Solubilization of Receptors for the Novel Ca\textsuperscript{2+}-Mobilizing messenger, Nicotinic Acid Adenine Dinucleotide Phosphate

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Running Title: Receptors for NAADP distinct from IP\textsubscript{3} and ryanodine receptors
Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca\(^{2+}\) mobilizing agent in a variety of broken and intact cell preparations. In sea urchin egg homogenates, NAADP releases Ca\(^{2+}\) independently of inositol trisphosphate or ryanodine receptor activation. Little, however, is known concerning the molecular target for NAADP. Here we report for the first time solubilization of NAADP receptors from sea urchin egg homogenates. Supernatant fractions, prepared following Triton X-100 treatment, bound \(^{32}P\)NAADP with similar affinity and selectivity as membrane preparations. Furthermore, the unusual non-dissociating nature of NAADP binding to its receptor was preserved upon solubilization. NAADP receptors could also be released into supernatant fractions upon detergent treatment of membranes prelabelled with \(^{32}P\)NAADP. “Tagged” receptors prepared in this way, were readily resolved by native gel electrophoresis as a single protein target. Gel filtration and sucrose density gradient centrifugation analysis indicates that NAADP receptors are substantially smaller than inositol trisphosphate or ryanodine receptors, providing further biochemical evidence that NAADP activates a novel intracellular Ca\(^{2+}\) release channel.
INTRODUCTION

Changes in cytosolic Ca\textsuperscript{2+} are indispensable for normal cell function (1). A multitude of cell surface Ca\textsuperscript{2+} channels have been characterized that mediate influx of Ca\textsuperscript{2+} from the extracellular space upon activation. These include voltage-operated Ca\textsuperscript{2+} channels, ligand-gated Ca\textsuperscript{2+} channels that are regulated directly by neurotransmitters and Ca\textsuperscript{2+} channels coupled to depletion of intracellular Ca\textsuperscript{2+} stores (1). In contrast, only two types of intracellular Ca\textsuperscript{2+} channels have been described to date: receptors for the second messenger inositol 1,4,5-trisphosphate (IP\textsubscript{3})\textsuperscript{1} and ryanodine receptors (2-4). The latter are modulated by the NAD metabolite, cyclic ADP-ribose (cADPR) (5, 6). Activation of these two pathways by a range of diverse extracellular stimuli evokes increases in cytosolic Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} stores that are largely independent of extracellular Ca\textsuperscript{2+}.

Although the subunit size of ryanodine receptors (~ 560 kDa) is almost twice that of IP\textsubscript{3} receptors (~ 300 kDa), these ubiquitous intracellular Ca\textsuperscript{2+} channels display several similarities in their structure and function. Multiple hydrophobic stretches of amino acids at the extreme C-terminus of both receptors form the transmembrane region encompassing the Ca\textsuperscript{2+} channel and it is within this region that the highest degree of sequence similarity between these proteins is found (7, 8). Both proteins assemble as tetrameric complexes with native molecular weights in excess of 1000 kDa (7, 9). Furthermore, IP\textsubscript{3} and ryanodine receptors are regulated by interactions with a range of accessory proteins including calmodulin (10, 11), the immunophilins, FKBP12 and calcineurin (12-14) and the cytoskeletal protein, ankyrin (15-17). Perhaps
the most important regulator of these Ca\textsuperscript{2+} channels is Ca\textsuperscript{2+} itself. Modest elevations in cytosolic Ca\textsuperscript{2+} are stimulatory resulting in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release whereas higher concentrations of Ca\textsuperscript{2+} inhibit Ca\textsuperscript{2+} release (18). Dual regulation of IP\textsubscript{3} and ryanodine receptors by their respective ligand and Ca\textsuperscript{2+} provides intricate control of channel function and is central to the generation of complex spatio-temporal Ca\textsuperscript{2+} signals (19).

