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Characterization of $NAADP^+$ Binding in Sea Urchin Eggs

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Nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) is a pyridine nucleotide which has been shown to release Ca^{2+} from intracellular membranes in echinoderms, *Ascidiae,* mammals, and plants. NAADP releases Ca^{2+} via a mechanism independent of ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors and the $NAADP^+$ receptor is likely to be located on a separate organelle. We have investigated the binding characteristics of $NAADP^+$ to its receptor in sea urchin egg homogenates. $NAADP^+$ binds to a saturable membrane-bound site with high affinity (K_d = 193 ± 35.7 pM). $NAADP^+$ associates to its receptor with a $t_{1/2}$ of approximately 7 min while dissociation does not occur during the time course of the experiment. Furthermore, NAD^+, $NAAD^+$, ADP, or ATP cannot displace $NAADP^+$ binding. The structurally related molecules $NADP^+$ and NADPH displayed a markedly lower affinity for the binding site with K_d's 500- and 25,000-fold higher than $NAADP^+$, respectively. This discrepancy between oxidized and reduced forms of $NADP^+$ might suggest that $NAADP^+$ signaling is itself regulated by the redox state of the cell.

Key Words: **calcium; NAADP; sea urchin; radioligand binding; pyridine nucleotides; inactivation; Ca^{2+}-release.**

Alongside the better known IP_3 and ryanodine receptors, evidence is emerging that a new Ca^{2+}-channel, gated by the pyridine nucleotide nicotinic acid adenine dinucleotide phosphate ($NAADP^+$), might be present on intracellular membranes [1, 2]. Most of the initial work has been conducted in sea urchin eggs, a model system that has proved useful in the study of both IP_3 and ryanodine receptors [3, 4]. It was in this system that cyclic adenosine 5′ diphosphate ribose (cADPR), a derivative of NAD^+, was first shown to release Ca^{2+} via ryanodine receptors [4, 5]. $NAADP^+$, which is structurally related to cADPR, has been shown to be a potent Ca^{2+}-release messenger both in sea urchin egg homogenates and in intact eggs, with an EC_{50} in the low nanomolar range [6–8]. Ca^{2+}-release by $NAADP^+$ is insensitive to agents which specifically block IP_3-induced (e.g., heparin) or cADPR-induced (e.g., 8-NH_2-cADPR) Ca^{2+}-release [6–8]. In contrast to these other two mechanisms, $NAADP^+$-induced Ca^{2+} release can be blocked by high concentrations of L-type Ca^{2+}-channel and K^+-channel antagonists [9]. Furthermore, while NAADP-induced Ca^{2+}-release displays homologous desensitization, it is not affected by pretreatment of homogenates with IP_3 or cADPR [6–8]. Taken together, these data strongly suggest that $NAADP^+$ releases Ca^{2+} via a novel and as yet unidentified mechanism.

It has been shown that $NAADP^+$ displays a unique inactivation phenomenon [8, 10]. In brief, concentrations of $NAADP^+$ that are too low to activate the Ca^{2+}-release mechanism are sufficient to prevent any further Ca^{2+}-release elicited by subsequent additions of supramaximal $NAADP^+$ concentrations. A crucial difference between the known Ca^{2+}-release mechanisms and that gated by $NAADP^+$ is the Ca^{2+}-store on which the mechanism resides [6, 11]. It is well accepted that IP_3 and cADPR release Ca^{2+} mainly from the endoplasmic reticulum, and that Ca^{2+} is loaded in these stores by a specific Ca^{2+}-ATPase. Pretreatment of homogenates with thapsigargin, an irreversible inhibitor of this ATPase, completely abolishes any IP_3- or cADPR-induced Ca^{2+}-release, while it does not affect responses to $NAADP^+$. This evidence, together with previous data which showed that microsomes responding to $NAADP^+$ could be physically separated from those responding to the other two messengers [4, 6], has led to the hypothesis that $NAADP^+$ does not release Ca^{2+} from the endoplasmic reticulum but from an as yet unidentified intracellular Ca^{2+} pool.

