NAADP binding to its target protein in sea urchin eggs requires phospholipids

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Mobilization of intracellular Ca^{2+} pools by NAADP (nicotinic acid–adenine dinucleotide phosphate) is becoming increasingly recognized as an important determinant of complex Ca^{2+} signals. However, the properties of the putative Ca^{2+} channel activated by NAADP are poorly defined. In the present study, we provide evidence that binding of NAADP to its target protein in sea urchin eggs requires phospholipids. Decreasing the level of protein-bound lipid in detergent extracts by either dilution of the preparation at a fixed detergent concentration or increasing the detergent concentration at a fixed protein concentration inhibited [³²P]NAADP binding. These effects were prevented by the addition of phospholipids, but not other related molecules, were reversible and were associated with a marked decrease in the apparent affinity of the target protein for its ligand. Additionally, we show that the extent of dissociation of NAADP–receptor ligand complexes during gel filtration in the presence of detergent correlates well with the extent of delipidation. Our data highlight the importance of the lipid environment for interaction of NAADP with its target protein.

Key words: calcium, nicotinic acid–adenine dinucleotide phosphate (NAADP), phospholipids, sea urchin, signal transduction.

INTRODUCTION

Mobilization of intracellular Ca^{2+} stores by messengers produced in response to extracellular cues such as hormones and transmitters is a ubiquitous signal transduction mechanism [1]. To date, the best characterized intracellular Ca^{2+} release channels are receptors for inositol trisphosphate [2,3] and the plant alkaloid ryanodine [4]. The latter are regulated by the NAD metabolite, cADP-ribose [5–7]. In addition, a number of studies have identified a novel Ca^{2+} mobilization pathway sensitive to NAADP (nicotinic acid–adenine dinucleotide phosphate) [5,7–12].

Although only in its infancy, current research focused on the actions of NAADP suggests that this derivative of NADP is a new Ca^{2+} mobilizing messenger [13]. NAADP is present in a range of cells [14–18], its cellular levels increase in response to extracellular stimuli that raise cytosolic Ca^{2+} levels [15,16,18], and desensitization of NAADP-induced Ca^{2+} release attenuates Ca^{2+} response to those stimuli (reviewed in [12]). In sea urchin (*Lytechinus pictus*) egg homogenates, it is clear, based on cross-desensitization experiments, a unique mode of ligand-induced inactivation and the insensitivity of Ca^{2+} release to bivalent cations, that NAADP appears to target Ca^{2+} channels that are distinct from inositol trisphosphate and ryanodine receptors (reviewed in [12]). Moreover, recent evidence suggests that these channels are located not on the endoplasmic reticulum but instead on novel lysosomal-like Ca^{2+} stores [19]. In mammalian cells, however, the situation is less clear. Manoeuvres that perturb inositol trisphosphate and/or ryanodine receptor function also attenuate NAADP-induced Ca^{2+} release in several cell types (e.g., see [20]). Although these effects are consistent with functional ‘chatter’ [8] between intracellular Ca^{2+} channels as originally proposed [20], a more direct effect of NAADP on established channels, in particular the ryanodine receptor [21–24], cannot be ruled out. It should be noted, however, that inositol trisphosphate and ryanodine receptor antagonists (in combination) also inhibit

NAADP-induced Ca^{2+} release in intact sea urchin eggs [25], a cell type where evidence for a dedicated NAADP-sensitive Ca^{2+} channel is ample and uncontested. Clarification of this issue will require molecular identification of the NAADP receptor.

Extending initial studies by Lee and colleagues [26], we have shown that [³²P]NAADP binds avidly to sea urchin egg homogenates, probably to a single site in a non-reversible manner [27]. The target protein has been solubilized and probably forms a high-molecular-mass complex [28]. Several lines of evidence suggest that this protein is related to the putative Ca^{2+} channel that mediates Ca^{2+} release in response to NAADP in this preparation. First, the rank order of potency of NAADP and NAADP analogues is similar in competition binding [29] and Ca^{2+} release [30] assays. Secondly, homogenate preparations that release Ca^{2+} in response to inositol trisphosphate and cADP-ribose, but not NAADP, fail to bind NAADP [27], and thirdly, the sensitivity of intracellular Ca^{2+} stores to NAADP is decreased in media containing low K^{+} concentrations, conditions under which binding of NAADP is partially reversible [31]. Whether NAADP binds directly to a Ca^{2+} channel is not known at present, an issue that will again require isolation (and functional expression) of the NAADP-binding protein(s).