Sea urchin egg homogenates contain readily accessible functional Ca\textsuperscript{2+} stores providing an ideal experimental tool for the study of intracellular Ca\textsuperscript{2+} release pathways (20). In this preparation, both IP\textsubscript{3} and cADPR effect robust release of sequestered Ca\textsuperscript{2+} through activation of IP\textsubscript{3} and ryanodine receptors, respectively. In addition, recent studies have uncovered a third intracellular Ca\textsuperscript{2+} release pathway activated by nicotinic acid adenine dinucleotide phosphate (NAADP) (21, 22). That this pathway is not modulated by changes in cytosolic Ca\textsuperscript{2+} clearly distinguishes it from IP\textsubscript{3}- and cADPR-mediated Ca\textsuperscript{2+} release (23, 24). Indeed, NAADP-induced Ca\textsuperscript{2+} mobilization is demonstrable in the presence of specific IP\textsubscript{3}/cADPR antagonists (21, 22), after depletion of IP\textsubscript{3} and ryanodine/ cADPR-sensitive Ca\textsuperscript{2+} stores with the Ca\textsuperscript{2+} ATPase inhibitor, thapsigargin (24) and also in subcellular fractions insensitive to IP\textsubscript{3} and cADPR (21, 25). The inactivation properties of this pathway are also somewhat unique. Concentrations of NAADP that are below the threshold for Ca\textsuperscript{2+} release are able to effect complete block of subsequent normally maximal NAADP challenge (26, 27). From radioligand experiments, NAADP appears to bind its receptor irreversibly (26, 28, 29) and this unusual property may underlie the unique inactivation of this pathway. These independent lines of
evidence from broken cell preparations support the notion that NAADP targets a novel intracellular Ca^{2+} channel.

Despite such discrete properties of NAADP sensitive Ca^{2+} release in broken cell preparations, the pharmacology of NAADP-induced Ca^{2+} signals in intact cells is much more complex. In sea urchin eggs (30) and starfish oocytes (31), NAADP-mediated Ca^{2+} signals can be inhibited by a combination of heparin and 8-amino cADPR, antagonists of IP_{3} and cADPR responses, respectively. Similarly, in pancreatic acinar cells, NAADP induces Ca^{2+} oscillations, reminiscent of those evoked by the brain-gut peptide, cholecystokinin, that are again sensitive to inhibitors of IP_{3} and cADPR (32). This apparent discrepancy in the pharmacology of NAADP-induced Ca^{2+} release in broken and intact cells can be explained if NAADP initiates a “trigger” Ca^{2+} increase in the intact cell that is then amplified by Ca^{2+}-induced Ca^{2+} release via IP_{3} and cADPR receptors. This effect is lost in biochemical experiments where normal cellular architecture is disrupted. Additionally, Ca^{2+} release from NAADP-sensitive Ca^{2+} stores in intact sea urchin eggs may also be shuttled to thapsigargin-sensitive Ca^{2+} stores resulting in sensitization of IP_{3} and ryanodine receptors and the generation of Ca^{2+} oscillations (33). Thus, this novel Ca^{2+}-mobilizing agent may serve to coordinate Ca^{2+} signals via interaction with other intracellular Ca^{2+} release channels (34). Indeed, blockade of NAADP receptors attenuates Ca^{2+} signals initiated by cholecystokinin in acinar cells (32), T-cell receptor activation in T-lymphocytes (35) and sperm in ascidian eggs (36), events that require functional IP_{3} and/or ryanodine receptors. That metabolism of NAADP is
regulated by Ca\textsuperscript{2+} in brain membranes (37) adds an additional point of regulation for the fine-tuning of Ca\textsuperscript{2+} dynamics.

Although the biochemical and molecular properties of IP\textsubscript{3} and ryanodine receptors are well defined, little is known concerning the nature of NAADP receptors. Here, we report for the first time, solubilization and characterization of NAADP receptors from sea urchin eggs. Our data provide evidence that NAADP targets a novel protein that is distinct from known intracellular Ca\textsuperscript{2+} release channels.
EXPERIMENTAL PROCEDURES

Receptor Solubilization – Sea urchin (Lytechinus pictus) egg homogenates (50% v/v) were prepared as described previously (27), washed twice by centrifugation (20,000 x g, 10 min) at 4°C in binding medium composed of 20 mM HEPES (pH 7.2), 250 mM potassium gluconate, 250 mM N-methyl D-glucamine and 1 mM MgCl₂. Washed homogenates (17% v/v) were solubilized by incubation with either Triton X-100, CHAPS or SDS (1%) for 60 min and centrifuged at 100,000 x g for 60 min. Supernatants were analysed for [³²P]NAADP binding (see below). Solubilization and centrifugation were performed at either 4°C (for Triton X-100 and CHAPS) or room temperature (for SDS).

