RESEARCH COMMUNICATION

Nicotinic acid–adenine dinucleotide phosphate mobilizes Ca^{2+} from a thapsigargin-insensitive pool

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Nicotinic acid–adenine dinucleotide phosphate (NAADP) is a novel intracellular Ca^{2+} releasing agent recently described in sea-urchin eggs and egg homogenates. Ca^{2+} release by NAADP is independent of that induced by either inositol trisphosphate (InsP_3) or cyclic adenosine dinucleotide phosphate (cADPR). We now report that in sea urchin egg homogenates, NAADP releases Ca^{2+} from a Ca^{2+} pool that is distinct from those that are sensitive to InsP_3 and cADPR. This organelle has distinct Ca^{2+} uptake characteristics: it is insensitive to thapsigargin and cyclopiazoic acid, but maintenance of the pool shows some requirement for ATP. Although the different Ca^{2+} pools have different characteristics, there appears to be some degree of overlap or cross-talk between the NAADP- and cADPR/InsP_3-sensitive Ca^{2+} pools. Ca^{2+}-induced Ca^{2+} release is unlikely to account for the apparent overlap between stores, since NAADP-induced Ca^{2+} release, in contrast with that stimulated by cADPR, is not potentiated by bivalent cations.

INTRODUCTION

Many cells, including sea-urchin eggs, possess multiple mechanisms for Ca^{2+} mobilization from internal stores [1], which may be evoked during cellular signalling. Inositol trisphosphate (InsP_3) and ryanodine (Ry) receptors are the two principal Ca^{2+}-release channels that have been characterized [2]. These two channels are regulated by InsP_3 and cyclic adenosine dinucleotide phosphate (cADPR) respectively, which are both potent Ca^{2+}-mobilizing agents and activators of sea-urchin eggs [3–6] and have been demonstrated to release Ca^{2+} from internal stores in a variety of mammalian cells [7,8]. InsP_3 and cADPR both contribute to the Ca^{2+} wave during the fertilization of sea-urchin eggs, since neither the InsP_3 receptor antagonist, heparin, nor antagonists of the cADPR/Ry receptor, Ruthenium Red or 8-amino-cADPR, are able to block the fertilization Ca^{2+}-wave alone, but their co-injection blocks both Ca^{2+} increases and egg activation by sperm [9,10].

Recently, nicotinic acid–adenine dinucleotide phosphate (NAADP) has been characterized as a third independent Ca^{2+}-releasing agent in sea-urchin eggs [11,12]. NAADP is even more potent than either InsP_3 or cADPR in releasing Ca^{2+} from intracellular stores in sea-urchin homogenates [11,12] and in intact eggs [12,13]. NAADP-induced release appears to operate via an InsP_3- and cADPR-independent mechanism, since there is no cross-desensitization between NAADP- and InsP_3/cADPR-induced Ca^{2+} release mechanisms in sea-urchin egg homogenates, and all three agents exhibit homologous desensitization [11,12]. Furthermore, heparin and 8-amino-cADPR, while blocking InsP_3- and cADPR-induced Ca^{2+} release respectively, have no inhibitory effects on NAADP-induced Ca^{2+} release [12], and NAADP does not affect $[^3H]$cADPR and $[^3H]$InsP_3 binding to sea urchin egg microsomes [11]. Although Ca^{2+} release by NAADP has been reported only in sea-urchin eggs to date, the ability of mammalian cells to synthesize and degrade this molecule has been shown in various rat tissues, including brain and liver [14]. Candidate enzymes for NAADP synthesis are ADP-ribosyl cyclases [15] and molecules related to the lymphocyte antigen CD38 [16], both of which are present in a variety of mammalian tissues [17,18]. Both of these enzymes promote the synthesis of cADPR from β-NAD^+, but they have also been shown to catalyse the synthesis of NAADP from its precursor β-$NADP^+$ by a base-exchange reaction in the presence of nicotinic acid at acidic pH [19]. These reports strongly suggest that NAADP is synthesized in mammalian cells and raise the possibility that it is also a ubiquitous Ca^{2+}-mobilizing agent.