Alongside the sea urchin egg model, there is now mounting evidence that $NAADP^+$ is a Ca^{2+}-releasing agent in other systems. $NAADP^+$ releases Ca^{2+} in intact starfish [12] and ascidian eggs [13], in plants [14], in rat pancreatic acinar cells [15] and in rat brain [16]. Investigation in these systems has also lead to the knowledge that $NAADP^+$-induced Ca^{2+}-release could act as a Ca^{2+}-trigger for the other Ca^{2+}-release messengers [15]. In starfish eggs, for example, $NAADP^+$-induced Ca^{2+}-release can be significantly reduced by pretreatment with inhibitors of both IP_3 and ryanodine receptors, but is not affected by pretreatment with one

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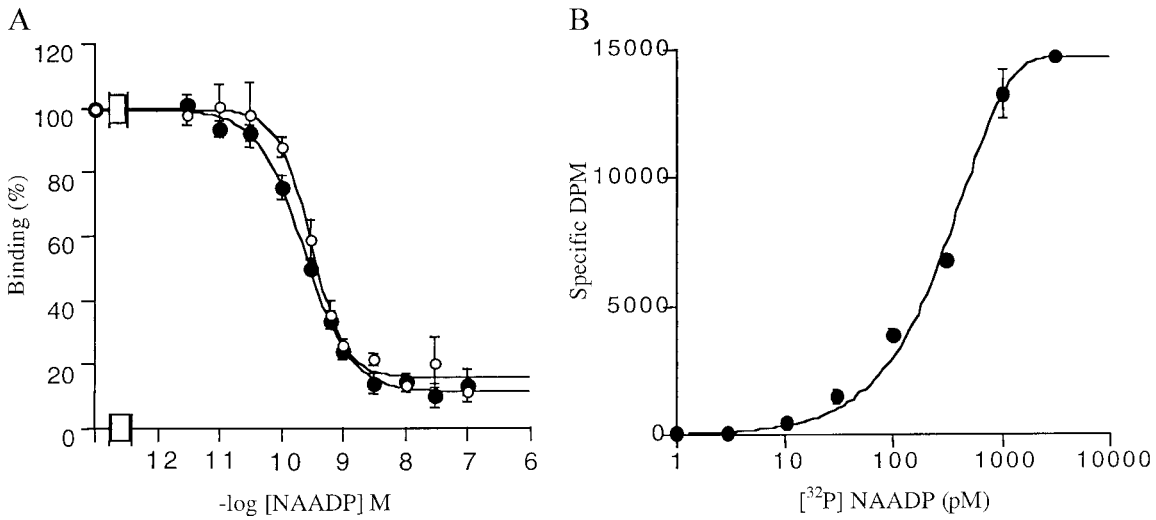

FIG. 1. NAADP$^+$ binds with high affinity to a saturable site. (A) Displacement of [^{32}P]NAADP$^+$ by cold NAADP$^+$ in sea urchin egg homogenate (closed circles) and microsomes (open circles) values are mean ± SEM of 12 determinations; (B) saturation curve using increasing concentrations of [^{32}P]NAADP$^+$. Nonspecific binding was calculated with 1 μM NAADP$^+$ values are mean ± SEM of 3 determinations.

of the two antagonists alone [12]. The trigger hypothesis is supported by the notion that in sea urchin eggs, NAADP$^+$-induced Ca^{2+}-release, unlike IP_3- and cADPR-induced responses, is not affected by extravesicular Ca^{2+}-concentrations [11, 17]. NAADP$^+$ has also been shown to induce Ca^{2+}-oscillations in sea urchin eggs, a phenomenon that might represent the sequential activation of multiple release mechanisms [18].

Considerable efforts have been made to understand whether NAADP$^+$ could be an endogenous messenger, i.e., whether it can be synthesized and degraded in cells [19]. It has been shown that the family of enzymes responsible for cADPR production and degradation, of which *Aplysia* ADP-ribosyl cyclase and CD38 are members, also catalyses a base-exchange reaction that converts NADP$^+$ to NAADP$^+$ in the presence of nicotinic acid [20]. Furthermore, it has recently been demonstrated that cADPR and NAADP$^+$ formation can be differentially modulated by cAMP- and cGMP-dependent pathways [21, 22]. Although not as well characterized, it has been shown that both sea urchin eggs and mammalian tissues can degrade NAADP$^+$ [23].