Many ion channels are modulated by their lipid environment. Direct effects of lipids include modulation of ion channels by lipid second messengers. For example, unsaturated fatty acids, such as AA (arachidonic acid), are thought to regulate certain members of the tandem-pore K^{+} channel family [32–35]. Similarly, sphingosine 1-phosphate, as well as activating cell-surface G-protein-coupled receptors [36], can also stimulate direct mobilization of endoplasmic reticulum Ca^{2+} stores via unidentified Ca^{2+} channels [37,38]. In addition, evidence suggest that several ion channels, such as inward rectifier K^{+} channels [39] and capsaicin receptors [40], bind phosphatidylinositol bisphosphate, and that changes in the levels of this lipid, following agonist stimulation, can exert regulatory influences. Ion channels may also be

Abbreviations used: AA, arachidonic acid; KCIIM, KCl-based, intracellular-like medium; KGIIM, potassium gluconate-based intracellular-like medium; LPC, lysophosphatidylcholine; NAADP, nicotinic acid–adenine dinucleotide phosphate; PA, palmitoleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPM, sphingomyelin.

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regulated by more stable associations with lipids. Ion flux through the nicotinic acetylcholine receptor, the prototypical member of the ligand-gated ion channel superfamily, shows an absolute requirement for acidic phospholipids and neutral lipids such as cholesterol [41]. This effect has been attributed to stabilization of secondary structure via distinct specific lipid-binding sites [42]. Ion channels can also be modulated by lipids indirectly through their effect on biophysical properties of the membrane such as fluidity and thickness [42]. Clearly then, a full appreciation of the functioning of ion channels requires knowledge of its interactions with lipids.

While much is known concerning the regulation of cell-surface ion channels by lipids, corresponding data for intracellular Ca^{2+} channels are scarce. In the present study, we show for the first time that phospholipids are required for binding of NAADP to its target protein in sea urchin eggs.

MATERIALS AND METHODS

Receptor solubilization

Sea urchin egg homogenates (50 %, v/v) were prepared as described previously [31] and were washed twice by centrifugation at 100 000 *g* for 5 min at 4 °C in KGluIM (potassium gluconate-based intracellular-like medium) composed of 250 mM potassium gluconate, 250 mM *N*-methyl-D-glucamine, 20 mM sodium Hepes (pH 7.2) and 1 mM MgCl_2 . Washed homogenates (8 %, v/v) were incubated with either Triton X-100 or CHAPS (1 %, w/v) for 60 min at 4 °C, and then centrifuged at 100 000 *g* for 60 min at 4 °C. Supernatant fractions containing soluble NAADP-binding protein were stored at –80 °C until required.

Radioligand binding to solubilized egg homogenates

[^{32}P]NAADP was prepared enzymatically from [^{32}P]NAD (1000 Ci/mmol) (Amersham Biosciences) as described previously [27]. Briefly, [^{32}P]NAD was first phosphorylated to [^{32}P]NADP and then converted into [^{32}P]NAADP by exchange of nicotinamide with nicotinic acid. Soluble samples were diluted 10-fold into KGluIM supplemented with [^{32}P]NAADP (0.5–1 nM) and γ -globulin (4 mg/ml) in either the absence or the presence of the indicated concentration of detergent and lipid. Stock solutions (50–100 mg/ml) of PC (phosphatidylcholine) (from egg yolk), LPC (lysophosphatidylcholine) (from egg yolk), PE (phosphatidylethanolamine) (from bovine brain), PS (phosphatidylserine) (from bovine brain), SPM (sphingomyelin) (from egg yolk), diacylglycerol (1-stearoyl-2-arachidonoyl-*sn*-glycerol), AA and PA (palmitoleic acid) (all from Sigma) were prepared in the appropriate detergent (10–20 %, w/v) and stored at –20 °C. Binding reactions were performed at room temperature for 30 min and terminated by precipitation of protein with 15 % (w/v) poly(ethylene glycol) (average molecular mass, 8000 Da) for 30 min at 4 °C. Samples were then centrifuged at 100 000 *g* for 5 min at 4 °C; the resulting pellets were washed with 15 % (w/v) poly(ethylene glycol) and dissolved in water for Cerenkov counting.