Radioligand Binding to Solubilized NAADP Receptors – [³²P]NAADP was prepared enzymatically from [³²P]NAD (1000 Ci/ mmol, Amersham Biosciences) as described previously (28, 38). Solubilized extracts were incubated in binding medium supplemented with [³²P]NAADP (0.2 nM) together with the indicated concentrations of unlabelled NAADP or the NAADP analogues, 2',3'-cyclic NAADP and 3'-NAADP (38, 39). Incubations were performed at room temperature for 1 - 3 h (final detergent concentration = 0.2 % w/v). γ-globulin (400 µg) was then added to samples and protein precipitated by incubation with 15% (w/v) polyethylene glycol (average molecular weight = 8000) for 30 - 90 min. Samples were then centrifuged at 12000 x g for 5 min, the resulting pellets washed with 15% (w/v) polyethylene glycol and dissolved in H₂O for Cerenkov counting. Specific binding (1000 – 2000 c.p.m./ incubation) was typically 70% of total binding.
Preparation and Solubilisation of Prelabelled NAADP receptors. - Sea urchin egg homogenates (0.1 - 25 % v/v) were incubated for 1 – 16 h at room temperature in binding medium supplemented with 1 - 2 nM [\(^{32}\)P]NAADP. Samples were subsequently washed twice in binding medium by centrifugation (2 min, 100,000 x g, 4°C). Washed membranes (17 % v/v) were then solubilized with the appropriate detergent as described for unlabelled homogenates.

Gel Electrophoresis - Homogenates (25 % v/v) were prelabelled with [\(^{32}\)P]NAADP in binding buffer (as described above) and subsequently washed and solubilized with Triton X-100 (1%) in a modified buffer composed of 20 mM HEPES (pH 7.2). This step was necessary since we found that binding buffer interfered with electrophoresis (not shown). Solubilized samples were separated on native 7.5% polyacrylamide gels (pH 8.8) at 4°C according to standard procedures (40). Buffers were supplemented with 0.2 % Triton X-100 to prevent protein precipitation. Samples were also subject to electrophoresis on 3-10 pH gradient gels (Bio-Rad) according to the manufacturers instructions. Gels were dried and apposed to Hyperfilm (Amersham Biosciences) at ~80°C for ~16 h prior to developing.

Gel filtration - Prelabeled NAADP receptors solubilised with Triton X-100- or CHAPS in binding medium (100 - 500 µl) were injected on to a Superdex 200 HR 10/30 column linked to an AKTA FPLC system (Amersham Biosciences) equilibrated with binding buffer supplemented with the appropriate detergent (1 %). Fractions (0.5 – 1 ml) were then collected (flow rate of 0.5 ml/ min) and analysed directly for radioactivity. Unlabelled
solubilized samples were also separated under identical conditions and individual fractions assayed for $[^{32}\text{P}]$NAADP binding using polyethylene glycol (see above). NAADP receptor migration was compared to the migration of the molecular weight markers, cytochrome-C (12.5 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), $\beta$-amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa).

**Sucrose Density Gradient Centrifugation** – Triton X-100-solubilized receptors prelabelled with their ligand (200 µl) were layered on to a 1.8 ml 5–20 % (w/ v) sucrose density gradient (prepared in 3% increments) in binding medium supplemented with 1% Triton X-100. Samples were centrifuged in a swing-out rotor at 166,000 x g for 3.5 h at 4°C. Fractions (195 µl) were collected from the top of the gradient and analysed for radioactivity. Unlabelled NAADP receptors were separated under identical conditions. In order to improve resolution of lower molecular weight proteins, prelabelled receptors (200 µl) were also layered on to larger 3.6 ml 5–20 % (w/ v) sucrose density gradients, centrifuged at 164,000 x g for 17 h at 4°C and fractionated as with smaller gradients.
RESULTS AND DISCUSSION