In this article, we have characterized NAADP$^+$ binding in sea urchin eggs homogenates, a necessary step to further validate the possibility that NAADP$^+$ acts as messenger in cells and an important technique to proceed toward the isolation of the receptor protein. We now report that NAADP$^+$ binds to a saturable site to a sea urchin egg membrane protein with an affinity of about 200 pM. NAADP$^+$ binding displays a $T_{1/2}$ of association of about 7 min, while no dissociation is detectable in the time course of the experiments (30 min). This affinity is consistent with the NAADP$^+$ concentration required for inactivation of the release mechanism. Since Ca^{2+}-release occurs at higher concentrations, the possibility that more than one binding site needs to be occupied simultaneously to elicit a response is a conceivable hypothesis. Further characterization of NAADP$^+$ binding has lead to the observation that NADP$^+$ has a more than 50-fold higher affinity than NADPH, suggesting that the redox state of the cell might be an important modulator of NAADP$^+$ signaling.

MATERIALS AND METHODS

Sea urchin egg homogenates. Homogenates of unfertilized *Lytechinus pictus* eggs (Marinus Inc., Long Beach, CA) were prepared as previously described [24] and stored at −80°C until use. For the present study, two separate preparations were used with similar results.

Synthesis of [^{32}P]-NAADP$^+$. [^{32}P] NAADP$^+$ was synthesized using an adaptation of the method described by [10]. Briefly, 12.5 μM [^{32}P]NAD$^+$ (800Ci/mmol; NEN, Hounslow, UK) and 2.5 mM ATP were incubated with 5 units NAD$^+$ kinase (Sigma, UK) for 1 h at 37°C in a buffer consisting of 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$ and 125 mM Tris, pH 7.5. The reaction was terminated by a ten-fold dilution into a buffer containing 200 mM sodium acetate and 100 mM nicotinic acid, pH 4.5. *Aplysia* ADP-ribosyl cyclase (Sigma, UK) was added to a final concentration of 5 μg/ml and the reaction incubated at 25°C for 3 h. [^{32}P]NAADP$^+$ was purified by HPLC using the protocol described by [20]. The trifluoroacetic acid in the [^{32}P]NAADP$^+$-containing fraction was then evaporated in a speed vacuum.

[^{32}P]NAADP$^+$ binding assays Binding of [^{32}P]NAADP$^+$ in sea urchin egg homogenates was determined using a centrifugation protocol. Binding experiments were carried out on ice in Intracellular Medium (Glu-IM; 250 mM Potassium gluconate, 250 mM *N*-methylglucamine, 20 mM Hepes, 1 mM $MgCl_2$, pH 7.2). [^{32}P]NAADP$^+$ was added at an approximate final concentration of 45 pM (10,000–15,000 CPM) and competing ligand was added to this mixture. The binding reaction was started by addition of sea urchin egg homogenate or fractions thereof (50 μg protein; final volume: 200 μl). Incu-

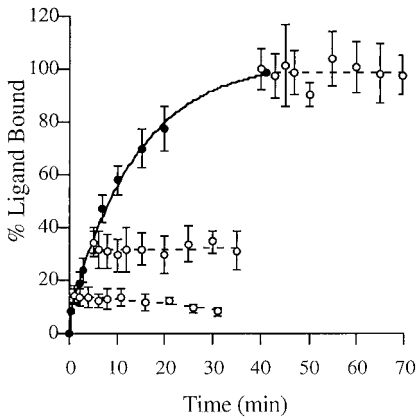

FIG. 2. Association (closed circles, solid line) and dissociation (open circles, dashed line) of [^{32}P]NAADP$^+$ to sea urchin homogenate. Dissociation was initiated by the addition of 1 μM cold NAADP$^+$. Values are mean ± SEM of 4 determinations.

bations were left on ice for 20 min and terminated by centrifugation at 20,000*g* for 5 min at 4°C. Microsomes were prepared by centrifuging whole homogenate at 10000g, discarding the pellet and centrifuging the supernatant at 100,000*g*. For binding, they were pelleted with the aid of polyethylene glycol (PEG). Briefly, after the 20-min incubation, 200 μl of 30% PEG (M.W. 8000) and 4 μl gammaglobulin (50 μg/ml) were added to the reaction and the tube vortexed. After 5 min, the tubes were centrifuged. The supernatant was aspirated and the pellet washed in 1 ml ice-cold Glu-IM. Pellets were then resuspended in 1 ml scintillation fluid and counted.