In some experiments (see Figures 1A and 1B), Triton X-100-solubilized preparations were serially diluted in binding medium [$\pm$ 1 % (w/v) Triton X-100 with or without phospholipid] before radioligand binding (final detergent concentration was 0.1 %, w/v). For reversibility experiments (see Figure 1C), solubilized egg homogenates were first diluted 4-fold with Triton X-100 (1 %, w/v), and then samples were adjusted to 1.5 % (w/v) Triton X-100 or 1.5 % (w/v) Triton X-100 + 2.5 mg/ml PC 5 min later using concentrated stock solutions such that the total incubation volume was increased < 5 %. Radioligand binding was compared with

samples that had not been diluted, but where the appropriate amount of detergent had been added. The final Triton X-100 concentration in this set of experiments was therefore 0.15 % (w/v).

Gel-filtration analysis

Sea urchin egg homogenates (2.5 %, v/v) were incubated for 30 min at room temperature in KGluIM supplemented with 0.1 nM [^{32}P]NAADP. Samples were subsequently washed and solubilized with Triton X-100 as described above for unlabelled homogenates. Radiolabelled NAADP samples (50 μl) were injected on to a Superdex 200 HR 10/30 column (Amersham Biosciences) linked to a HPLC system (Waters). Fractionation was performed at room temperature (flow rate, 0.5 ml/min) in either KGluIM or KCIIM (KCl-based intracellular-like medium) composed of 250 mM KCl and 20 mM sodium Hepes, pH 7.2, in either the absence or the presence of PC (1 mg/ml). Both media were supplemented with Triton X-100 (1 %, w/v). In some experiments, unlabelled soluble extracts were incubated with [^3H]PC (0.02 nM, 66 Ci/mmol) (Amersham Biosciences) for 5 min before separation. Collected fractions were analysed directly for radioactivity by Cerenkov (for [^{32}P]NAADP) or scintillation (for [^3H]PC) counting. NAADP receptor migration was compared with that of apoferritin (molecular mass of 443 kDa) determined by measuring the absorbance of the column eluate at 280 nm.

Data analysis

All data are expressed as means $\pm$ S.E.M. from *n* independent experiments.

RESULTS

Effect of dilution at a fixed detergent concentration on [^{32}P]NAADP binding to solubilized sea urchin egg homogenates

We have reported previously the successful solubilization of NAADP-binding sites from sea urchin egg homogenates [28]. In the present study, homogenates solubilized with the non-ionic detergent Triton X-100 were serially diluted at a fixed detergent concentration (1 %, w/v) and binding of [^{32}P]NAADP was examined. Results from a typical experiment (Figure 1A) indicate that [^{32}P]NAADP binding to diluted samples clearly deviates from the linear relationship expected between maximal binding and protein concentration (broken line). From several experiments of this type, the amount of [^{32}P]NAADP bound at arbitrary concentrations of 1 and 0.125 was 12 ± 1 (*n* = 6) and 0.38 ± 0.26 (*n* = 3) fmol/incubation respectively; the latter value being only 27 ± 12 % of that expected following an 8-fold dilution [$(12 \pm 1)/8 = 1.5 \pm 0.1$ fmol/incubation; Figure 1B]. Since the detergent concentration remained constant in these experiments, we considered the possibility that the inhibitory effect of dilution on [^{32}P]NAADP binding was due to the resulting decrease in the protein/lipid ratio of the preparation. Binding of [^{32}P]NAADP to soluble preparations was therefore examined in the presence of exogenous phospholipid. When PC (the most abundant phospholipid of biological membranes) was included during dilution, [^{32}P]NAADP binding was strictly linear (Figure 1A). Thus, following an 8-fold dilution in the presence of PC, binding of [^{32}P]NAADP from three independent experiments was 1.6 ± 0.3 fmol/incubation and therefore close (109 ± 7 %) to the theoretical value of 1.5 fmol/incubation (see above). [^{32}P]NAADP binding to solubilized preparations that had been diluted 4-fold with detergent was similar whether PC was added during or 5 min after dilution (Figure 1C); respective average values were 24 ± 1 % and 26 ± 1 % of control (undiluted) incubations (*n* = 3). Taken together, these data reveal

Figure 1 Effect of dilution at a fixed detergent concentration on [32 P]NAADP binding to solubilized sea urchin egg homogenates

