Modulation of NAADP (nicotinic acid–adenine dinucleotide phosphate) receptors by K^+ ions: evidence for multiple NAADP receptor conformations

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NAADP (nicotinic acid–adenine dinucleotide phosphate) mediates Ca^{2+} release from intracellular Ca^{2+} stores in a wide variety of cell types. In sea urchin eggs, subthreshold concentrations of NAADP can cause full inactivation of NAADP-induced Ca^{2+} release, an effect that may be related to the ability of the target protein to bind its ligand in an essentially irreversible manner. In the present study, we found that K^+ ions inhibit dissociation of NAADP from sea urchin egg homogenates. In low K^+-containing media, an addition of excess unlabelled NAADP effectively displaced bound radioligand whereas dilution of radioligand initiated only partial dissociation. The inhibitory effects of K^+ on dissociation of NAADP were concentration dependent, reversible and persisted after detergent solubilization. Lowering $[K^+]$ of the medium decreased the sensitivity of NAADP receptors for their ligand in stimulating Ca^{2+} release, but it did not affect inactivation of NAADP-induced Ca^{2+} release by subthreshold concentrations of NAADP. Our results are consistent with the observation of multiple conformations of the NAADP receptor that are readily revealed in low K^+-containing media.

Key words: NAADP, calcium, sea urchin, desensitization, fertilization.

INTRODUCTION

Many extracellular stimuli mediate their cellular effects through manipulation of cytosolic $[Ca^{2+}]$ [1]. Not surprisingly, such changes in cytosolic $[Ca^{2+}]$ are complex. Indeed, the observed temporal and spatial inhomogeneity in the Ca^{2+} signal is thought to confer the required specificity in order for the same ion to mediate a vast array of cellular processes [1]. In many cells, such Ca^{2+} signals are driven by mobilization of intracellular Ca^{2+} stores through activation of InsP_3 [inositol (1,4,5)-trisphosphate] [2,3] and ryanodine receptors [4] by the endogenous ligands InsP_3 [5] and cyclic ADP ribose (cADPR) [6–8] respectively. Recent studies in a variety of cells demonstrated a new mechanism of intracellular Ca^{2+} release regulated by the NADP analogue, NAADP (nicotinic acid–adenine dinucleotide phosphate) [6,8–10]. In broken cell preparations, NAADP-induced Ca^{2+} release is insensitive to inhibitors of InsP_3 and ryanodine receptors [11,12]. Moreover, unlike Ca^{2+} release mediated by InsP_3 and cADPR, NAADP-induced Ca^{2+} mobilization is not regulated by cytosolic Ca^{2+} [13,14] and is demonstrable following depletion of endoplasmic reticulum Ca^{2+} stores with the Ca^{2+} ATPase inhibitor, thapsigargin [14,15]. These results suggest that NAADP targets a novel intracellular Ca^{2+}-release channel located on a distinct Ca^{2+} store. Indeed, we have provided evidence that NAADP receptors are substantially smaller than the known intracellular-Ca^{2+}-release channels [16] and are probably expressed on acidic lysosomal-like Ca^{2+} stores [17]. Taken together, with the recent demonstrations of changes in cellular NAADP levels evoked by extracellular stimuli [18–20], these studies provide strong evidence that NAADP serves as a novel Ca^{2+}-mobilizing second messenger.

Several lines of independent evidence have pointed to an interplay between NAADP receptors and receptors for InsP_3 and cADPR in the generation of complex Ca^{2+} signals [9,21]. Increases in Ca^{2+} in response to NAADP are amplified probably through Ca^{2+}-induced Ca^{2+} release via InsP_3 and/or ryanodine receptors in pancreatic acinar cells [22], starfish oocytes [23], sea urchin eggs [24] and smooth-muscle cells [25]. In addition, Ca^{2+} oscillations in response to NAADP in sea urchin eggs were considered to be driven by release of Ca^{2+} from an NAADP-sensitive pool and subsequent uptake and release of Ca^{2+} from InsP_3 and cADPR-sensitive Ca^{2+} stores [15]. NAADP may thus co-ordinate the activity of other intracellular Ca^{2+} channels by triggering Ca^{2+} release, a process probably important in regulating Ca^{2+}-dependent events such as second-messenger-mediated neurotransmitter release [26]. Stimulation of metabolism of NAADP by Ca^{2+} [27] provides additional scope for the fine-tuning of NAADP-mediated Ca^{2+} signals.

A further unusual property of NAADP receptors, at least in sea urchin egg homogenates, is the ability of the target protein to bind its ligand in an essentially irreversible manner [28–30]. Thus, unlabelled NAADP effectively competes with tracer concentrations of the radioligand only when egg homogenates are exposed to the two ligands simultaneously but is ineffective once association of the radioligand is initiated. It has been proposed that this kinetic behaviour underlies the somewhat unusual inactivation properties of the channel [28]. Exposure of egg homogenates to even subthreshold concentrations of NAADP can render the homogenates completely refractory to subsequent challenge with maximal concentrations of NAADP [28,31]. Such regulation may form the basis of a simple spatial and temporal memory [6]. Indeed, local release of NAADP in intact sea urchin eggs shapes the spatial organization of Ca^{2+} increases in response to subsequent global release of NAADP [32]. Intriguingly, desensitization persists indefinitely in sea urchin egg homogenates [31], even though the desensitization of NAADP-induced Ca^{2+} release in intact eggs appears to be reversible [32]. These results indicate that, probably, there are mechanisms that exist within the egg that modulate the inactivation of NAADP receptors. In mammalian cells, inactivation of NAADP receptors appears to proceed by fundamentally different means. Although,

Abbreviations used: cADPR, cyclic ADP ribose; Kglu, potassium gluconate; IM, intracellular-like medium lacking Kglu; InsP_3, inositol (1,4,5)-trisphosphate; Naglu, sodium gluconate; NAADP, nicotinic acid–adenine dinucleotide phosphate.