The nature of the NAADP-sensitive Ca^{2+} store is unknown. The fractionation of sea urchin egg homogenates on Percoll gradients resolves the InsP_3- and cADPR-sensitive Ca^{2+} stores to the microsomal band, while NAADP-induced Ca^{2+}-releasing activities are scattered throughout various fractions [12]. Although it is clear that the NAADP-sensitive Ca^{2+} release mechanism is distinct from cADPR and InsP_3 release mechanisms, we have investigated the possibility that the NAADP-sensitive Ca^{2+} release mechanism may reside on a separate internal store. We show that the pharmacology of Ca^{2+} sequestration into NAADP-sensitive Ca^{2+} pools differs from that of InsP_3- and cADPR-sensitive pools, since the microsomal Ca^{2+}-uptake inhibitor, thapsigargin, while functionally removing InsP_3- and cADPR-sensitive Ca^{2+} pools, leaves the NAADP-sensitive Ca^{2+} pool intact. However, NAADP-induced Ca^{2+} release and that evoked by InsP_3 or cADPR are non-additive, suggesting either direct or indirect communication between the different Ca^{2+} pools.

Abbreviations used: NAADP, nicotinic acid–adenine dinucleotide phosphate; InsP_3, inositol trisphosphate; cADPR, cyclic adenosine dinucleotide phosphate; Ry, ryanodine; IM, intracellular medium; ER, endoplasmic reticulum; CICR, Ca^{2+}-induced Ca^{2+} release; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone.

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The present data supports the hypothesis that NAADP-sensitive Ca^{2+} pools are distinct from InsP_3 and Ry receptor-sensitive Ca^{2+} stores, with the possibility that the NAADP-induced Ca^{2+} release mechanism is located on a distinct organelle, but that there is some degree of overlap or cross-talk between NAADP- and cADPR/InsP_3-sensitive Ca^{2+} pools.

MATERIALS AND METHODS

Ca^{2+} release assays

Homogenates [2.5 % (w/v)] of unfertilized *Lytechinus pictus* eggs (Marinus Inc., Long Beach, CA, U.S.A.) were prepared as described previously [4], and Ca^{2+}-loading was achieved by incubation at 17 °C for 3 h in an intracellular medium (IM) consisting of 250 mM potassium gluconate, 250 mM *N*-methylglucamine, 20 mM Hepes (pH 7.2), 1 mM $MgCl_2$, 1.0 mM ATP, 10 mM phosphocreatine, 10 units/ml creatine phosphokinase, 1 μg/ml oligomycin, 1 μg/ml antimycin, 1 mM sodium azide and 3 μM fluo-3. Free Ca^{2+} concentration was measured by monitoring fluorescence intensity at excitation and emission wavelengths of 490 nm and 535 nm respectively. Fluorimetry was performed at 17 °C using 500 μl of homogenate in a Perkin-Elmer LS-50B fluorimeter. Additions were made in 5 μl volumes and all chemicals were added in IM containing 10 μM EGTA. Basal concentrations of Ca^{2+} were typically between 100 and 150 nM. Sequestered Ca^{2+} was determined by monitoring the decrease in fluo-3 fluorescence during microsomal loading and by measuring Ca^{2+} release in response to ionomycin (5 μM), and was constant between experiments. Ca^{2+} calibrations were performed for each condition tested in each experiment.

[³H]cADPR binding

[³H]cADPR binding was determined in sea-urchin homogenates as described by Chini et al. [11]. In brief, homogenates were diluted to a concentration of 2 mg/ml in IM containing 1 mM EGTA, and incubated with 20 nM [³H]cADPR for 10 min at 4 °C. Non-specific binding was assessed with 10 mM cADPR. Binding was terminated by filtration (fibreglass GF/B filters) under vacuum and the filters were rapidly washed twice in ice-cold IM. Radioactivity retained on the filters was determined using standard scintillation counting techniques.