(A) Non-linear binding of [32 P]NAADP to diluted solubilized egg homogenates. Typical experiment (performed in triplicate; means $\pm$ S.D.) showing specific binding of a saturating concentration of [32 P]NAADP (0.5–1 nM) to varying concentrations of egg homogenates solubilized with Triton X-100 following a 10-fold dilution of the preparation in detergent-free binding medium (final Triton X-100 concentration = 0.1%, w/v). Solubilized preparations were used directly for radioligand binding or first serially diluted (2–16-fold) in binding medium + 1% (w/v) Triton X-100 with (○) or without (●) 2.5 mg/ml PC, to yield arbitrary receptor concentrations of 1 and 0.5–0.125 respectively. Results from several experiments of this type ($n=3$) are shown in (B), where data are expressed as a percentage of that expected, assuming a linear relationship between receptor concentration and [32 P]NAADP binding (broken line). (C) Inhibitory effects of dilution on [32 P]NAADP binding are reversible. Binding of [32 P]NAADP to soluble preparations that had been diluted 4-fold (receptor concentration = 0.25) with Triton X-100 (1%, w/v) either in the absence (Dilute) or presence of PC added simultaneously (Dilute + PC) or 5 min after dilution (Dilute $\rightarrow$ PC). See the Materials and methods section for further details. Data are normalized to binding to undiluted samples (receptor concentration = 1). AU, arbitrary units.

a potential requirement for phospholipids in binding of NAADP to its target protein and that the inhibitory effect of lipid removal on [32 P]NAADP binding is fully reversible.

Figure 2 Effect of detergent concentration at a fixed dilution on [32 P]NAADP binding to solubilized sea urchin egg homogenates

(A) Triton X-100 inhibits [32 P]NAADP binding. Binding of [32 P]NAADP to Triton X-100-solubilized extracts (arbitrary receptor concentration = 1) was determined in the presence of Triton X-100 at the indicated concentrations in either the absence (●) or presence of PC (○). Results are from at least four independent experiments. (B) Similar experiments using CHAPS-solubilized samples. (C) Inhibitory effects of Triton X-100 on [32 P]NAADP binding are reversible. Binding of [32 P]NAADP was examined to Triton X-100-solubilized samples adjusted to a submaximal inhibitory concentration of detergent (0.4%, w/v) and compared with samples where further detergent was added (after a 5 min incubation) to a final concentration of 1.6% (w/v) in either the absence (0.4% $\rightarrow$ 1.6%) or presence of 4 mg/ml PC (0.4% $\rightarrow$ 1.6% + PC). Data are normalized to [32 P]NAADP binding at 0.1% (w/v) Triton X-100.

Effect of detergent concentration at a fixed dilution on [32 P]NAADP binding to solubilized sea urchin egg homogenates

In a converse set of experiments, radioligand binding to a fixed receptor concentration was examined at increasing detergent concentrations. As shown in Figure 2(A), Triton X-100 inhibited binding of [32 P]NAADP to soluble samples. The concentration of Triton X-100 that caused 50% inhibition (IC_{50}) was 0.17%

(w/v). The inhibitory effects of Triton X-100 on [32 P]NAADP binding were prevented by PC (Figure 2A). Thus decreasing the protein/lipid ratio of the preparation by this independent means also inhibits binding of NAADP to its target protein, an effect prevented by exogenous phospholipid. Additionally, we examined the effects of the zwitterionic detergent CHAPS on [32 P]NAADP binding to soluble preparations. As with Triton X-100, CHAPS inhibited [32 P]NAADP binding in the absence, but less so in the presence, of PC (Figure 2B). The inhibitory effects of CHAPS (in the absence of added phospholipid) were less marked than Triton X-100 ($IC_{50} = 1.4\%$, w/v).

We examined the effects of phospholipid on [32 P]NAADP binding after changing the protein/lipid ratio by the addition of high concentrations of Triton X-100. As in Figure 2(A), 0.4% (w/v) Triton X-100 inhibited [32 P]NAADP binding by $40 \pm 5\%$ (Figure 2C). Binding was reduced by $75 \pm 11\%$ following further addition of Triton X-100 (to a final concentration of 1.6%) 5 min after the first addition (Figure 2C). In parallel incubations, binding of [32 P]NAADP following subsequent addition of detergent in the presence of PC was similar to control incubations where the protein/lipid ratio was unperturbed (final detergent concentration, 0.1%) (Figure 2C). These data indicate that the effects of delipidation by high concentrations of detergent are reversible.