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similar to sea urchin eggs, mammalian NAADP receptors can inactivate before opening, this appears to occur only at high (μM) concentrations of NAADP, giving rise to 'bell-shaped' concentration–effect relationships [19,22,25,33,34]. Radioligand-binding studies also show clear differences between the sea urchin egg and mammalian cells. For example in mouse brain [35] and rat heart [36], binding of NAADP is fully reversible. Kinetic considerations of NAADP binding are therefore important as they may provide mechanistic insights into the quite remarkable process by which NAADP receptors inactivate.

In the present study, we have examined the kinetics of NAADP binding to NAADP receptors from sea urchin eggs. We provide evidence that K^+ ions reversibly inhibit dissociation of bound [^{32}P]NAADP in a concentration-dependent manner, an effect which is preserved after detergent solubilization. In low K^+-containing media, we demonstrate that high concentrations of NAADP are more effective in dissociating a bound radioligand when compared with radioligand dilution, the effects being associated with a decrease in the ability of submaximal concentrations of NAADP to stimulate Ca^{2+} release. Our results are consistent with the existence of interconvertible conformations of the NAADP receptor which bind their ligands in either a reversible or irreversible manner.

METHODS

Preparation of sea urchin egg homogenates

Sea urchins (*Lytechinus pictus*) were obtained from Marinus (Long beach, CA, U.S.A.). Eggs were harvested in Instant Ocean® aquarium salt solution (specific gravity, 1.02–1.023; Sigma) after intracoelomic injection of 500 mM KCl. The eggs were dejellied by passing them through a nylon mesh (85 μm) and washed (by centrifugation) sequentially into Ca^{2+}-free artificial sea water (470 mM NaCl/27 mM $MgCl_2$/28 mM $MgSO_4$/10 mM KCl/2.5 mM $NaHCO_3$/1 mM EGTA, pH 8; two washes) and nominally Ca^{2+}-free artificial sea water (Ca^{2+}-free artificial sea water without EGTA; two washes) at 4 °C. The eggs were then resuspended (50 %, v/v) into KgluIM [intracellular-like medium containing Kglu (potassium gluconate)], comprising 250 mM Kglu, 250 mM *N*-methyl D-glucamine, 1 mM $MgCl_2$ and 20 mM Hepes (pH 7.2, with acetic acid) supplemented with an ATP-regenerating system (1 mM ATP, 10 mM phosphocreatine and 10 units/ml creatine phosphokinase) and a protease inhibitor cocktail (EDTA-free; Roche, Lewes, U.K.). Homogenization was performed at 4 °C using a Dounce homogenizer (tight-fitting plunger, six strokes). Homogenates were immediately centrifuged at 11 600 ***g*** for 8 s to remove cortical granules and stored at − 80 °C before use.

Radioligand binding to membrane-bound NAADP receptors

Homogenates were diluted 100–500-fold to a final concentration of 0.1–0.5 % (v/v) and incubated with enzymically prepared [^{32}P]NAADP (1000 Ci/mmol; 80 pM–1 nM) [29] at room temperature for 1–17 h as indicated. Bound and free radioligand were separated by rapid filtration through glass-fibre filters using a cell harvester. Radioactivity associated with the filters was determined by Cerenkov counting. Radioligand binding was performed in KgluIM (see above for composition) or in an intracellular-like medium lacking Kglu (IM; composition: 250 mM *N*-methyl D-glucamine, 1 mM $MgCl_2$ and 20 mM Hepes, pH 7.2). In some experiments, binding was performed in a media composed of 20 mM Hepes (pH 7.2) and Kglu, sodium gluconate (Naglu), KCl or NaCl (250 mM).

Receptor solubilization

Sea urchin egg homogenates were washed twice by centrifugation (100 000 ***g***, 5 min) at 4 °C into KgluIM, IM or Kglu. Washed homogenates (17 %) were solubilized by incubation with 1 % (w/v) Triton X-100 for 60 min at 4 °C and centrifuged at 100 000 ***g*** for 60 min. Supernatant fractions containing soluble NAADP receptors, stored at − 80 °C before use, were incubated with [^{32}P]NAADP (0.1 nM) at room temperature for 1–3 h (final detergent concentration, 0.1 %, w/v). γ-Globulin (400 μg) was then added to samples, and protein was precipitated by incubation with 15 % (w/v) poly(ethylene glycol) (mean molecular mass, 8 kDa) for 30 min at 4 °C. Samples were then centrifuged at 100 000 ***g*** for 5 min, and the resulting pellets were washed with 15 % (w/v) poly(ethylene glycol) and dissolved in water for Cerenkov counting.

Gel-filtration analysis of prelabelled NAADP receptors

Sea urchin egg homogenates (2.5 %) were incubated for 16 h at room temperature in KgluIM supplemented with 1–2 nM [^{32}P]NAADP. Samples were subsequently washed twice in KgluIM by centrifugation (2 min, 100 000 ***g***, 4 °C) and solubilized as described for unlabelled homogenates. NAADP receptor–radioligand complexes (200 μl) were injected on to a Superdex 200 HR 10/30 column (Amersham Biosciences) linked to an HPLC system (Waters). Fractionation was performed at room temperature (flow rate of 0.5 ml/min) in either Kglu or Naglu supplemented with 1 % Triton X-100. Collected fractions (1 ml) were analysed directly for radioactivity. The migration of NAADP receptor was compared with the migration of apoferritin (molecular mass, 443 kDa) and free [^{32}P]NAADP.

