NAADP induces pH changes in the lumen of acidic Ca²⁺ stores

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NAADP (nicotinic acid–adenine dinucleotide phosphate)-induced Ca²⁺ release has been proposed to occur selectively from acidic stores in several cell types, including sea urchin eggs. Using fluorescence measurements, we have investigated whether NAADP-induced Ca²⁺ release alters the pH_L (luminal pH) within these acidic stores in egg homogenates and observed their prompt, concentration-dependent alkalization by NAADP (but not β-NAD⁺ or NADP). Like Ca²⁺ release, the pH_L change was desensitized by low concentrations of NAADP suggesting it was secondary to NAADP receptor activation. Moreover, this was a direct effect of NAADP upon the acidic stores and not second-

ary to increases in cytosolic Ca²⁺ as it was not mimicked by IP₃ (inositol 1,4,5-trisphosphate), cADPR (cyclic adenine diphosphoribose), ionomycin, thapsigargin or by direct addition of Ca²⁺, and was not blocked by EGTA. The results of the present study further support acidic stores as targets for NAADP and for the first time reveal an adjunct role for NAADP in regulating the pH_L of intracellular organelles.

Key words: Acridine Orange, calcium signalling, luminal pH (pH_L), inositol 1,4,5-trisphosphate (IP₃), cyclic ADP-ribose (cADPR), sea urchin egg.

INTRODUCTION

The ER (endoplasmic reticulum) is established as the primary Ca²⁺ store for the intracellular messengers IP₃ (inositol 1,4,5-trisphosphate) and cADPR (cyclic ADP-ribose) [1,2]. Its properties have been well defined with respect to protein synthesis and ionic movements, particularly in relation to Ca²⁺ [1,2], but more recently, other subcellular organelles have been ascribed a dynamic Ca²⁺ storage role; both the Golgi [3] and acidic/lysosome-related organelles [4–8] release Ca²⁺ in response to IP₃ and NAADP (nicotinic acid–adenine dinucleotide phosphate) respectively. However, such organelle selectivity of these second messengers is not universally accepted, and some controversy surrounds whether acidic vesicles represent a unique NAADP-sensitive store [9–12].

While the ER and Golgi accumulate Ca²⁺ as a direct consequence of P-type Ca²⁺-ATPase activities {e.g. SERCA (sarco-endoplasmic reticulum Ca²⁺-ATPase) and secretory pathway Ca²⁺-ATPase [3]}, the NAADP-sensitive acidic stores are refilled in a more indirect manner, driven by the proton gradient generated by a V-type H⁺-ATPase (and speculatively supported by Ca²⁺/H⁺ exchange in animals [6,13,14], by analogy with plants and yeast [15,16]). Inhibitors of the V-type H⁺-ATPase such as bafilomycin A thereby abrogate NAADP-sensitive Ca²⁺ release upon dissipation of the H⁺ gradient, a situation also mimicked by protonophores [5–7,17,18].

The proposed acidic nature of the NAADP-sensitive Ca²⁺ store prompted us to investigate whether the pH of these stores changes during Ca²⁺ signalling. We have used fluorescence techniques in sea urchin egg homogenates, an excellent model system for investigating acidic vesicles and Ca²⁺ stores [6], to show, for the first time, that NAADP is unique in increasing the pH_L (luminal pH) of acidic stores directly, independent of changes in cytosolic Ca²⁺. The results of the present paper strengthen the argument for acidic stores as a target for NAADP and reveal a novel role for NAADP in their pH regulation.

MATERIALS AND METHODS

Reagents

NAADP was enzymatically synthesized [19] or purchased from Sigma–Aldrich. IP₃ and bafilomycin A1 were from LC Laboratories. cADPR, GPN (glycyl-L-phenylalanine β-naphthylamide), nigericin, FCCP [carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone], thapsigargin and valinomycin were all obtained from Sigma–Aldrich. Acridine Orange, LysoSensorTM Green DND-189 and Fluo-3 (K⁺ salt) were from Molecular Probes (Invitrogen). Ionomycin was from Calbiochem–Novabiochem (Merck Biosciences).