Materials

cADPR was synthesized as previously described [15]. Fluo-3 was obtained from Calbiochem and NAADP from RBI (St. Albans, U.K.). Bafilomycin A_1 was obtained from LC Laboratories (Bingham, U.K) and [³H]cADPR from Amersham (Amersham, UK). All other chemicals were from Sigma.

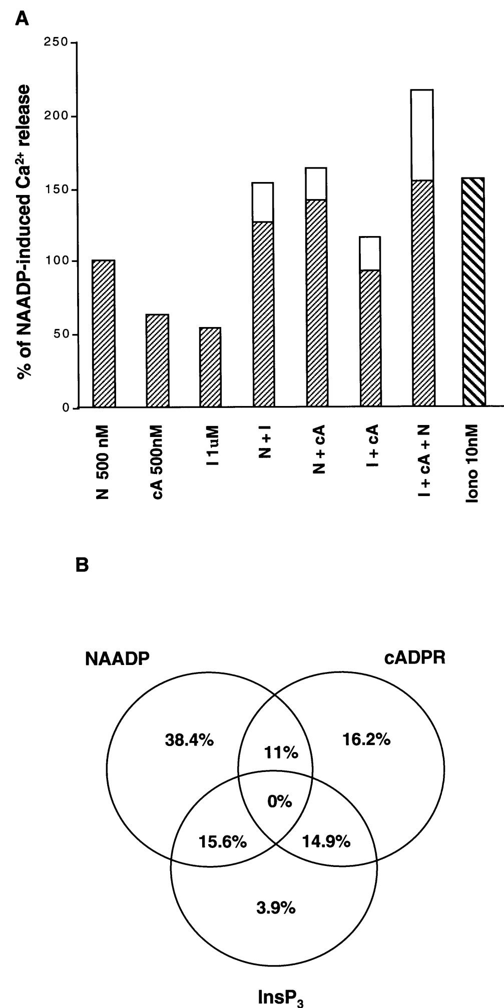

Figure 1 (A) Ca^{2+} release by Ca^{2+}-mobilizing agents and (B) distribution of agonist-sensitive Ca^{2+} pools in sea urchin egg homogenates

(**A**) Ca^{2+} release by maximal concentrations of the three Ca^{2+}-mobilizing agents, NAADP, cADPR and InsP_3 in sea urchin egg homogenates. NAADP (500 nM) released 7.3 ± 0.3 nmol of Ca^{2+}. Values are medians of 6–12 determinations. Hatched bars show actual release, while unfilled bars represents the additional Ca^{2+} release expected if release by two or more agents added together was additive. N, NAADP; cA, cADPR; I, InsP_3; Iono, ionomycin. (**B**) Venn-diagram of the distribution of agonist-sensitive Ca^{2+} pools, derived from the data in (**A**), showing the extent of overlap between different Ca^{2+} pools.

RESULTS AND DISCUSSION

In sea urchin egg homogenates, NAADP mobilized a larger Ca^{2+} pool than either InsP_3 or cADPR. A maximal concentration of NAADP (500 nM) typically released 6–10 nmol of Ca^{2+} in different experiments from separate batches of sea urchin egg homogenates. This was significantly more Ca^{2+} than was released by maximal concentrations of either cADPR (500 nM) or InsP_3 (1 μM), which were typically in the ranges 3.6–6 and 3–5 nmol of Ca^{2+} respectively. The ratio between the release by NAADP and by the other two agonists was similar between experiments.