Solubilization of NAADP Receptors - We and others have previously reported binding of $[^{32}P]$NAADP to membrane preparations derived from sea urchin eggs (26, 28, 29). In the present study, washed sea urchin egg homogenates were treated with the non-ionic detergent Triton X-100 and supernatants, following ultracentrifugation, analysed for $[^{32}P]$NAADP binding. Binding of $[^{32}P]$NAADP was readily detected following Triton X-100 solubilization (yield = 153 ± 31 %, n = 3). From isotope dilution experiments, $[^{32}P]$NAADP binding to solubilized receptors was inhibited by NAADP (IC$_{50}$ = 1 ± 0.3 nM, n = 8) and the NAADP analogues, 2',3'-cyclic NAADP (IC$_{50}$ = 3 ± 1 nM, n = 3) and 3'-NAADP (IC$_{50}$ = 8 ± 4 nM, n = 3; Fig. 1A). The rank order of potency for solubilized NAADP receptors (NAADP>2',3'-cyclic NAADP>3'-NAADP) is therefore the same as that reported previously for membrane-bound NAADP receptors (38) and correlates closely with the rank order of potency of NAADP and its analogues in stimulating Ca$^{2+}$ mobilization from sea urchin egg homogenates (39).

An intriguing feature of $[^{32}P]$NAADP binding to sea urchin egg homogenates, but notably not mammalian brain (38) or heart (41) membranes, is its apparent non-dissociating nature (26, 28, 29). As originally described by Lee and colleagues (26), an excess of unlabelled NAADP competes with $[^{32}P]$NAADP if added simultaneously with the radioligand but has no effect once association is initiated (28, 29). In converse experiments, we have shown that if sea urchin egg homogenates are incubated first with unlabelled NAADP prior to addition of radioligand, an apparent leftward-shift in
competition-curves is observed compared to experiments in which labelled and unlabelled NAADP are added together (28). These data are again inconsistent with a reversible interaction. As shown in Fig. 1B, addition of unlabelled NAADP (1 µM) following prior incubation of solubilized receptors with radioligand was without effect on binding of [32P]NAADP over the time-course of the experiment (90 min). Thus, sea urchin egg NAADP receptors, whether membrane-bound or soluble, appear to bind their ligand in an essentially irreversible manner.

The data from Fig. 1 show that the binding characteristics of membrane-bound and solubilized NAADP receptors are similar. Indeed, binding of [32P]NAADP to Triton X-100 extracts was inhibited by high concentrations (1 M) of NaCl but unaffected by pH in the range 6 - 8 (data not shown), again consistent with previous studies using membrane preparations (28). Taken together, our data indicate that detergent treatment of sea urchin egg homogenates results in solubilization of functional NAADP receptors.

Solubilization of NAADP Receptor-ligand Complexes - Since binding of NAADP to both membrane-bound and solubilized receptors does not readily dissociate, we determined whether membrane-bound NAADP receptors could be first “tagged” with their ligand and then solubilized as a receptor-ligand complex. Sea urchin egg homogenates were incubated with [32P]NAADP and membranes washed by centrifugation inorder to remove unbound radioligand. [32P]NAADP binding to sea urchin egg homogenates was similar whether determined by this centrifugation method (14.8 ± 1 fmol/µl homogenate, n = 3) or filtration (10.3 fmol/µl homogenate, n = 2).
These data indicate that washing does not induce dissociation of radioligand validating the use of this assay for the preparation of labelled membranes. Simultaneous addition of unlabelled NAADP together with radioligand during prelabelling of sea urchin egg homogenates (0.1 % v/v) reduced the levels of tagged receptors following subsequent centrifugation in concentration-dependent manner (Fig. 2A). The IC$_{50}$ value for NAADP in these protection assays (2 ±0.6, n = 3) is similar to the apparent affinity of membrane-bound (28) and solubilized (Fig. 1A) NAADP receptors. At higher homogenate concentrations (25% v/v), we noted that the apparent affinity of NAADP receptors determined by centrifugation (16 ±2 nM, n = 3) and also by filtration (data not shown) was somewhat reduced (Fig. 2A). Accordingly, reduced levels of [32P]NAADP binding to a fixed amount of sea urchin egg homogenate was evident when incubations were performed at lower dilutions (Fig. 2B). Clearly then, binding of NAADP versus homogenate concentration is non-linear (Fig. 2B, inset), an effect which may be due to increased NAADP metabolism at higher protein concentrations. Preincubation of sea urchin egg homogenates with a low concentration of NAADP prior to addition of radioligand reduced subsequent tagging to a greater extent than when the same concentration of NAADP was incubated together with the radioligand (Fig. 2C). In addition, incubation of a normally maximal concentration of NAADP was without effect on tagging if added after the radioligand during preincubation. These effects, which were similar at low and high concentrations of sea urchin egg homogenates during labelling (Fig. 2C), further indicate that NAADP does not dissociate from its
receptor following washing by centrifugation confirming again our results obtained by conventional filtration assays (28).