RESULTS AND DISCUSSION

To characterize [^{32}P]NAADP$^+$ binding in sea urchin egg homogenates, initial experiments were designed to establish conditions for optimal analysis. As reported previously [10], NAADP$^+$ binding was detectable in Glu-IM buffer at 0°C, and therefore these two parameters were kept constant. To avoid ligand depletion, i.e., a proportion in excess of 20% of labeled ligand bound to the receptor, protein concentration was titrated to 50 μg of protein per tube. Furthermore, since initial experiments showed that NAADP$^+$ bound avidly to the receptor, radiolabeled ligand was used at a concentration of <100 pM. Experiments using higher amounts of radioligand yielded similar results to the ones described in this manuscript. Nonspecific binding was calculated by the addition of 100 nM to 1 μM NAADP$^+$ (values were approximately similar). Under these conditions, NAADP appeared to bind with a K_d of approx 193 ± 35.7 pM (values ranged between 100 and 244 pM; $n = 12$) and a B_{max} of 230 ± 27.5 fmol/mg (Fig. 1A). Furthermore, the Hill slope calculated from these data is close to the unity (1.18 ± 0.086). Scatchard plot analysis also confirmed that NAADP$^+$ appeared to bind to a homogenous population of sites. These experiments were performed on whole homogenate, and the presence of soluble proteins that bind NAADP$^+$ could have interfered with the free ligand concentration. To exclude this possibility, experiments were performed on a purified membrane fraction (membranes that pellet at >10,000 g). In these experiments, the K_d and the Hill slope were not significantly different to the whole homogenate (K_d = 280 ± 38.2 pM, Hill slope = 1.595 ± 0.23) (Fig. 1A), while, as expected, the B_{max} was significantly increased (629.5 ± 72 fmol/mg).

The K_d obtained in this study is consistent with the IC_{50} necessary to elicit half-maximal inactivation of the NAADP$^+$ receptor, while it is about 100-fold lower than the EC_{50} required for Ca^{2+}-release in this system. Such a discrepancy is consistent with the hypothesis that the NAADP$^+$ channel possesses more than one NAADP$^+$ binding site, and while fully liganded receptors elicit Ca^{2+}-release, partially liganded receptors undergo inactivation. This hypothesis is also consistent with stopped flow data, where it was suggested that a receptor undergoes an all-or-none inactivation [25]. A putative mechanism by which NAADP$^+$ could both elicit inactivation and activation via the same receptor can be hypothesized by drawing a parallel with the nicotinic receptor [26]. In brief, the NAADP$^+$ binding site could exist in two affinity states. When unbound the receptor exists in a low affinity state, but would shift to a high affinity conformation upon NAADP$^+$ binding. Equilibrium binding analysis will only record the latter, since the low affinity state will be transient. The possibility that our ligand concentrations, though, are well below the affinity of a distinct binding site responsible for NAADP$^+$-induced Ca^{2+}-release which therefore remains undetected. To test whether we could detect a second binding site at higher concentrations of radioligand, we performed experiments using increasing concentrations of radioligand (nonspecific binding was calculated by using 1 μM cold NAADP$^+$). Under these conditions, binding was satu-

TABLE 1

Displacement of [^{32}P]NAADP$^+$ Binding by Structural Analogues and Ca^{2+}-Mobilizing Agents

Analogue	% [^{32}P]NAADP$^+$ bound
	100 ± 1.3
NAADP$^+$ 1 μM	0.95 ± 0.098
ADP 1 μM	99 ± 1.5
ATP 1 μM	101 ± 2.3
NAAD$^+$ 1 μM	87 ± 5.0
NAD$^+$ 1 μM	103 ± 0.55
NADP$^+$ 1 μM	13 ± 5.2
NADPH 1 μM	51 ± 3.2
IP_3 1 μM	94 ± 3.4
cADPR 100 nM	72 ± 1.2
cADPR 1 μM	19 ± 0.30
cADPR 10 μM	9.2 ± 6.1

Note. Values are mean ± SEM of 3 determinations. NADP$^+$ was purified once using HPLC. 45 pM [^{32}P]NAADP$^+$ was used as the labeled ligand in these experiments.

rable (Fig. 1B), with a calculated K_d similar to that calculated by the previous method (381 ± 36.1 pM). Linearization of these data by Scatchard analysis suggested that only one population of sites was present. These data would therefore suggest that $NAADP^+$ binds to just a single homogeneous population of sites.