Since cADPR- and InsP_3-sensitive pools are thought to be part of the endoplasmic reticulum (ER) [20], we examined whether there was appreciable overlap between these pools and the pool that was sensitive to NAADP, which, if the case, may favour an ER location for NAADP-sensitive pools. To address the question of whether the pools from which the Ca^{2+} is released by the three agonists showed significant overlap, co-additions were performed and the total amount of Ca^{2+} release ascertained. Figure 1(A) shows that when any two agonists are co-added their effect is less than additive. The finding that cADPR- and InsP_3-induced Ca^{2+} release are non-additive effects is in accordance with a previous report [21], although in the present experiments the extent of overlap is significantly less. We found that Ca^{2+} release evoked by NAADP in combination with either cADPR or InsP_3 was similarly non-additive, although to a lesser extent, despite the previous apparent separation of NAADP-and cADPR/InsP_3-sensitive Ca^{2+} pools on Percoll gradients [12]. When all three agonists were co-added, the Ca^{2+} released was equivalent to the amount of Ca^{2+} released by a maximal

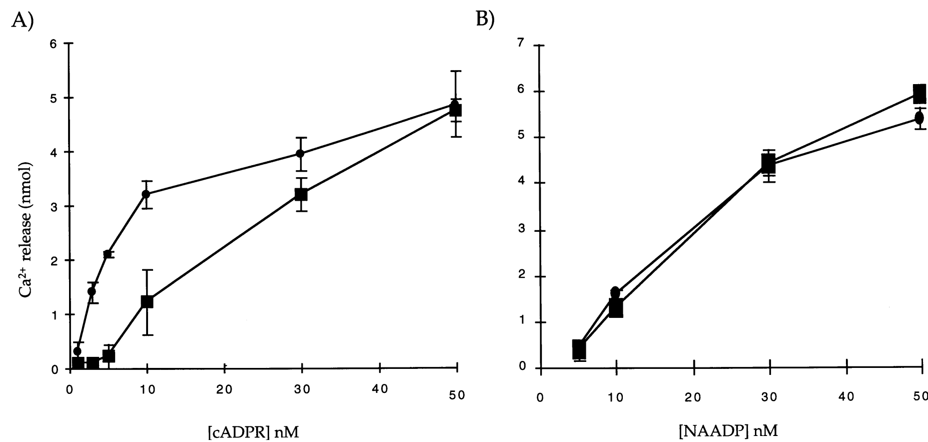

Figure 2 Effect of Sr^{2+} ions on Ca^{2+} release induced by sub-maximal concentrations of cADPR (A) or NAADP (B)

Homogenates were pretreated with Sr^{2+} (●; 100 μM), which itself caused no Ca^{2+} release, and cADPR (**A**) or NAADP (**B**) was added 30 s later and the resultant maximal Ca^{2+} release recorded. ■, Control. Concentrations of agonists higher than 50 nM were not potentiated or augmented by Sr^{2+}. Values are means $\pm$ S.E.M. of 6–9 determinations.

concentration of the Ca^{2+} ionophore, ionomycin (10 μM). A role for mitochondrial pools in Ca^{2+} mobilization by NAADP, InsP_3 or cADPR in sea-urchin homogenates can be ruled out, since high concentrations of mitochondrial inhibitors (oligomycin, antimycin, sodium azide), acting at different targets in the respiratory/electron-transport chain, are added during the preparation (see the Materials and methods section). Since Ca^{2+} release by NAADP, InsP_3 and cADPR together can account for all the releasable Ca^{2+} from intracellular non-mitochondrial Ca^{2+} stores in the sea-urchin egg, this may suggest that no further Ca^{2+}-mobilizing agents remain to be discovered in the sea-urchin egg. The effects of the three Ca^{2+}-releasing agents on sea-urchin egg Ca^{2+} stores revealed that a total of just over half the non-mitochondrial Ca^{2+} (58.5 %) could be independently released by a single agonist alone, while the remainder was sensitive to more than one agonist (Figure 1B), with no Ca^{2+} pool releasable by all three agonists (Figure 1B). Extrapolating these data to intact eggs, the results may in part explain the redundancy of cADPR and InsP_3 in the generation of fertilization Ca^{2+}-waves, since a substantial portion of stored Ca^{2+} is accessible to more than one agent. However, the largest pool is sensitive to NAADP alone (38.4 %; Figure 1B).