In the next set of experiments, we treated membranes labelled with [32P]NAADP with the Triton X-100, the zwitter-ionic detergent CHAPS and the ionic detergent, SDS. All three detergents resulted in efficient release of radioligand from prelabelled membranes into supernatant fractions following ultracentrifugation whereas buffer alone was without effect (Fig. 3A,C). In order to determine whether released radioligand following solubilization of prelabelled membrane remained bound to its receptor or had dissociated due to detergent treatment, we precipitated fractions with polyethylene glycol and analysed the resulting pellets for radioactivity (Fig. 3B,C). Following solubilization with Triton X-100 and CHAPS, 63 ± 13 % and 74 ± 11 % (n = 3) of the radioactivity in supernatant fractions could be precipitated, respectively, indicating that binding of [32P]NAADP to its receptor is preserved. In contrast, recovery after SDS treatment was minimal (4 ± 1 %, n = 3, Fig. 3C). Thus, under the latter denaturing conditions, supernatant radioactivity is likely free, unbound radioligand. These data, together with the finding that high salt concentrations initiate partial dissociation of bound [32P]NAADP to membrane preparations (28), provide evidence that irreversible binding of [32P]NAADP to NAADP receptors is not due to covalent modification of the target protein.

Having defined solubilization conditions that retain binding of [32P]NAADP to its receptor, we analysed prelabelled NAADP receptors by native polyacrylamide gel electrophoresis. Autoradiograms following
separation of labelled membranes solubilized with Triton X-100 revealed that a significant proportion of the radioactivity migrated as a band distinct from the dye-front (Fig. 4A). These data further suggest that $^{32}$PNAADP remains bound to its receptor following solubilization of labelled membranes with mild detergents. Additionally, $^{32}$PNAADP appears to bind only a single protein under these conditions. However, dissociation of $^{32}$PNAADP from its receptor was also evident (Fig. 4A). We also analysed NAADP receptor migration by electrophoresis on pH gradient gels (Fig. 4B). From these experiments the isoelectric point of prelabelled NAADP receptors was 5.9 ± 0.3 ($n = 3$).

Molecular Size Determination of NAADP Receptors - Although NAADP receptor-ligand complexes could be resolved by native polyacrylamide gel electrophoresis (Fig. 4), separation of proteins by this method is dependent not solely on size but also net charge, and is therefore not well suited for determining molecular size. SDS (which eliminates the endogenous charge of proteins) could not be included during electrophoresis since it induced ligand dissociation (Fig 3C). The molecular size of NAADP receptors was therefore determined by gel filtration. With this method, previous studies have demonstrated that both IP$_3$ and ryanodine receptors, (which assemble as large tetrameric complexes) migrate to fractions corresponding to molecular weights of ~1000 and 2000 kDa, respectively (42, 43). In contrast, we provide evidence that NAADP receptors are substantially smaller. Fractionation of prelabelled NAADP receptors solubilized in the presence of Triton X-100 on Superdex 200 indicated a molecular weight of 471 kDa ($n = 3$, Fig. 5A, closed
circles). Similar results were obtained following fractionation of unlabelled homogenates (Fig. 5A, inset). In these “post-labelling” experiments, NAADP receptor migration was determined conventionally by analysing the individual fractions for $[^{32}P]$NAADP binding as in Fig. 1. Similar results were also obtained when gel filtration was performed in the presence of a protease inhibitor cocktail (data not shown). Migration of prelabelled NAADP receptors solubilized with CHAPS (Fig. 5B) revealed that the molecular weight was consistently smaller (408 kDa, $n=3$) than that determined in the presence of Triton X-100 (471 kDa, $n=3$). Gel filtration of the NAADP receptor in the presence of CHAPS, where total protein distribution could be determined in parallel (Fig. 5B, dotted line), indicated a $9.3 \pm 0.4$-fold ($n=3$) enrichment of NAADP receptors in the peak fraction.