Homogenate preparation

Sea urchin eggs from *Lytechinus pictus* were harvested by intracoelomic injection of 0.5 M KCl, collected in ASW (artificial sea water: 435 mM NaCl; 40 mM MgCl₂; 15 mM MgSO₄; 11 mM CaCl₂; 10 mM KCl; 2.5 mM NaHCO₃ and 20 mM Tris base, pH 8.0) and de-jellied by passage through 100 μm nylon mesh (Millipore). Egg homogenates were prepared at 4 °C and used as described previously [19]. Essentially, eggs were washed four times in Ca²⁺-free ASW (the first two washes containing 1 mM EGTA) and then washed in GluIM (gluconate intracellular-like medium: 250 mM potassium gluconate; 250 mM N-methylglucamine; 20 mM Hepes and 1 mM MgCl₂, pH 7.2). Eggs were then homogenized in GluIM supplemented with 2 mM Mg-ATP, 20 units/ml creatine phosphokinase, 20 mM phosphocreatine and a CompleteTM EDTA-free protease inhibitor tablet (Roche). The homogenate (50 %, v/v) was centrifuged at 13 000 g at 4 °C for 10 s and the supernatant stored at –70 °C. On the day of use, an aliquot (500 μl) of homogenate was sequentially diluted in equal volumes of GluIM (containing the ATP regenerating system above) over a period of 3 h at 17 °C to give a 2.5 % (v/v) final concentration.

Abbreviations used: ASW, artificial sea water; cADPR, cyclic adenine diphosphoribose; ER, endoplasmic reticulum; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GPN, glycyl-L-phenylalanine β-naphthylamide; GluIM, gluconate intracellular-like medium; IP₃, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid–adenine dinucleotide phosphate; NICR, NAADP-induced Ca²⁺ release; pH_L, luminal pH; RFU, relative fluorescence units; SERCA, sarco-endoplasmic reticulum Ca²⁺-ATPase.

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Fluorimetry

All fluorimetry was conducted at 17°C in a microcuvette containing a magnetic stir bar mounted in a PerkinElmer LS-50B fluorimeter. Ca^{2+} release was routinely measured in homogenates with 3 μM Fluo-3 (excitation 506 ± 3 nm, emission 526 ± 4 nm) which was calibrated using the standard equation $[\text{Ca}^{2+}] = K_d \times (F - F_{\min}) / (F_{\max} - F)$, using a K_d of 0.4 μM ; $F_{\min}$ (where F is fluorescence) and $F_{\max}$ were determined by the addition of 0.5 mM EGTA and 10 mM Ca^{2+} respectively at the end of each run [20].

To assess whether Acridine Orange loading of homogenates affected NAADP-induced Ca^{2+} release, we measured Ca^{2+} with the spectrally-distinct Fura-2 (3 μM ; excitation 340 ± 3 nm, emission 520 ± 3 nm) and using a K_d of 0.224 μM . In preliminary *in vitro* experiments in GluIM, excitation and emission spectra of 3 μM Fura-2 were generated in the absence or presence of ≤ 10 μM Acridine Orange in order to verify optimal wavelengths for minimal interference. In homogenate, the Fura-2 signal was calibrated on every run as for Fluo-3 (see above). Note that basal $F_{\min}$ and $F_{\max}$ values were unaffected by the presence of even 10 μM of Acridine Orange, with the observations being $98 \pm 6\%$, $96 \pm 6\%$ and $93 \pm 5\%$ of the experiments without Acridine Orange ($n = 7$; $P > 0.2$).

To routinely assess a vesicular pH gradient, 1–10 μM Acridine Orange was added to each cuvette immediately before each run and allowed to equilibrate (5–10 min), while the dye partitioned into acidic vesicles, as indicated by a gradual fall in fluorescence (see Figure 5A). Acquisition wavelengths were the same as for Fluo-3. In some experiments, 10 μM LysosensorTM Green DND-189 was used as an alternative to Acridine Orange (excitation 443 ± 4 nm and emission 505 ± 3 nm). The ordinate of each representative trace is scaled as RFU (relative fluorescence units), except those for Figures 6(C) and 6(D), which were normalized to their minimum and maximum fluorescence changes.