Measurement of NAADP-induced Ca^{2+} release

Sea urchin egg homogenates (50 %) were sequentially diluted (approx. 2-fold at 30 min intervals) to a final concentration of 1.25 % with KgluIM or a similar medium in which Kglu was replaced with the same concentration of Naglu. Media were supplemented with an ATP-regenerating system (to promote uptake of Ca^{2+} into intracellular Ca^{2+} stores; see above for composition) and the Ca^{2+} sensitive dye, fluo-3 (to monitor [Ca^{2+}] of the medium; 3 μM). Fluorescence was measured using a Fluorimetric Imaging Plate Reader [FLIPR (Molecular Devices, Wokingham, U.K.)] at the University College London FLIPR facility (Department of Physiology, University College London, London, U.K.). Briefly, after decreasing [Ca^{2+}] of the medium to steady state (approx. 4 h after initiating Ca^{2+} uptake), aliquots of sea urchin egg homogenates (230–250 μl) were manually transferred to a 96-well plate and known volumes (typically 200 μl) were automatically injected (by an integrated 96-well pipettor) into a black-walled clear-based 96-well imaging plate (Becton Dickinson, Oxford, U.K.) containing concentrated agonist stock solutions (typically 2 μl). In some experiments, homogenates were pretreated with subthreshold concentrations of NAADP (3 pM–10 nM) or vehicle for 25 min before injection. fluo-3 was excited using the 488 nm line of an argon laser (Coherent Inc., Ely, U.K.) and the emitted fluorescence (510–560 nm) was collected every 1.5 s with a thermoelectrically cooled charge-coupled-device camera. Comparing fluorescence of untreated homogenates that had been automatically injected into the imaging plate with the fluorescence of an equivalent volume dispensed manually, directly into the imaging plate, indicated that the injection procedure itself did not significantly increase fluo-3 fluorescence (results not shown). The latter results were averaged over the first 30 s of recording to give basal

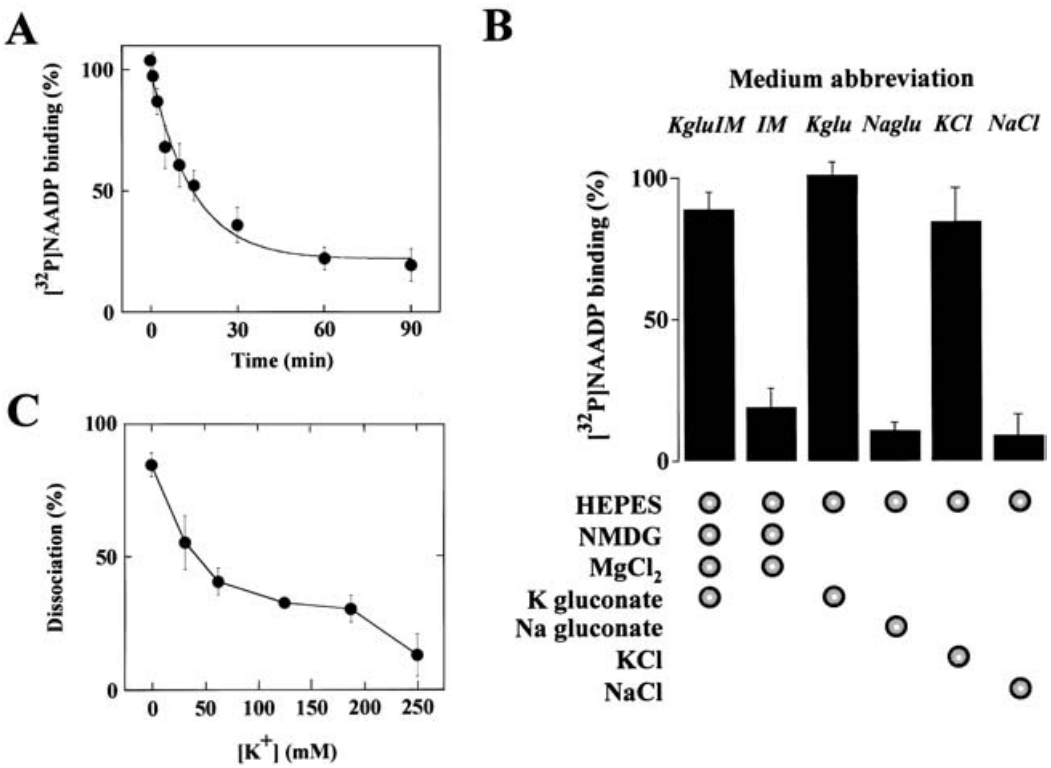


Figure 1 K^+ inhibits dissociation of NAADP from its receptor in sea urchin egg homogenates

(**A**) Sea urchin egg homogenates were incubated with [^{32}P]NAADP (80 pM) for 1 h followed by 1 μM unlabelled NAADP (designated time = 0 on graph) for the times shown before separation of bound and free radioligand ($n = 6$). Experiments were performed in IM. Data are normalized to binding of [^{32}P]NAADP before addition of unlabelled NAADP and corrected for non-specific binding (determined by the simultaneous addition of radioligand and 1 μM NAADP). (**B**) Sea urchin egg homogenates were incubated with [^{32}P]NAADP for 1 h and the binding of radioligand was determined after a 90 min incubation with 1 μM unlabelled NAADP ($n = 3$–5). Experiments were performed in media (abbreviated and italicized) containing 20 mM Hepes and the indicated salts (250 mM; except $MgCl_2 = 1$ mM). (**C**) Sea urchin egg homogenates were incubated with [^{32}P]NAADP for 1 h followed by 1 μM unlabelled NAADP for 90 min in media containing 20 mM Hepes and various concentrations of Kglu ($n = 3$).

fluorescence values (F_0) used to normalize the results (F) obtained subsequently (see Figure 5). In some experiments, fluorescence values (F) were converted into [Ca^{2+}] using the following equation:

$$[Ca^{2+}] = K_d^*(F - F_{min})/(F_{max} - F)$$

where F_{min} and F_{max} are the minimal (determined in the presence of 1 mM EGTA) and maximal (determined in the presence of 5 mM Ca^{2+} and 10 μM ionomycin) fluorescence values for the dye respectively and K_d is the dissociation constant of fluo-3 for Ca^{2+}, which, after correction for the high ionic strength of the media, was assumed to be 1.1 μM [37].