We next determined the molecular weight of NAADP receptors by sucrose density gradient centrifugation (Fig. 6). As with gel filtration, both IP$_3$ and ryanodine receptors migrate as high molecular weight complexes and are accordingly recovered in “heavy” fractions with this technique (7, 9). Prelabelled NAADP receptors were therefore layered onto a 5 - 20% sucrose gradient and fractions analysed for radioactivity following ultracentrifugation (Fig 6A). In contrast to the results from gel filtration experiments, where NAADP receptors coeluted with apoferretin (molecular weight 443 kDa, Fig. 5), the estimated molecular size of prelabelled NAADP receptors with this method was substantially smaller. Thus, whereas apoferretin migrated to fraction $5.2 \pm 0.2$ ($n=7$), NAADP receptors prelabelled with $[^{32}P]$NAADP at a homogenate concentration of either 25 % v/v or 0.1 % v/v migrated earlier to
fractions 2.4 ± 0.3 (n = 5) and 2.7 ± 0.4 (n = 3), respectively (Fig. 6A, closed circles, pooled data from all 8 experiments). This distribution is more similar to that of alcohol dehydrogenase (2.7 ± 0.4, n = 3; molecular weight 150 kDa). Again, similar results were obtained when NAADP receptor migration was determined by post labelling of the collected fractions with [32P]NAADP following separation of unlabelled NAADP receptors (peak fraction = 2.5, n = 2; Fig. 6A, open circles). These data further highlight the usefulness of the prelabelling method for tracking NAADP receptors during purification.

Migration of prelabelled NAADP receptors on glycerol density gradients was also the same as sucrose density gradients (data not shown). In order to obtain a more accurate molecular weight by sucrose density gradient centrifugation, we performed experiments on larger gradients following prolonged centrifugation (Fig. 6B). From these experiments, the molecular weight of prelabelled NAADP receptors was estimated to be 120 ± 2 kDa. (n = 3). Inclusion of an excess of unlabelled NAADP during incubation of homogenates with [32P]NAADP, resulted in substantial reduction in radioactivity following solubilization and fractionation, confirming the specificity of binding (Fig. 6B, open circles).

The surprising discrepancy between the calculated molecular weight of NAADP receptors by gel filtration (Fig. 5) and sucrose density gradient centrifugation (Fig. 6) was further analysed by combining the two techniques in sequence (Fig. 7, insets). Separation of partially purified NAADP receptors following gel filtration on sucrose gradients, indicated that prelabelled NAADP receptors again coeluted with alcohol dehydrogenase (Fig. 7A, n = 3).
and were thus substantially smaller than that estimated by gel filtration (Fig. 5). Conversely, prior separation of NAADP receptors on sucrose density gradients followed by purification of peak fractions by gel filtration, indicated that NAADP receptors were substantially larger than when analysed on sucrose density gradients (Fig. 6). In these experiments, NAADP receptors comigrated with apoferritin (Fig. 7B, n = 3) as did crude solubilized samples (Fig. 5A). One possibility to reconcile the results from gel filtration and sucrose density gradient experiments is that NAADP receptors form oligomeric complexes that are dissociated by the latter technique in a reversible manner. Although the mechanism underlying this effect is not known at present, this feature is not shared by known intracellular Ca\textsuperscript{2+} channels, suggesting that NAADP binds to a distinct, smaller target. Indeed, under the present conditions optimized for solubilization of NAADP receptors, we were unable to solubilize ryanodine receptors with Triton X-100 following prelabelling of homogenates with \[^{3}H\]ryanodine (data not shown).