Effect of delipidation on the binding properties of the [32 P]NAADP target protein

We next examined in more detail the interaction of PC and related molecules with the NAADP receptor. Inhibition of NAADP binding by Triton X-100 (1.6%, w/v) was prevented by PC in a concentration-dependent manner, with the half-maximal effect occurring at approx. 2 mg/ml (Figure 3A). Similar results were obtained with LPC (which lacks one of the fatty acid side chains), although this lipid was less effective than PC (Figure 3A). In contrast, the sphingosine-based phospholipid SPM afforded little protection against high detergent (Figure 3A). Similarly, diacylglycerol (which lacks the head group) and non-esterified ('free') fatty acids also failed to protect against delipidation by Triton X-100 (Figure 3B). Indeed, both AA and PA caused further inhibition of [32 P]NAADP binding to delipidated preparations. In similar experiments, the effect of Triton X-100 was partly prevented by the polar head group phosphocholine (Figure 3C), but relatively high concentrations were required compared with PC when taking into account the differences in the molecular mass of the two compounds. In contrast, choline had little effect (Figure 3C). Finally, a second neutral phospholipid, PE, and the negative phospholipid, PS, were tested. Like PC, both completely prevented inhibition by Triton X-100 (Figure 3D). Taken together, these data show that phospholipids are specifically required for binding of NAADP to its target protein.

Inhibitory effects of delipidation on [32 P]NAADP binding are due to a decrease in the apparent affinity of the target protein for its ligand

To determine the mechanism by which lipid removal inhibits NAADP binding to its target protein, we performed saturation binding analysis in the presence of a low and a high concentration of Triton X-100. As shown in Figure 4, delipidation of the preparation was associated with a decrease in the apparent affinity of the target protein for its ligand. At a detergent concentration of 0.1% (w/v), the apparent affinity determined by curve fitting of the data to a one-site model was 100 ± 3 pM ($n = 5$). This value was clearly increased in the presence of 1.6% (w/v) detergent, although the data could not be accurately fitted by the same relationship, since saturation was not observed. However, the use

Figure 3 Inhibitory effects of delipidation on [32 P]NAADP binding are specifically prevented by phospholipids

(A–C) Lack of effect of phospholipid-related molecules on detergent-mediated inhibition of [32 P]NAADP binding. Binding of [32 P]NAADP to solubilized samples was determined in the presence of 1.6% (w/v) Triton X-100 and the indicated concentration of test compound. (A) Effect of PC (●), LPC (○) and SPM (▽). (B) Effect of diacylglycerol (DAG, ●) and two unsaturated fatty acids, PA (○) and AA (▽). (C) Effect of the head group constituents phosphocholine (PhC, ●) and choline (Cho, ○). (D) Other phospholipids prevent detergent-mediated inhibition of [32 P]NAADP binding. Similar experiments to (A–C) using PC, PS or PE (4 mg/ml), or no phospholipid (–). All data (from at least three experiments) are expressed relative to binding of [32 P]NAADP determined at 0.1% (w/v) Triton X-100.

of higher concentrations of radioligand was precluded, since the ratio of non-specific to total binding approached unity. The absolute level of [32 P]NAADP binding at the highest concentration of

Figure 4 Inhibitory effects of delipidation on $[^{32}\text{P}]\text{NAADP}$ binding are due to a decrease in the apparent affinity of the target protein for its ligand

Binding of various concentrations of $[^{32}\text{P}]\text{NAADP}$ to Triton X-100-solubilized preparations at a final detergent concentration of 0.1 % (●) or 1.6 % (○). Data are from at least three experiments.

ligand tested (3 nM) was similar at Triton X-100 concentrations of 0.1 % (w/v) (6.3 ± 1 fmol/incubation; $n = 5$) and 1.6 % (w/v) (6 ± 1 fmol/incubation; $n = 3$).