In vitro concentration–fluorescence curves for Acridine Orange and LysosensorTM Green DND-189 were generated in GluIM at pH 5.0, which more closely mimics the pH of acidic vesicles. Excitation and emission wavelengths were as described above. Fluorescein was used as a control dye as it does not readily undergo self-quenching, a phenomenon inferred from a non-linear relationship between concentration and fluorescence. Fluorescein fluorescence was centred upon the same wavelengths as those used for Acridine Orange. For each dye, the results were normalized to the fluorescence of a 1 μM dose, which offered a good signal-to-noise ratio.

Confocal laser scanning microscopy

Intact sea urchin eggs adhering to poly-lysine-coated glass coverslips were loaded with 10 μM Acridine Orange for 15–20 min at room temperature (20°C) and placed on the stage of a Zeiss LSM 510 Meta confocal microscope equipped with a 40 $\times$ oil immersion objective (NA 1.3), and maintained at room temperature in ASW. Micropipettes for pressure injection were back-filled with 0.5 M KCl containing 200 μM AlexaFluor 647 Dextran as an injection marker either without (control) or with 100 μM NAADP (~ 1 μM intracellular concentration). The excitation/emission wavelengths for acquisition were 488 nm/505–530 nm (Acridine Orange) 633 nm/645–719 nm (AlexaFluor 647 Dextran). The whole-cell mean Acridine Orange fluorescence was normalized to the initial basal value (F_0) and the results expressed as the $\Delta F/F_0$.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Where two data sets were compared, Student's *t* test was used, whereas multiple groups

were analysed using ANOVA and a Tukey–Kramer post-test. Results were paired where appropriate and significance assumed at $P < 0.05$.

RESULTS

Characterization of pH_L dyes

Changes in the pH_L within acidic vesicles can be monitored in sea urchin egg homogenates using fluorescent dyes that selectively partition into these stores as a function of pH_L. Acridine Orange is a weak base that accumulates in these vesicles and is well known to undergo self-quenching upon aggregation [21], whereas the properties of LysosensorTM Green DND-189 are less well defined. Therefore, we first investigated the relationship between dye concentration and fluorescence *in vitro*; dyes that do not readily self-quench exhibit a near linear relationship, as exemplified by fluorescein which was used as a control ($r^2 = 0.988$; $P < 0.001$; Figure 1A, main panel). By contrast, Acridine Orange markedly departed from linearity, and displayed a bell-shaped curve over the range used *in situ*, as did LysosensorTM Green DND-189, consistent with both pH_L dyes undergoing self-quenching (Figure 1A). Excitation and emission spectra with different concentrations of Acridine Orange or LysosensorTM Green DND-189 also confirmed that this non-linearity was independent of wavelength (results not shown).

Before investigating pH_L changes, we first checked that Acridine Orange-loading of acidic vesicles did not interfere with NAADP-sensitive Ca^{2+} responses, therefore we measured NICR (NAADP-induced Ca^{2+} release) in the presence or absence of the pH_L dye. As Figures 1(B) and 1(C) show, NAADP evoked a substantial Ca^{2+} release (Figure 1B, black trace). Addition of 10 μM Acridine Orange (the highest concentration used) did not affect basal Fura-2 fluorescence, and while there was a tendency for NICR to be slightly reduced after Acridine Orange loading (Figure 1B, grey trace), this did not attain statistical significance ($n = 7$, $P > 0.05$). Indeed, we have shown previously that the closely related compound 9-amino acridine had only a small effect upon NICR even at 100 μM [22]. We therefore conclude that Acridine Orange-loading does not overly perturb the NAADP-sensitive Ca^{2+} stores.