RESULTS

Addition of an excess of unlabelled NAADP (1 μM) to sea urchin egg homogenates that had been preincubated with [^{32}P]NAADP in an intracellular-like medium lacking Kglu (see the Methods section) revealed that binding of [^{32}P]NAADP was largely reversible (Figure 1A). After a 90 min incubation with unlabelled NAADP (1 μM), binding of [^{32}P]NAADP in IM was decreased to 19 ± 7% ($n = 5$, Figures 1A and 1B) of that before addition, with a first-order dissociation rate constant (k_{-1}) of 0.07 min^{-1} ($t_{1/2} = 10$ min, $n = 6$). Confirming previous studies, binding of NAADP was not readily reversed when experiments were performed in KgluIM (Figure 1B). These results suggest that Kglu inhibits dissociation of NAADP from its receptor.

To determine whether the effect of Kglu on the kinetics of NAADP binding was specific or a non-specific effect of ionic strength, dissociation experiments were performed in several simplified K^+- or Na^+-based media (Figure 1B). As with KgluIM, dissociation of NAADP from its receptor was inhibited when experiments were performed in a pH-buffered medium containing only 250 mM Kglu (Kglu, Figure 1B). In contrast, dissociation was readily observed in 250 mM Naglu (Figure 1B). Similarly, bound [^{32}P]NAADP was dissociated by excess NAADP in a pH-buffered medium containing only NaCl but not KCl (250 mM; KCl, NaCl, Figure 1B). These results strongly suggest that K^+ ions are both necessary and sufficient to inhibit dissociation of NAADP from its receptor. This inhibitory effect of K^+ was half-maximal at a concentration of approx. 50 mM (Figure 1C).

We next examined the effects of radioligand dilution on the kinetics of [^{32}P]NAADP binding (Figure 2). In contrast with substantial dissociation of bound NAADP by excess unlabelled NAADP in low K^+-containing media (Figure 1), dilution (11-fold) of sea urchin egg homogenates that had been prelabelled with 80 pM [^{32}P]NAADP in IM induced only partial dissociation (Figure 2A, $k_{-1} = 0.05$ min^{-1}, $t_{1/2} = 13$ min, $n = 4$). Compared with control incubations, binding of [^{32}P]NAADP was decreased to 58 ± 5% ($n = 7$), 90 min after dilution (Figures 2A and 2B). The extent of dissociation was not increased further by higher (up to 73-fold) dilution of the incubations (Figure 2A, inset). Dilution of sea urchin egg homogenates with KgluIM did not induce appreciable dissociation of pre-bound [^{32}P]NAADP (Figure 2B). Dilution was also without effect on [^{32}P]NAADP

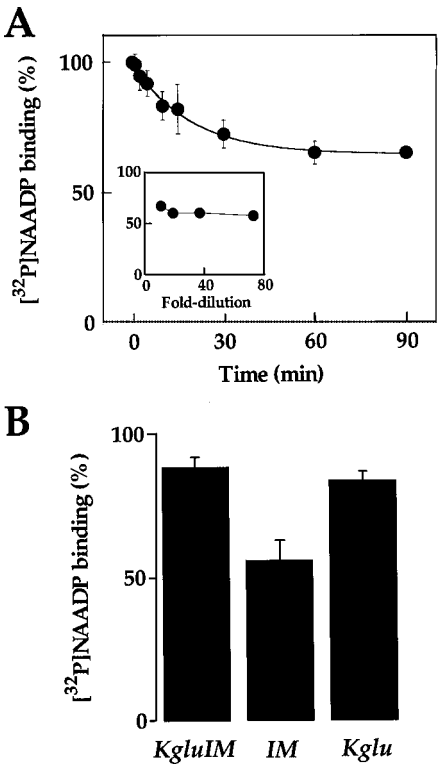


Figure 2 Effect of radioligand dilution on dissociation of NAADP from its receptor

(**A**) Sea urchin egg homogenates were equilibrated with [^{32}P]NAADP (80 pM) in IM for 60 min, then diluted 11-fold in the same medium without adding the radioligand. Reactions were continued for various times before separation of bound and free ligand. The inset compares the effect of larger radioligand dilutions on the extent of dissociation after a 90 min incubation. (**B**) Effect of K^+ on dissociation of NAADP initiated by radioligand dilution (11-fold, 90 min). Mean results from 3 to 7 independent experiments have been presented.