It has been reported that cADPR modulates Ca^{2+}-induced Ca^{2+} release (CICR) in sea urchin egg homogenates [6,22,23]. To determine whether this effect was specific to cADPR or was shared by the other pyridine nucleotide, NAADP, and whether Ca^{2+} release from one pool could trigger Ca^{2+} release from another, and thus account for the apparent non-additivity of the different Ca^{2+} release mechanisms, the effect of the bivalent cation strontium (Sr^{2+}) on the different Ca^{2+}-release mechanisms was studied. As previously reported, Sr^{2+} can act as a surrogate for Ca^{2+} in potentiating cADPR-induced Ca^{2+} release, with the advantage that it does not alter fluo-3 fluorescence and so any fluorescence changes observed are entirely due to stimulation of Ca^{2+} release (Figure 2A; see also [23]). If the NAADP-sensitive release is modulated by bivalent cations, Sr^{2+} should potentiate its action. Sr^{2+} did not significantly augment NAADP-induced Ca^{2+} release (Figure 2B). Furthermore, caffeine (1 mM), another agent that potentiates CICR via an Ry-sensitive mechanism, did not potentiate NAADP-induced Ca^{2+} release, although it enhanced cADPR-induced Ca^{2+} release (results not shown; Lee [23]). In addition, it has been reported that Mg^{2+}, an inhibitor of CICR, while blocking cADPR-induced Ca^{2+} release, does not alter that induced by NAADP [24]. Since it has been previously reported that Sr^{2+} ions and caffeine are also ineffective at potentiating InsP_3-induced Ca^{2+} release [23], it appears that in sea urchin egg homogenates, CICR is a property only of the cADPR/Ry-sensitive Ca^{2+} release mechanism, and thus cannot account for the extensive overlap between the different Ca^{2+} stores present. It therefore appears that the overlap between the pools is not due to CICR but is most likely due to a small physical overlap.

To distinguish between the different Ca^{2+} pools, egg homogenates were incubated for 1 h with various agents that interfere with intracellular Ca^{2+} sequestration, and then challenged with maximal concentrations of InsP_3, cADPR or NAADP. As expected, when homogenates were pre-treated with ionomycin (10 μM) none of the three agonists were able to release further Ca^{2+}. This could not be explained by dye saturation, since further addition of Ca^{2+} still produced a detectable increase in fluorescence (results not shown).

When homogenates were treated with a supra-maximal concentration of thapsigargin (10 μM), a potent and selective inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}/Mg^{2+}-ATPases [25,26], the Ca^{2+} level slowly rose to a plateau value after about 50 min, after which no resequestration was observed (Figure 3). Ca^{2+} release by cADPR and InsP_3, added after 1 h of incubation with thapsigargin (10 μM), was significantly reduced to less than 20 % of release in the absence of thapsigargin (Figure 3 and Table 1). This result is in agreement with previous reports that both InsP_3- and ryanodine-sensitive stores are sensitive to thapsigargin [27]. In contrast, release by NAADP was unaffected by pre-treatment with thapsigargin (Figure 3 and Table 1). Cyclopiazoic acid, another selective inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}/Mg^{2+}-ATPase [28] had a similar effect in substantially reducing Ca^{2+} release by InsP_3 and cADPR but not affecting Ca^{2+} release by NAADP (Table 1). These results provide strong evidence that the NAADP-induced Ca^{2+} release mechanism is located on stores that are distinct from the ER Ca^{2+}-release channels gated by InsP_3 and cADPR.

We therefore explored the effects of agents that inhibit Ca^{2+}-sequestration mechanisms on membranes other than those of the ER. Although plasma membranes should not contribute to Ca^{2+} release in the sea-urchin homogenate, the possibility that they