NAADP-induced pH_L changes in acidic vesicles

Using these dyes to measure pH_L changes in homogenates, 1 μM NAADP elicited a prompt, but sustained, alkalization indicated by an increase (dequenching) of Acridine Orange fluorescence when compared with water vehicle (Figures 2A and 2C; as a percentage of the maximal 10 mM NH_4Cl response: vehicle, 4.5 ± 1.0 ; NAADP, 19.5 ± 1.6 ; $P < 0.001$; $n = 36$ –45). Qualitatively similar results were seen with both 1 and 10 μM Acridine Orange. This was not a dye artefact, as similar results were obtained with 10 μM LysosensorTM Green DND-189 (Figure 2B), where NAADP elicited a response that was $26 \pm 9\%$ of the maximum ($n = 3$) compared with the lack of response observed with vehicle ($4 \pm 3\%$, $P < 0.05$ compared with NAADP). However, the superior Acridine Orange signal-to-noise ratio was routinely used. The prompt alkalization was apparently selective for NAADP as the same concentration of other related nucleotides β -NAD⁺ and NADP failed to reproduce the effect (Figure 2D).

The observation that changes in Acridine Orange fluorescence were truly emanating from an acidic Ca^{2+} store was supported further by additional data. First, the increase evoked by NH_4Cl was not merely a consequence of vesicular disruption which would be detected as an increase in $[\text{Ca}^{2+}]$ and which was barely

Figure 1 Characterization of pH_L dyes *in vitro* and effects upon NAADP-dependent Ca²⁺ release

(A) Increasing concentrations of fluorescein (□), Acridine Orange (○) and Lysosensor™ Green DND-189 (●) were added to GluIM (pH 5.0) in a cuvette and the fluorescence was measured. Fluorescence at each concentration is expressed as a fraction of the fluorescence at the 1 μM value for each dye, and the inset depicts a magnified view of the Acridine Orange and Lysosensor™ Green DND-189 values with the same units for the ordinate and abscissa. Results are expressed as means ± S.E.M. for six separate determinations. (B) Effect of Acridine Orange upon NAADP-induced Ca²⁺ release in homogenates, as measured with Fura-2. NAADP responses in the absence (black trace) or presence (grey trace) of 10 μM Acridine Orange (AO) added where indicated. (C) Bar graph summarizing the results of (B), showing means ± S.E.M. of seven experiments.

detectable with Fluo-3 ($\Delta[\text{Ca}^{2+}]$ of 6 ± 1 nM; $n = 7$). Secondly, fluorescence did not arise from mitochondrial vesicles (e.g. those inverted during homogenization), because there was no effect of 5 μg/ml oligomycin with or without 2 μg/ml antimycin A (results not shown). Thirdly, the K⁺/H⁺ ionophore, nigericin, evoked an increase in fluorescence in both Acridine Orange- and Lysosensor™ Green DND-189-loaded homogenates (Figure 2A,

Figure 2 Characterization of the pH_L of acidic Ca²⁺ stores

Sea urchin egg homogenates were loaded with 1–10 μM Acridine Orange (A,C and D) or 10 μM Lysosensor™ Green DND-189 (B). Homogenates were treated with 1 μM NAADP, 1 μM β-NAD⁺, 1 μM NADP, 100 μM GPN, 10 mM NH₄Cl or 2 μM nigericin (Nig) where indicated. Traces are representative of at least three preparations.

2 μM, $70 \pm 5\%$, $n = 3$; Figure 2B, $95 \pm 5\%$, $n = 3$). The increase in fluorescence induced by the cathepsin C substrate and lysosomotropic peptide GPN ($75 \pm 3\%$, $n = 9$; Figures 2A and 2C) additionally confirmed the labelling of a lysosome-related organelle. Responses were non-additive (Figures 2A–2C).

In contrast to nigericin, the electrogenic protonophore, FCCP (2 μM), failed to evoke much of an alkalization (Figures 3A and 3C). However, a high concentration (20 μM) slowly increased the Acridine Orange signal (up to $74 \pm 11\%$ after approx. 45 min; $n = 2$; results not shown). However, this weak protonophore effect can be attributed to the membrane potential of the organelle, well known to limit the H⁺ movements facilitated by the electrogenic V-ATPase and by FCCP [23,24]. In naïve homogenate, the K⁺ ionophore valinomycin, presumably by dissipating the inhibitory membrane potential, promoted further vesicle acidification and a fall in Acridine Orange fluorescence (Figure 3B), which has been observed in other systems [24]. Subsequent addition of 2 μM FCCP now evoked a rapid, robust increase in fluorescence (Figure 3B), which was significantly larger and 45 ± 9 -fold faster than in the absence of valinomycin ($P < 0.05$; Figures 3A and 3C), and the combination was effective at alkalizing when added in either order (cf. Figure 3) [24]. We conclude that Acridine Orange detects pH_L changes from well-sealed acidic vesicles.