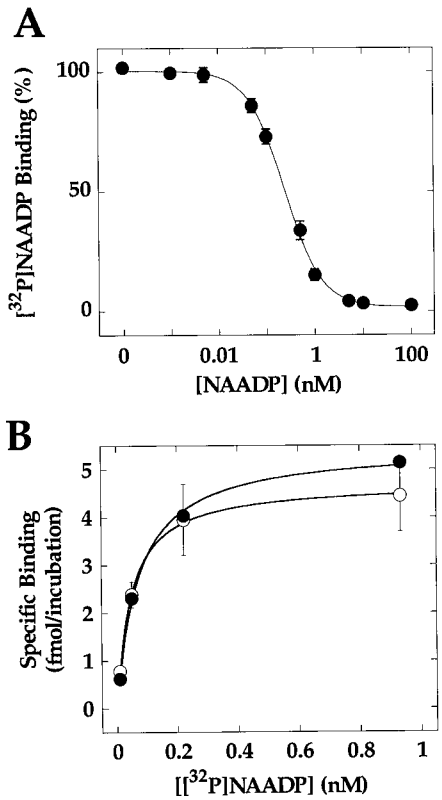


Figure 3 Equilibrium binding properties of NAADP receptors in low K^+-containing media

(**A**) Isotope dilution: sea urchin egg homogenates were incubated with [^{32}P]NAADP (80 pM) together with the indicated concentration of unlabelled NAADP for 3–5 h in IM before separation of bound and free radioligand ($n=3$). (**B**) Saturation analysis: sea urchin egg homogenates were incubated with various concentrations of [^{32}P]NAADP for 17 h in a medium composed of 20 mM Hepes and 250 mM Naglu (●, $n=3$) or Kglu (○, $n=3$).

binding when experiments were performed in Kglu (Figure 2B).

The experiments so far used sea urchin egg homogenates that were prepared in K^+-containing medium (see the Methods section) and subsequently diluted to adjust the [K^+] as indicated. These results suggest that the inhibitory effects of K^+ ions on dissociation of NAADP are reversible. Additional experiments were performed to investigate further the extent of reversibility. In the first set of experiments, sea urchin egg homogenates were labelled with [^{32}P]NAADP in KgluIM and then subsequently diluted (50-fold) into the same medium or a K^+-free medium to decrease the [K^+] to 5 mM. Dilution into KgluIM had no effect on [^{32}P]NAADP binding; binding was $93 \pm 9\%$ ($n=3$) of that before its addition. However, dilution of labelled homogenates into K^+-free media induced partial dissociation of radioligand reducing NAADP binding to $52 \pm 4\%$ ($n=3$). Residual binding after dilution under the latter conditions was similar to that when labelling and dilution were performed in IM ($58 \pm 5\%$, $n=7$; Figure 2), further suggesting that the inhibitory effect of K^+ on dissociation of NAADP from its receptor is reversible. In the second set of experiments, NAADP dissociation was determined in KgluIM using egg homogenates that had first been incubated in IM, conditions under which bound [^{32}P]NAADP can be dissociated by unlabelled NAADP or by dilution (Figures 1 and 2). These experiments revealed that [^{32}P]NAADP binding was insensitive to the addition of an excess of unlabelled NAADP or to radioligand dilution. Bound [^{32}P]NAADP, after these treatments, was $80 \pm 8\%$ and $89 \pm 17\%$ respectively of that relative to control incubations ($n=3$). Thus, modulation of the dissociation kinetics of NAADP receptors by K^+ ions is readily reversed after repeated manipulation of [K^+] of the medium.

We characterized the equilibrium binding properties of NAADP receptors in low K^+-containing media (Figure 3). From isotope dilution experiments in IM (Figure 3A), it was found that NAADP bound with high affinity ($K_d = 0.16 \pm 0.03$ nM, $n=3$) to a single class of sites ($n_H = 1.1 \pm 0.1$, $n=3$). Saturation analysis in Naglu indicated that the B_{max} value was similar to that determined in Kglu (Figure 3B). These results suggest that both reversible and irreversible NAADP binding sites are present at stoichiometric levels.

In the next set of experiments, we examined the dissociation kinetics of NAADP binding to soluble NAADP receptors (Figure 4). Sea urchin egg homogenates were washed with KgluIM, IM or Kglu and solubilized with the non-ionic detergent, Triton X-100 (1%). The reversibility of [^{32}P]NAADP binding was then determined by the addition of an excess of unlabelled NAADP. Confirming our previous study, binding of NAADP to soluble receptors was essentially irreversible when experiments were performed in KgluIM [16] (Figure 4A). However, bound [^{32}P]NAADP was dissociated by unlabelled NAADP in IM but not in Kglu (Figure 4A). Thus, as observed in membrane preparations, K^+ ions also inhibit dissociation of NAADP from soluble NAADP receptors.

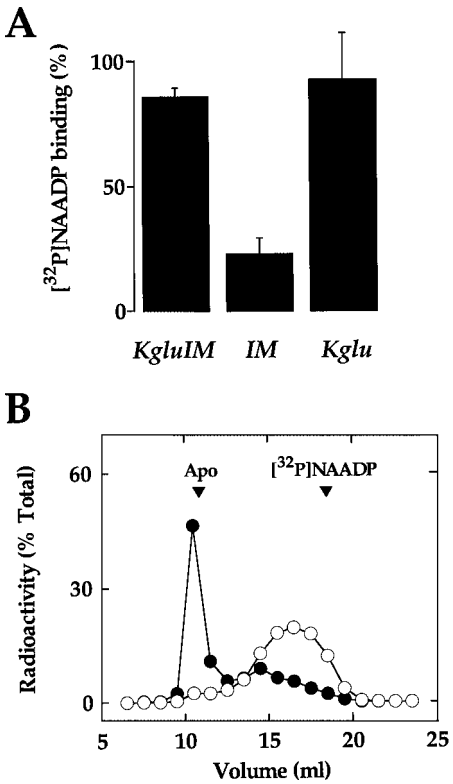


Figure 4 Effect of K^+ on dissociation of NAADP from soluble NAADP receptors

(**A**) Soluble NAADP receptors were incubated with [^{32}P]NAADP in KgluIM, IM or Kglu for 1 h, and dissociation of radioligand after a 90 min incubation with 1 μM NAADP was determined. (**B**) Sea urchin egg homogenates were incubated with [^{32}P]NAADP in KgluIM for 1 h and unbound radioligand removed by centrifugation. Labelled membranes were solubilized with Triton X-100 and supernatant fractions after ultracentrifugation were subjected to gel-filtration analysis in Kglu (●) or Naglu (○) supplemented with 1 % Triton X-100. For comparison, apoferritin (443 kDa) and [^{32}P]NAADP were fractionated under similar conditions (arrow heads). Mean results from two independent experiments are displayed.