In summary, we have for the first time characterized the binding properties of NAADP receptors solubilized from sea urchin eggs. Solubilized NAADP receptors bound their ligand with the appropriate affinity and selectivity. Furthermore, binding of \[^{32}P\]NAADP to detergent extracts is not readily reversible indicating that this unusual property is likely to be intrinsic to the NAADP receptor. We also show that NAADP receptors can be tagged with their ligand and solubilized intact thereby providing a convenient means of tracking NAADP receptors during purification. Results from gel filtration and sucrose density gradient centrifugation experiments indicate that
NAADP receptors are significantly smaller than IP₃ and ryanodine receptors. Our data provide further evidence that NAADP mediates Ca²⁺ mobilization via a novel pathway.

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ABBREVIATIONS

The abbreviations used are: cADPR, cyclic ADP-ribose; IP₃, inositol trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate.
FIG 1. [³²P]NAADP binding to solubilized sea urchin egg homogenates. A, Competitive [³²P]NAADP displacement. Washed sea urchin egg homogenates were solubilized with Triton X-100 (1 %) and binding of [³²P]NAADP (0.2 nM) to supernatant fractions determined in the presence of the indicated concentrations of unlabelled NAADP (open circles, n = 8), 2',3'-cyclic NAADP (closed circles, n = 3) and 3'-NAADP (squares, n = 3) added simultaneously. B, Irreversible [³²P]NAADP binding. Solubilized preparations were incubated with [³²P]NAADP (0.2 nM) for 3 h followed by 1 µM unlabeled NAADP (designated t = 0 on graph) for the times shown prior to separation of bound and free radioligand (n = 3). Simultaneous addition of NAADP (1 µM) with the radioligand reduced total binding to 19 ± 6 % (n = 3).

FIG 2. Prelabelling of NAADP receptors with [³²P]NAADP. A, Sea urchin egg homogenates were incubated with [³²P]NAADP (0.8 nM) together with the indicated concentration of unlabelled NAADP and subsequently washed in binding medium to remove unbound ligand. Incubations were performed with either 0.1 % (closed circles) or 25 % (open circles) v/v homogenates. B, C Effect of homogenate concentration on [³²P]NAADP binding. A fixed amount of sea urchin egg homogenate (1 µl) was prelabelled with [³²P]NAADP at the indicated dilution (incubation volume = 4 – 1024 µl). Inset, Data from B expressed as [³²P]NAADP binding per unit incubation volume. C, Prelabelling of NAADP receptors with radioligand ([³²P]) in the simultaneous (+) presence of a submaximal and maximal concentration of unlabelled NAADP as indicated. Membranes were also incubated with unlabelled NAADP before
and after addition of radioligand as depicted by arrows. Binding was performed with 0.1% (left) or 25% (right) v/v sea urchin egg homogenates and is expressed relative to binding of \[^{32}\text{P}]\text{NAADP}\) in the absence of unlabelled NAADP (100%).

**FIG. 3** Solubilization of intact NAADP receptor-ligand complexes. A, Sea urchin egg homogenates were incubated with \[^{32}\text{P}]\text{NAADP} (1-2 nM) for 1 h and free ligand removed by centrifugation. Labelled membranes were then incubated with binding buffer with or without the indicated detergent (1%) for 60 min (1). Samples were centrifuged (100,000 x g, 60 min) and radioactivity in the supernatant (sup.) quantitated (B). Data are expressed relative to total membrane-bound radioactivity added prior to solubilization. Supernatant samples were also incubated with 15% polyethylene glycol (PEG) in order to precipitate protein (2). Radioactivity associated with the recovered pellets (expressed relative to total radioactivity present in incubations prior to precipitation) following centrifugation (12000 x g, 5 min) is shown in C.