Role of V-ATPase in regulating pH_L in sea urchin egg

The proposed importance of V-ATPase activity for the NAADP Ca²⁺ response [6] prompted us to more directly investigate its role in regulating pH_L. To our surprise, a maximal concentration of the V-ATPase inhibitor, bafilomycin A1, had little effect upon resting Acridine Orange fluorescence over a period of 8–11 min

Figure 3 Valinomycin is required for an FCCP-induced alkalinization

FCCP ($2 \mu\text{M}$) and valinomycin ($10 \mu\text{M}$) were added to Acridine Orange-loaded homogenates where indicated (**A** and **B**). (**C**) Summarizes the fluorescence changes in (**A**) and (**B**) and shows the means $\pm$ S.E.M. for 7 or 8 experiments. Open bars refer to the protocol in (**A**) (FCCP addition first), whereas the closed bars refer to the protocol in (**B**) (FCCP added second).

Figure 4 Effect of bafilomycin A1 upon NAADP-evoked pH_i changes

Bafilomycin A1 ($1 \mu\text{M}$), NAADP ($1 \mu\text{M}$) and NH_4Cl (10 mM) were added to Acridine Orange-labelled homogenates where indicated (**A** and **B**). Traces are drawn on a common scale depicted in (**A**). (**C**) Summarizes the changes in fluorescence (ΔF) in (**A**) and (**B**), and are shown as the means $\pm$ S.E.M. of 9–12 determinations expressed as a percentage of the maximal NH_4Cl response. Baf Postinc, responses when bafilomycin A1 was added after NAADP; Baf Preinc, responses when bafilomycin A1 was added before NAADP.

(Figures 4B and 4C) when we had expected to observe the unmasking of an H^+ -leak pathway cf. [25,26]. Nonetheless, bafilomycin A1 was apparently active because it significantly enhanced the subsequent NAADP-induced response ($P < 0.001$; Figure 4B compared with Figure 4A), consistent with NAADP acting upon a bafilomycin A1-sensitive compartment [6]. Addition of bafilomycin A1 after NAADP (Figure 4A) had a marginally larger effect than bafilomycin A1 in resting homogenate ($P < 0.05$).

To understand why bafilomycin A1 had a minimal effect upon pH_i when added alone, we first hypothesized that the vesicular membrane potential acted as a brake upon passive H^+ efflux, as it had upon protonophore-induced efflux (Figure 3). Therefore, we added bafilomycin A1 after a 3 min treatment with valinomycin (Figure 5B). Reassuringly, bafilomycin A1 immediately arrested the valinomycin-induced acidification, confirming that this decrease was mediated by a V-ATPase, but, in spite of this block, there was still no substantial increase in fluorescence (Figures 5B and 5D) and the increase that there was only occurred at a slow rate (Figure 5E).

We therefore speculated that these acidic vesicles are very 'tight' and do not express a sizeable H^+ -leak pathway; we overcame this by introducing an exogenous, low-level leak pathway with a minimal amount of FCCP (100 nM). A 20-fold lower concentration than used in the experiments shown in Figure 3 had little effect upon Acridine Orange fluorescence in naïve homogenate (Figures 5A, 5D open bar and 5E open bar) or upon the valinomycin-evoked acidification rate (Figures 5C and 5E hatched bars; note that FCCP was added 3 min after valinomycin cf. bafilomycin A1). The latter result, in particular, confirmed that 100 nM FCCP is sufficiently low so as not to offset the V-ATPase.