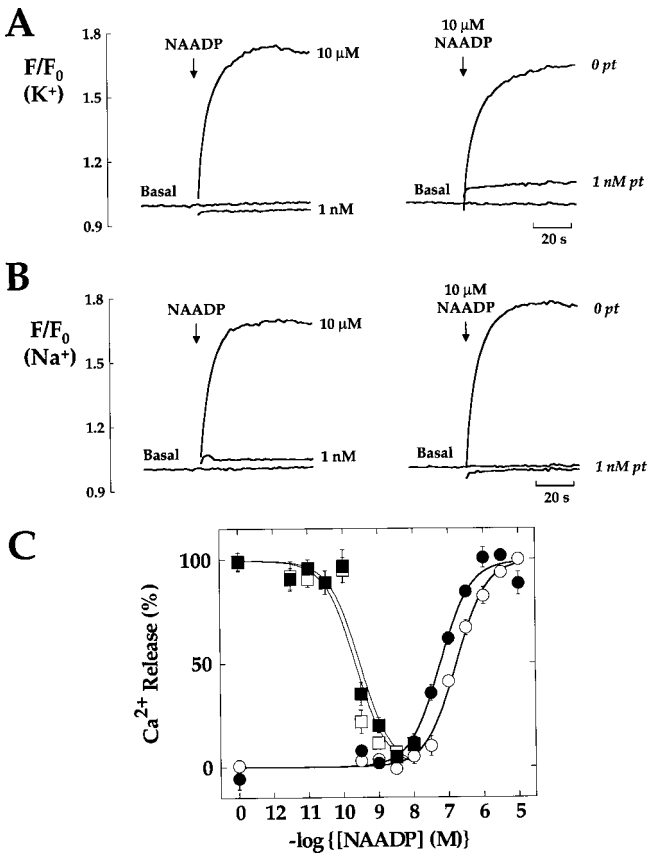


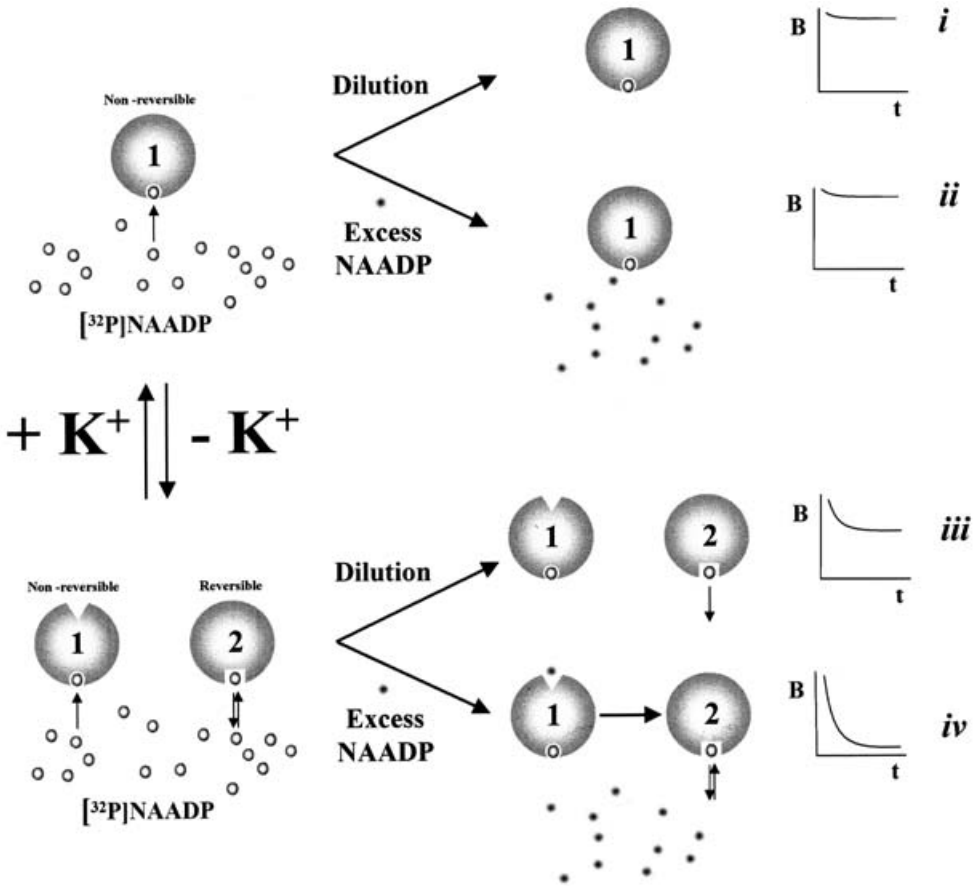
Figure 5 Effect of K^+ on activation and inactivation of Ca^{2+} release by NAADP

(**A**, **B**) Sea urchin egg homogenates were incubated with an ATP-regenerating system and [Ca^{2+}] of the medium monitored using the fluorescent Ca^{2+} indicator fluo-3. Experiments were performed either in KgluIM (**A**) or a similar medium in which Kglu was substituted with Naglu (**B**). The left-hand-side traces show the effect of a subthreshold (1 nM) and maximal concentration (10 μM) of NAADP on fluo-3 fluorescence. The right-hand-side traces show Ca^{2+} release in response to NAADP (10 μM) from homogenates that had been pretreated (pt) for 25 min with either 1 nM NAADP or vehicle (0) before stimulation. Traces marked 'Basal' were unstimulated. (**C**) Effect of the indicated concentration of NAADP in stimulating Ca^{2+} release (circles) and inactivating the response to 10 μM NAADP after pretreatment (squares). Experiments were performed in K^+ (filled symbols) or Na^+ (open symbols) based medium according to the procedures outlined in (**A**) and (**B**) respectively. Mean results from 6 to 8 independent experiments have been presented.