**FIG. 4.** Gel electrophoresis of NAADP receptor-ligand complexes. Sea urchin egg homogenates were labelled with \[^{32}\text{P}]\text{NAADP} either in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of unlabelled NAADP (100 µM) and solubilized with Triton X-100 following removal of unbound ligand. A, Samples were separated on a native 7.5% polyacrylamide gel (pH 8.8) and the resulting autoradiogram shown. Lanes 2 and 4 were loaded with twice the amount of protein as lanes 1 and 3. These data show that a significant
proportion of soluble radioactivity following prelabelling/detergent treatment is associated with a single protein (“Bound”) likely reflecting binding of \[^{32}\text{P}]\text{NAADP}\) to its receptor. Dissociated ligand in the dye-front is marked (“Free”). B, Separation of prelabelled NAADP receptors on 3 – 10 pH gradient gels. Comparison with the migration of prestained markers (left, indicates that the isoelectric point (pI) of NAADP receptor-ligand complexes is ~ 6. Lanes contained equal amounts of protein. Data are representative of at least 3 experiments.

FIG. 5. **Gel Filtration analysis of NAADP Receptors.** Sea urchin egg homogenates (25% v/v) were incubated with \[^{32}\text{P}]\text{NAADP}\) and solubilized (following washing) with either 1% Triton X-100 (A) or CHAPS (B). Samples were then subject to FPLC using a Superdex 200 HR column. Resulting fractions (0.5 ml) were analysed directly for radioactivity (closed circles, \(n = 3\)). The inset compares this prelabelling protocol (“PRE”, \(n = 3\)) to determine the molecular size of NAADP receptors with that obtained by fractionation of unlabelled solubilized samples and assaying of the collected fractions (1 ml) for \[^{32}\text{P}]\text{NAADP}\) binding (“POST”, \(n = 2\)). The dotted line in B is the associated total protein profile determined by monitoring absorbance at 280 nm. Migration of molecular weight markers is shown at the top of each trace (error bars were smaller than the symbols).

FIG. 6 **Separation of NAADP receptors by sucrose density gradient centrifugation.** Sea urchin egg homogenates (0.1% and 25% v/v) were
incubated either in the presence (closed circles) or absence (open circles) of 
$[^{32}P]$NAADP and solubilized (following washing) with 1\% Triton X-100.
Samples were layered on to either a 1.8 ml (A) or 3.6 ml (B) 5 - 20 \% sucrose
density gradient. Following ultracentrifugation, the distribution of
prelabelled ("PRE", n = 8) and unlabelled ("POST", n = 2) NAADP receptors,
in fractions (195 µl) collected from the top of the gradient, was determined as
in Fig. 5.

**FIG. 7** Sequential separation of prelabelled NAADP receptors by gel
filtration and sucrose density gradient centrifugation. The insets are
schematics of the experimental protocol. Prelabelled NAADP receptors
(solubilized in Triton X-100) were either separated first by gel filtration (G) as
in Fig. 5A and then the peak fraction analysed by sucrose density gradient
centrifugation (S) as in Fig. 6 (A) or the converse experiment in which
prelabelled NAADP receptors were subject to sucrose density gradient
centrifugation prior to gel filtration analysis (B). Data are expressed as means
± s.e.m. from at least 3 independent experiments.
**Fig. 2**

A. Graph showing specific binding (%), with [NAADP] (nM) on the x-axis.

B. Graph showing specific binding (fmol/incubation), with [Homogenate] (%) on the x-axis.

C. Bar chart showing [32P]NAADP binding (% total), with different conditions and concentrations indicated.
A

[\textsuperscript{32}P]Membranes + Detergent → sup. x g → sup. → PEG + pellet

B

\(n = 6\) \(n = 8\) \(n = 7\) \(n = 3\)

C

\(n = 3\) \(n = 3\) \(n = 3\)

Fig. 3
Fig. 4

A

Bound

Free

B

pI

7.5 —

7.1 —

7.0 —

4.7 —
Fig. 5
Fig. 6
Fig. 7
Solubilization of receptors for the novel Ca2+-mobilizing messenger, nicotinic acid adenine dinucleotide phosphate

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