It was only under these conditions (collapsed membrane potential with low-level H^+ leak) that bafilomycin A1 could now evoke a substantial alkalinization (Figures 5C–5E). This alkalinization was observed only when all three agents were added together, in which ever order (Figure 5), although the absolute kinetics varied somewhat. These experiments strongly suggest that naïve (basal) sea urchin egg acidic vesicles are very 'tight' with respect to H^+ . Moreover, the magnitude of the fluorescence increase confirmed that bafilomycin A1-sensitive acidic vesicles account for at least 80 % of the Acridine Orange labelling (Figure 5D) calculated as a percentage of the NH_4Cl response; Figure 5(A) illustrates the equivalence of the NH_4Cl response and the degree of Acridine Orange quenching upon uptake into acidic vesicles. This also held for Figures 5(B) and 5(C) and results not shown, such that NH_4Cl attained $104 \pm 0\%$, $108 \pm 1\%$ and $107 \pm 2\%$ of the initial Acridine Orange fluorescence respectively for Figures 5(A–C).

Properties of NAADP-induced pH_i changes

To characterize more thoroughly the NAADP pH_i response, we investigated its concentration-response relationship. Increasing the concentration of NAADP resulted in a graded increase in fluorescence (Figures 6A and 6B). The relatively small signal-to-noise ratio precluded an exact determination of the EC_{50} , but an estimate of $10\text{--}30 \text{ nM}$ agrees well with the EC_{50} of NAADP for Ca^{2+} release in sea urchin egg homogenates [27,28].

In sea urchin egg, NAADP-induced Ca^{2+} release shows a profound self-inactivation when preincubated with low NAADP concentrations [4], so we next assessed whether Acridine Orange changes also exhibited this phenomenon. In parallel, we first

Figure 5 Effect of membrane potential, H^+ leak and V-ATPase activity on pH_L

(A, B and C) Fluorescence traces of homogenates labelled with $10 \mu M$ Acridine Orange (AO). Addition of AO, $100 nM$ FCCP (F), $1 \mu M$ bafilomycin A1 (B or Baf), $10 \mu M$ valinomycin (V or Val) and $10 mM$ NH_4Cl were effected where shown. The bar charts show means $\pm$ S.E.M. for 8–10 experiments for the amplitudes (D) and initial kinetics (E) of the respective responses. Amplitudes are expressed as the change in fluorescence (ΔF) as a percentage of the maximum change with NH_4Cl and rates as fluorescence units/s (U/s).

confirmed that application of $5 nM$ NAADP for 5–10 min inhibited the Ca^{2+} response elicited by $1 \mu M$ NAADP in the same preparations (control: $197 \pm 12 nM$; desensitized: $53 \pm 10 nM$; $P < 0.001$; $n = 4-5$; note the small response in the desensitized homogenate reflects Ca^{2+} contamination of the NAADP and therefore underestimates the degree of inactivation). Accordingly, $5 nM$ NAADP elicited a very small response itself, but profoundly reduced that to a subsequent addition of $1 \mu M$ NAADP when compared with the control (Figures 6C–6E). This strongly suggests that NAADP is affecting pH_L changes via activation of the NAADP receptor, for which self-inactivation is diagnostic.

NAADP-induced pH_L responses and transmembrane ion movements

To go some way towards examining the role of ion fluxes across the vesicle membrane during NAADP-induced pH_L changes, we investigated the effect of several ionophore and bafilomycin A1 combinations (Table 1). Note that the inevitable non-linearity of the Acridine Orange signal means that one must exercise caution in comparing the absolute fluorescence changes when NAADP is added upon an elevated baseline (i.e. an apparent decrease in the Acridine Orange change with NAADP does not necessarily reflect a real decrease in the alkalization response).