We had previously taken advantage of the non-dissociating nature of NAADP binding to both membrane-bound and soluble NAADP receptors to 'tag' NAADP receptors with their ligand, thus providing a convenient means of tracking NAADP receptors after solubilization and fractionation [16]. This technique was used in the present study to determine whether the inhibitory effects of K^+ on NAADP dissociation to soluble NAADP receptors were reversible. Egg homogenates were incubated with [^{32}P]NAADP in KgluIM and membranes were subsequently washed in KgluIM to remove the unbound ligand. These prelabelled membranes were then solubilized with Triton X-100 and supernatant fractions containing NAADP receptor–radioligand complexes were recovered. Analysis of prelabelled receptors by gel filtration in a K^+-based medium revealed that most of the radioactivity co-eluted with apoferritin (Figure 4B, closed circles); free NAADP eluted at later times (elution volume = 18.5 ml, $n = 2$). These results confirm our previous finding that [^{32}P]NAADP remains bound to its receptor during gel filtration and that NAADP receptor–ligand complexes migrate as high molecular mass complexes [16]. In contrast, when tagged NAADP receptors were analysed by gel filtration in a Na^+-based medium, radioactivity was broadly distributed, peaking at an elution volume of 16.5 ml ($n = 2$, Figure 4B, open circles). These results are consistent with dissociation of NAADP from its receptor during fractionation in the presence of Na^+ but not K^+. Thus stabilization of soluble NAADP-receptor–ligand complexes by K^+ ions is fully reversible.

In the final set of experiments, we examined the effects of K^+ on NAADP-induced Ca^{2+} release. In KgluIM, sea urchin egg homogenates effectively decreased the [Ca^{2+}] of the medium to 154 ± 26 nM ($n = 3$) in the presence of an ATP-regenerating system. Subsequent addition of NAADP (10 μM) stimulated robust Ca^{2+} release, whereas lower concentrations of NAADP (1 nM) were ineffective (Figure 5, left). Although the lower concentration of NAADP did not result in detectable Ca^{2+} release, homogenates that were pretreated with 1 nM NAADP failed to respond to subsequent addition of 10 μM NAADP (Figure 5, right). Thus, confirming previous studies, high concentrations of NAADP activate sea urchin egg NAADP receptors resulting in Ca^{2+} release, whereas lower concentrations cause inactivation [28,31].

Substitution of Kglu with Naglu (final [K^+] = 6.25 mM) did not prevent Ca^{2+} uptake into intracellular Ca^{2+} stores or



Scheme 1 Proposed model for the existence of multiple NAADP receptor conformations

We propose that at least two conformations of the NAADP receptor exist (large circles with labels 1, 2), one of which binds NAADP in an irreversible manner (site 1). In the presence of K^+ (top), conformation 1 is stabilized such that after occupancy with [^{32}P]NAADP (open circles), binding is insensitive to either radioligand dilution or excess unlabelled NAADP (filled small circles). This is schematically depicted in graphs i and ii respectively, where 'B' represents bound radioactivity and 't' time. In the absence of K^+ (bottom), both receptor conformations are revealed. Under these conditions, radioligand dilution affects dissociation from site 2 only, such that binding is partially reversible (iii). In the presence of excess NAADP, we propose the stabilization of site 2 (possibly through binding of NAADP to a third low-affinity site represented as a triangle) such that binding is fully reversible (iv). See text for more details.

affect Ca^{2+} release in response to a maximal concentration of NAADP. At steady state, [Ca^{2+}] of the medium (44 ± 12 nM, $n = 3$) was similar to that in KgluIM (see above) and the peak fluorescence increases (F/F_0; see the Methods section) stimulated by 10 μM NAADP were 1.8 ± 0.1 and 2 ± 0.1 in KgluIM and the substituted medium respectively ($n = 6$, Figure 5B, left). Low K^+-containing media also supported inactivation of NAADP receptors by prior treatment with 1 nM NAADP (Figure 5B, right). Analysis of the complete concentration–effect relationship in the two media revealed that EC_{50} for NAADP-stimulated Ca^{2+} release was substantially increased from 62 ± 8 nM ($n = 8$) in KgluIM to 175 ± 20 nM ($n = 8$) after substitution of K^+, to a 3 ± 0.4-fold decrease in sensitivity (Figure 5C, circles). In marked contrast, K^+ did not affect the sensitivity of NAADP receptors to inhibition by subthreshold concentrations of NAADP (Figure 5C, squares); IC_{50} values for attenuation of Ca^{2+} release in response to 10 μM NAADP were 226 ± 30 and 305 ± 41 pM in media containing high and low K^+ respectively ($n = 6$).

DISCUSSION

Several studies have demonstrated that binding of [^{32}P]NAADP to sea urchin egg homogenates is not readily reversible [28–30]. We noted that all previous experiments were performed in a similar intracellular-like medium (referred to as KgluIM in the present study). We now show for the first time that K^+ ions reversibly inhibit dissociation of NAADP from its receptor in sea urchin eggs such that in low K^+-containing media both reversible and irreversible components of NAADP binding are revealed. A decrease in [K^+] of the medium resulted in a decrease in the sensitivity of NAADP receptors for their ligand in activating Ca^{2+} release.