To further characterize binding and to test whether different affinities could be detected under nonequilibrium binding, we performed association and dissociation experiments. 45 pM $NAADP^+$ associated to the receptor with a $t_{1/2}$ of about 7 min at 0°C (Fig. 2). It has been previously shown that $NAADP^+$ binding is slowly reversible, a situation that could represent the high affinity state of the receptor [10]. Therefore, we decided to perform dissociation kinetics at different times after incubation. If the shift in affinity is a slow process, we could expect that the K_{off} of binding would change over time. [^{32}P]$NAADP^+$ molecules bound did not substantially dissociate even when incubated for just one minute before the addition of 1μM displacing ligand (Fig. 2). As a control experiment, $NAADP^+$ dissociation was investigated by dilution instead of addition of cold ligand. $NAADP^+$ did not dissociate from the receptor after 20 min (data not shown). These data suggest that if our hypothesis is correct, and a shift in affinity accounts for the discrepancy between the concentrations required for activation and inactivation, this shift is rapid. It is likely that the development of fluorescent ligands and the use of stopped-flow methods will be necessary to allow detection of the phenomenon. Furthermore, the data on dissociation are consistent with previous rapid-kinetic experiments, where the existence of only fully desensitized or nondesensitized receptors (in contrast to partially desensitized) was suggested [25].

To further investigate $NAADP^+$ binding, we analyzed the effect of closely related molecules on binding. As expected, IP_3 did not displace $NAADP^+$ binding, while, rather surprisingly, cADPR, a loosely-related pyridine nucleotide, which releases Ca^{2+} via ryanodine receptors, displaced $NAADP^+$ binding at a concentration of 1 μM (Table 1). Neither nicotinic acid adenine dinucleotide ($NAAD^+$), nor NAD^+ appeared to possess any significant affinity for the receptor, supporting functional evidence that suggests that the phosphate charge on $NAADP^+$ is crucial for receptor recognition [27]. Initial studies with $NADP^+$, the molecule most closely related to $NAADP^+$, suggested that it had a high affinity for the receptor. Since it has been previous shown that commercially available $NADP^+$ is contaminated with $NAADP^+$, we then further purified $NADP^+$ by HPLC [4, 6, 28]. "One time" purified $NADP^+$ displaced $NAADP^+$ binding with a K_d of approximately 107 nM (Fig. 3). This affinity, though, could still be an overestimation, since it has been shown that further purification of $NADP^+$ leads to a further decrease in activity of the commercially available compound [28].

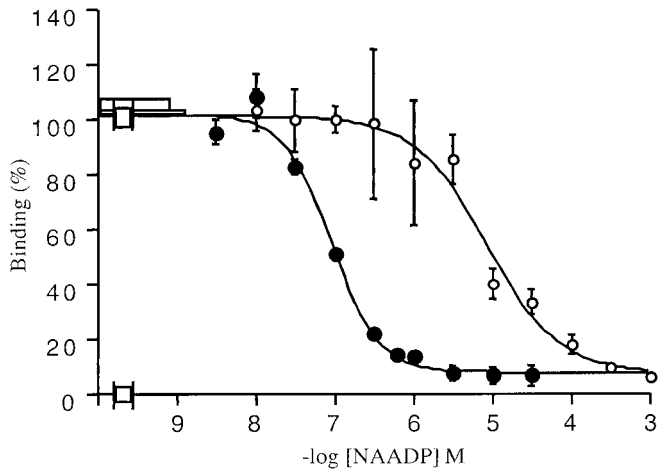

FIG. 3. $NADP^+$ (closed circles) and NADPH (open circles) have lower affinity than $NAADP^+$ for the $NAADP^+$ binding site. $NADP^+$ was purified one time on HPLC before performing experiments. Values are mean ± SEM of 6–9 determinations.

In sharp contrast, NADPH displaced binding with a K_d of approximately 5.5 μM (Fig. 3). The ability of $NADP^+$ to displace $NAADP^+$ binding contrasts previous data, but it is likely that this discrepancy is due to the amount of radioligand present in the incubation medium [10]. It is interesting to note, though, that $NADP^+$ has at least 500-fold lower affinity for the receptor than $NAADP^+$. Since it is possible that NAADPH might exist in cells, it is likely that the difference in affinity between $NADP^+$ and NADPH will be maintained, if not enhanced between $NAADP^+$ and its reduced analogue. If this were the case, the redox state of the cell could play a dual role in controlling the $NAADP^+$-sensitive mechanism: (i) it would control the concentration of $NADP^+$, which has been shown to be a precursor of $NADP^+$; and (ii) it would control the $NAADP^+$/NAADPH ratio.

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