Delipidation of solubilized sea urchin egg homogenates by gel filtration

Regulation of NAADP binding to its target protein by phospholipids was explored further by separating soluble samples by gel

filtration, a technique commonly employed to delipidate detergent extracts. Since binding of NAADP to its target protein is not reversible [26,27,43], homogenates can be labelled with $[^{32}\text{P}]\text{NAADP}$, unbound ligand readily removed by centrifugation and soluble protein–ligand complexes recovered following detergent treatment [28]. Analysis of the ‘tagged’ NAADP-binding protein by gel filtration in KGLuIM supplemented with 1 % (w/v) Triton X 100 resulted in the recovery of radioactivity as a single peak that co-eluted with apoferritin (molecular mass of 440 kDa) (Figure 5A), as reported in [28]. In contrast, when gel filtration was performed in KCIIM using preparations that were otherwise labelled and solubilized in an identical manner, two peaks of radioactivity were recovered (Figure 5B). The first peak corresponded to that when gel filtration was performed in KGLuIM, reflecting bound radioactivity. The remainder of the radioactivity was broadly distributed. Radioactivity associated with these fractions were not recoverable by poly(ethylene glycol) precipitation (Figure 5B, inset). These data indicate the presence of free $[^{32}\text{P}]\text{NAADP}$ probably generated as a result of dissociation of protein–ligand complexes during fractionation.

In the light of the phospholipid requirement for binding of NAADP to its target protein identified above (Figures 1 and 2), we explored the possibility that the observed difference in bound radioactivity during gel filtration in the two media was a consequence of differential delipidation of the soluble preparation. We therefore analysed the phospholipid content of the collected fractions. In both media, the majority of the phospholipids were recovered in late fractions, consistent with separation of protein

Figure 5 Delipidation of solubilized sea urchin egg homogenates by gel filtration

Triton X-100-solubilized sea urchin egg homogenates labelled with $[^{32}\text{P}]\text{NAADP}$ before solubilization (A and B) or $[^3\text{H}]\text{PC}$ after solubilization (C and D) were separated by gel filtration in KGLuIM (A and C) or KCIIM (B and D), and radioactivity of the collected fractions was determined. Both media were supplemented with 1 % (w/v) Triton X-100. The inset in (B) shows the distribution of recovered radioactivity following precipitation of the collected fractions with poly(ethylene glycol). The triangle represents the elution of apoferritin (molecular mass of 443 kDa). Data are from at least three experiments.

Figure 6 Gel-filtration analysis of solubilized sea urchin egg homogenates in the presence of PC

[32 P]NAADP-labelled sea urchin egg homogenates solubilized with Triton X-100 were separated by gel filtration in KgluIM (●) or KCIIM (○) as in Figure 5, except that PC (1 mg/ml) was included in the gel filtration medium. Data are from three experiments.

and lipids in the presence of detergent (Figures 5C and 5D). In addition, a second minor fraction of phospholipids was detected in earlier-eluted fractions. When separation was performed in KgluIM, the early phospholipid pool co-eluted with NAADP-binding sites (Figure 5C). This was not the case in the presence of KCIIM (Figure 5D). Thus fractions enriched in [32 P]NAADP also contained significant levels of phospholipid following fractionation in KgluIM, but not KCIIM. Clearly then, binding of NAADP to its target protein during gel filtration correlates well with phospholipid content.

To provide further evidence that dissociation of protein–ligand complexes in KCIIM was due to delipidation of the NAADP target protein, we examined the effect of including phospholipid during gel filtration on the distribution of [32 P]NAADP (Figure 6). When samples were separated in KCIIM in the presence of PC, ligand dissociation was prevented (Figure 6, ○). Thus, whereas in its absence, two peaks of radioactivity were observed (Figure 5B), only a single species was recovered in the presence of PC. PC, however, had little effect on NAADP binding to its target protein following fractionation in KgluIM (Figure 6, ●). Taken together, these data provide additional evidence that binding of NAADP to its target is critically dependent upon the presence of phospholipids.

DISCUSSION

In the present study, we have shown that decreasing the level of lipid associated with soluble sea urchin egg protein inhibits binding of NAADP to its target protein. We used three independent means to effect this change: (i) decreasing protein concentration (by dilution) at a fixed detergent concentration, (ii) increasing the detergent concentration at a fixed protein concentration, and (iii) gel filtration in the presence of detergent. Inhibition of NAADP binding by all three methods was prevented by addition of phospholipids. Taken together, our results uncover a phospholipid requirement for the binding of NAADP to its target protein.