We demonstrate that in low K^+-containing media, an excess of unlabelled NAADP effectively dissociates bound radioligand, whereas dissociation of radioligand induced by dilution is much less effective (Figures 1 and 2). On the basis of these results, we propose the existence of multiple NAADP binding sites. In the absence of K^+, two populations of NAADP binding sites are occupied by radioligand. [^{32}P]NAADP binds to one of these sites in an essentially irreversible manner (site 1), whereas binding to the other site (site 2) is slowly reversible (Scheme 1, bottom). Under these conditions, radioligand dilution will affect dissociation of radioligand from site 2 only (Scheme 1, iii). That addition of excess NAADP results in almost complete dissociation of a radioligand (Scheme 1, iv), can be explained if high concentrations of NAADP convert NAADP receptors in conformation 1 to conformation 2 such that ligand binding is

now fully reversible. This may occur through occupancy of a putative low-affinity NAADP binding site. In the presence of K^+ ions, conformation 1 is stabilized such that binding is insensitive to both high NAADP concentrations and radioligand dilution and is thus irreversible (Scheme 1, i and ii) [28–30]. We favour the existence of interconvertible NAADP binding sites as opposed to K^+-mediated occlusion of reversible binding sites since saturation analysis was independent of $[K^+]$ (Figure 3). If decreasing $[K^+]$ resulted in the appearance of additional reversible binding sites, then the maximal density of the binding sites would be expected to increase under these conditions. Additionally, although in K^+-deficient media the extent of radioligand dissociation was greater when initiated by the addition of an excess of NAADP compared with radioligand dilution, the rates of dissociation were similar ($t_{1/2} = 10$ and 13 min for dissociation initiated by unlabelled NAADP and dilution respectively; Figures 1 and 2). This somewhat peculiar form of negative co-operativity, which is clearly different from that reported for binding of NAADP to cardiac microsomal preparations [36], is again consistent with dissociation from a homogeneous population of binding sites.

In sea urchin egg homogenates, low concentrations of NAADP, which are below the threshold for Ca^{2+} release, have been reported to inactivate responses to subsequent challenges with normally maximal concentrations of NAADP [28,31]. These findings are confirmed in the present contribution (Figure 5). Studies demonstrating essentially irreversible binding of [^{32}P]NAADP to sea urchin egg homogenates [28–30] have prompted speculation that this binding site represents an inactivated (closed) conformation of the NAADP receptor. We directly tested this hypothesis by comparing concentration–response curves for inhibition of Ca^{2+} release by low, subthreshold concentrations of NAADP in high and low K^+-containing media, having established that under the latter conditions, reversible components of NAADP binding are revealed (Figures 1 and 2). Despite the substantial changes in the kinetics of dissociation of NAADP from its receptor on decreasing $[K^+]$ of the medium, no apparent change in the ability of NAADP to promote inactivation was observed (Figure 5). Since during preincubation of homogenates with low concentrations of ligand in the absence of K^+, binding of NAADP is likely to proceed by both reversible and irreversible means (Figure 2), it is quite possible that the latter, if related to inactivation, is sufficient to attenuate subsequent Ca^{2+} release. However, on subsequent addition of a normally maximal concentration of NAADP, binding of NAADP to its receptor is largely reversible (Figure 1). But clearly this quite drastic change in kinetics of NAADP binding did not re-sensitize inactivated receptors. Again it is possible that a small fraction of the total population of NAADP receptors (not readily amenable to characterization by the current methods) continue to bind their ligand irreversibly thereby promoting inactivation, even in the absence of K^+ and in the presence of high concentrations of NAADP. Alternatively, a simpler and, perhaps, more likely explanation of our results is that [^{32}P]NAADP binds to a site related to opening and not closing of NAADP receptors. Indeed, in the absence of K^+, we demonstrate that the reversible conformation of the receptor identified by radioligand binding is associated with a decrease in the sensitivity of NAADP-sensitive Ca^{2+} stores for their ligand (Figure 5C). This is expected since any dissociation of NAADP from its receptor would decrease the probability of receptor occupancy, i.e. as we have previously suggested, irreversible binding of NAADP to its receptor (in the presence of K^+) serves to enhance sensitivity [29]. Furthermore, our results showing differential modulation of the concentration–effect relationship by K^+, for activation and inactivation of Ca^{2+} release by NAADP, also suggest that the two processes are mediated independently.

Modest increases in $[K^+]$ have been reported to occur during fertilization of sea urchin eggs [38]. Such changes, however, are unlikely to affect the kinetics of NAADP dissociation from its receptor since dissociation is maximally inhibited by 250 mM $[K^+]$ (Figure 3), which corresponds to the basal $[K^+]$ in sea urchin eggs [39]. Decreases in cellular K^+ levels are much more likely to regulate NAADP receptors. Of note are substantial decreases in cytosolic $[K^+]$ that occur during apoptosis [40]. For example, in populations of dying thymocytes, the average cytosolic $[K^+]$ can be decreased to as low as 50 mM [41], a concentration well within the range to affect dissociation kinetics of NAADP receptors ($IC_{50} \sim 50$ mM, Figure 1C). This raises the possibility that NAADP receptor function may be altered during programmed cell death. Indeed, apoptosis is demonstrable in sea urchin oocytes, eggs and early embryos [42,43], and is a key event during oocyte maturation in mammals [44].

In summary, we demonstrated that K^+ ions inhibit dissociation of NAADP from its receptor in sea urchin eggs and provide evidence for multiple NAADP receptor conformations. Our definition of experimental conditions in which both reversible and irreversible components of NAADP binding are revealed should prove useful in further studies, delineating their role in the activation and inactivation of Ca^{2+} release mediated by this novel Ca^{2+}-mobilizing messenger.

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