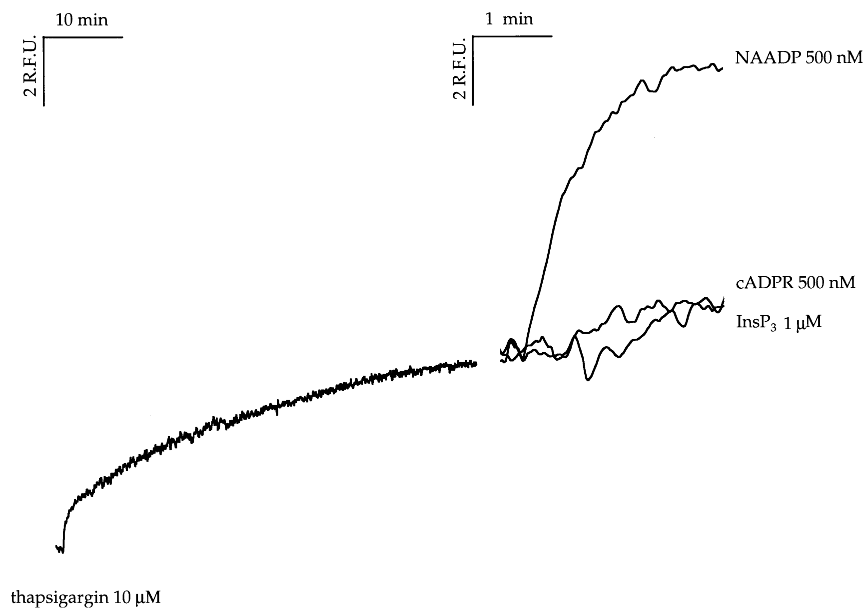

Figure 3 Representative fluorimetric trace of Ca^{2+} release by NAADP, InsP_3 or cADPR after addition of 10 μM thapsigargin

Plateau fluorescence was observed after 50 min in the presence of thapsigargin. NAADP, InsP_3 or cADPR was added after 1 h to separate homogenate aliquots. Note that the time scale is different in the two parts of the Figure to accentuate the difference in kinetics of NAADP-, InsP_3- and cADPR-induced Ca^{2+} release after thapsigargin treatment. R.F.U. is relative fluorescence units representing the fluo-3 fluorescence changes observed.

Table 1 Effect of different Ca^{2+}-uptake inhibitors on maximal Ca^{2+} release by NAADP, cADPR and InsP_3

Values are means ± S.E.M. of 6–15 determinations in 2–5 separate experiments. Results are expressed as a percentage of maximal Ca^{2+} release (100%) obtained in the absence of Ca^{2+}-uptake inhibitors.

Inhibitor	NAADP (500 nM)	cADPR (500 nM)	InsP_3 (1 μM)
Thapsigargin (10 μM)	93 ± 7.6	22 ± 2.2	21 ± 5.4
Cyclopiazoic acid (10 μM)	93 ± 4.0	40 ± 6.5	33 ± 2.1
Vanadate (10 mM)	102 ± 7.1	47 ± 9.51	19 ± 8.0
CCCP (10 μM)	87 ± 4.0	12 ± 6.2	46 ± 8.9
Apyrase (10 units/ml)	48 ± 3.8	46 ± 8.8	6.8 ± 1.8

would reseal and therefore create artifactual microsomes was evaluated by using vanadate, an inhibitor of both the ER and plasma membrane Ca^{2+}-pumps [29], since it has been reported that it is a more potent inhibitor of the plasma membrane type pump [30,31]. Pretreatment with sodium orthovanadate did not modify the Ca^{2+}-release properties of NAADP and InsP_3 (Table 1). In contrast, Ca^{2+} release by cADPR was decreased by 50%. Since InsP_3- and cADPR-sensitive Ca^{2+}-release mechanisms appear to reside on the same stores they should share the same uptake mechanisms, thus the effect of vanadate is probably not due to specific action on a Ca^{2+} pump but to a direct effect of vanadate, or one of the species formed by this compound in solution [32], on the cADPR receptor. A precedence for this is that decavanadate, one of the species formed in solution, inhibits InsP_3-induced Ca^{2+} release in endocrine cells [33] and InsP_3-binding to its receptor [34], and it is therefore possible that another form of oligovanadate, or decavanate itself, blocks the cADPR-gated channel in the sea urchin. However, vanadate does not appear to act at the cADPR-binding site, since [^{3}H]cADPR-binding to sea urchin egg microsomes was not significantly altered in the presence of 10 mM orthovanadate. Specific binding to egg microsomes obtained with this radioligand was 1008 ± 229 d.p.m./mg of protein and 1122 ± 347 d.p.m./mg of protein (results given ± S.E.M.; $n = 5$, for both determinations) in the presence or absence of orthovanadate (10 mM) respectively. Therefore vanadate does not interfere with cADPR-binding to its receptor, but rather could act at the level of the Ca^{2+} channel itself.

The protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), when used at high concentrations (100 μM), completely abolished Ca^{2+} release by NAADP, cADPR and InsP_3 (results not shown), while lower concentrations (10–50 μM) selectively reduced cADPR- and InsP_3-sensitive Ca^{2+} release (Table 1). Since, as mentioned above, all experiments were performed in the presence of mitochondrial inhibitors, it is unlikely that CCCP exerts its effects on agonist-induced Ca^{2+} release by uncoupling oxidative phosphorylation. An alternative explanation is that CCCP, at high concentrations, acts as a non-specific ionophore, releasing Ca^{2+} from the ER. Since NAADP-sensitive Ca^{2+}-stores appear to be more resistant to CCCP, this further discriminates between the site of NAADP action and the sites sensitive to InsP_3 and cADPR. This may suggest differential accessibility of the NAADP-sensitive pool to CCCP, or a different membrane composition of this store that is less susceptible to protonophore insertion.

To test whether maintenance of the NAADP-sensitive Ca^{2+} pool was dependent on ATP, homogenates were incubated for 1 h with a high concentration of apyrase (10 units/ml), which possesses a high ATPase activity. During the incubation, Ca^{2+} levels rose steadily (results not shown), demonstrating that Ca^{2+} homoeostasis in the egg homogenate is a dynamic process which requires the presence of ATP. Ca^{2+} release by all three agonists was affected by apyrase, since the Ca^{2+} release by

NAADP, cADPR and InsP_3 was dramatically reduced in its presence. NAADP-induced Ca^{2+} release was affected least, since only 50% of the release was abolished (Table 1). This further exemplifies differences between NAADP-sensitive Ca^{2+} pools and those regulated by cADPR or InsP_3. These data may suggest: (1) that the Ca^{2+} sequestration mechanism of the NAADP-sensitive pool is less dependent on ATP than are the other pools; (2) that other sequestration mechanisms may also be operating here; or (3) that the pool is less labile, perhaps due to a lower background activity of a Ca^{2+} leak pathway.

To further investigate the localization of the NAADP-sensitive pool, we examined the effects of drugs known to interfere with Ca^{2+} storage by other organelles. Homogenates were pre-incubated with brefeldin A, a drug which selectively disassembles the Golgi complex [35], since it has been reported that pre-incubation with this drug is able to reduce Ca^{2+} storage in LLC-PK1 cells [35]. In the sea-urchin homogenate, brefeldin A did not significantly affect Ca^{2+} release by either of the three agonists tested (results not shown). Bafilomycin A_1, an antibiotic which has been reported to block Ca^{2+}/H^+ exchange in vacuoles of *Trypanosoma brucei*, where the presence of an acidocalcisome has been suggested [36], was also ineffective in modifying release by NAADP, InsP_3 or cADPR (results not shown). These results suggest that that the Golgi complex, or a putative acidocalcisome [36], are unlikely to be the sites of the Ca^{2+}-mobilizing actions of NAADP.

In conclusion, this study extends previous reports suggesting that the novel Ca^{2+}-releasing compound NAADP acts upon a different Ca^{2+}-release mechanism from those modulated by InsP_3 and cADPR. We suggest that NAADP not only activates a distinct Ca^{2+}-release mechanism, but that the site of this mechanism may be located on a distinct organelle with different characteristics from the ER both in terms of Ca^{2+} release and uptake.

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