding to its receptor are unique and are probably an important means to increase the sensitivity of detecting cellular NAADP levels.

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