Delipidation of solubilized preparations by addition of a high detergent markedly inhibited [32 P]NAADP binding (Figure 2), allowing us to readily probe the specificity of the observed protective effects of PC by comparison with various lipids and related molecules (Figure 3). LPC, which is formed from PC by cleavage of one of the fatty acid side chains, was less effective than PC in preventing the inhibitory effects of delipidation on binding of [32 P]NAADP (Figure 3A). These data indicate that hydrophobic

interactions with lipids and the target protein are required for ligand binding. SPM, however, afforded little protection against the effects of detergent (Figure 3A), which, although it is not a glycerol-based phospholipid, shares the same head group as PC and adopts a similar conformation. Clearly then, the effects of PC are specific. Furthermore, neither non-esterified fatty acids, diacylglycerol, which lacks a polar head group, nor head groups alone (at least at equivalent molar concentrations) were able to prevent the inhibitory effects of high detergent (Figures 3B and 3C). These data indicate contributions of both the head group and lipid tail of PC in stabilizing binding of NAADP. However, the head group requirement was relatively non-specific, since both PS and PE also prevented delipidation. Inhibition (rather than potentiation) of [32 P]NAADP binding to delipidated preparations by AA is interesting, as this fatty acid has previously been shown to inhibit NAADP-induced Ca^{2+} release from sea urchin egg homogenates [44].

Fractionation of target protein–ligand complexes by gel filtration using KCIIM results in significant dissociation of NAADP (Figure 5B); similar experiments using KgluIM do not (Figure 5A). This difference was apparent despite the fact that concentration of K^{+} in the two media (250 mM) was identical and was shown previously to maximally stabilize binding of NAADP to its target [31]. Detailed analysis of phospholipid content, however, revealed clear differences under the two experimental paradigms. While we have no explanation at present as to why the migration of phospholipids should differ, the clear correlation between ligand dissociation and phospholipid content is entirely consistent with a role for lipids in the recognition of NAADP by its target protein.

As reported previously [28], and confirmed in the present study (Figures 5 and 6), from gel-filtration analysis, the native molecular mass of the NAADP-binding protein is 400–500 kDa. In contrast, size estimates from sucrose-density-gradient centrifugation is substantially smaller (approx. 120 kDa) [28]. Sedimentation of proteins by the latter method is dependent upon the buoyant molar mass of the particle [45]. Since the partial specific volume of lipid is greater than protein, the molecular mass of proteins in protein–lipid complexes would tend to be underestimated when using water-soluble proteins of known molecular mass to calibrate density gradients. Association with lipids might therefore explain the apparent difference in molecular mass of the target protein determined by the two techniques.

Scant information is available regarding the regulation of intracellular Ca^{2+} -release channels by surrounding membrane lipids. PC, PS and PE have little effect on binding of inositol trisphosphate to partially purified liver (predominantly type II) inositol trisphosphate receptors [46], whereas phosphatidylinositol bisphosphate inhibits binding to both liver and recombinant type I membrane-bound inositol trisphosphate receptors [46,47]. Ryanodine binding to cardiac sarcoplasmic reticulum vesicles is inhibited by prior treatment with phospholipase A2 (which cleaves single fatty acid residues from phospholipids), an effect reversed by PC, PS and PE [48]. These data are consistent with a requirement for phospholipids in maintaining binding of ryanodine to its receptor. Indeed, ryanodine binding to soluble type I ryanodine receptors is inhibited by CHAPS in the absence, but not in the presence, of PC [49,50]. The phospholipid dependence of ligand binding of NAADP to its target identified in the present study is therefore similar to that of ryanodine receptors.

The mechanism whereby removal of phospholipids inhibits binding of NAADP to its target protein in sea urchin eggs is not known at present. One possibility is that delipidation disrupts the quaternary structure of a protein complex that is essential for ligand binding. Indeed, high-affinity ryanodine binding to ryanodine receptors depends upon contributions from each subunit within

a tetrameric assembly [51]. In contrast, individual subunits of the inositol trisphosphate receptor bind inositol trisphosphate independently [52], a property consistent with the reported insensitivity of ligand binding to phospholipids for this family of intracellular Ca^{2+} channels [48].

In summary, we show that phospholipids are required in order for NAADP to bind to its target protein. Preservation of the ligand-binding properties of the putative NAADP receptor via an appropriate lipid environment should aid in the design of strategies for the purification of this most unusual intracellular Ca^{2+} -release channel.

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