NAADP still elicited a response in the presence of a high concentration of FCCP, albeit slightly reduced, and whilst valinomycin alone had no effect upon the magnitude of the NAADP response (Table 1; $P > 0.9$), it did enhance the kinetics (time-to-peak: NAADP alone, $179 \pm 29 s$; NAADP plus valinomycin, $72 \pm 7 s$; $P < 0.01$). When valinomycin was included with FCCP, the subsequent NAADP response was essentially the same as with FCCP alone (with no effect upon kinetics; results not shown). One possible explanation for these results is that NAADP predominantly alters pH_L by a mechanism other than by changing

H^+ leaks or K^+ fluxes. By contrast, bafilomycin A1 enhanced the NAADP response (Figure 4 and Table 1) and even more so in the presence of valinomycin and FCCP (conditions where V-ATPase activity is enhanced; Figure 3). These results confirm that the bafilomycin A1-sensitive V-ATPase is a major player in modulating dynamic pH_L changes, but is unlikely to be a target for NAADP.

Cytosolic Ca^{2+} and pH_L changes

We next asked whether NAADP-induced changes in pH_L were related to a direct effect of NAADP upon vesicular Ca^{2+} fluxes, e.g. a fall in the luminal $[Ca^{2+}]$ [29] and/or an increase in Ca^{2+} at the cytosolic face [30] when Ca^{2+} is released from the acidic store. As a first step, we elevated the extravesicular $[Ca^{2+}]$ (i.e. the cytosolic face) in different ways, initially by comparing NAADP with other Ca^{2+} -releasing messengers that release Ca^{2+} from mostly neutral stores [5,6]. In spite of the fact that the Ca^{2+} responses to messengers were not significantly different when measured in parallel $\{\Delta[Ca^{2+}]$ (% NAADP): cADPR, 64 ± 16 ; IP_3 , 74 ± 23 ; $P > 0.1$; ANOVA; $n = 3\}$, neither IP_3 nor cADPR could mimic the alkalization induced by NAADP ($P < 0.001$; Figures 7A, 7B and 7E) and, indeed, there was no significant difference between the vehicle, cADPR and IP_3 responses ($P > 0.05$).

We also released Ca^{2+} from the ER in other ways. First, the Ca^{2+} ionophore, ionomycin (which preferentially releases Ca^{2+} from neutral but not acidic stores [31]), which evoked a substantial Ca^{2+} response in homogenate that was $203 \pm 10\%$ ($n = 6$) of the corresponding NAADP response; however, this robust increase in extravesicular Ca^{2+} did not translate into a substantial pH_L response (Figures 7C and 7E; $P < 0.001$ compared with NAADP), and was not significantly different from IP_3 or cADPR ($P > 0.05$), whereas a subsequent addition of NAADP evoked the characteristic alkalization ($12 \pm 1\%$ of NH_4Cl , $P < 0.001$

Figure 6 Characterization of NAADP receptor-mediated pH_L responses

(A) Traces of Acridine Orange fluorescence in homogenates illustrating the relationship between pH_L and NAADP concentration (10, 30, 100 and 1000 nM). (B) Summarizes the data in (A), showing the means $\pm$ S.E.M. of 7 or 8 experiments normalized to the final 10 mM NH_4Cl response. To investigate the effect of self-inactivation, traces depict typical Acridine Orange responses to 1 μ M NAADP alone (C) or after desensitisation by incubation with 5 nM NAADP (D) on a common time scale (see bar) and normalized to the maximal and minimum responses. (E) Shows amplitudes (means $\pm$ S.E.M.) of responses to 1 μ M NAADP alone (Ctrl, open bar), 5 nM NAADP alone (filled bar, Desens), and 1 μ M NAADP subsequent to 5 nM NAADP (filled bar, Desens); results are expressed as a percentage of the final 10 mM NH_4Cl response for 7–12 experiments.

Table 1 Effect of ionophores and bafilomycin A1 upon NAADP-induced pH_L responses

Acridine Orange-loaded homogenate was stimulated by 1 μ M NAADP, either after no treatment (control) or after preincubation with the indicated agents: 20 μ M FCCP, 1 μ M bafilomycin A1 (Baf), 10 μ M valinomycin alone (Val), 10 μ M valinomycin plus 2 μ M FCCP, or 10 μ M valinomycin plus 2 μ M FCCP plus bafilomycin A1. Results are normalised to NAADP control responses and expressed as means $\pm$ S.E.M. for the number of replications given in parentheses. * $P < 0.02$, *** $P < 0.001$ compared with NAADP controls. Elevated baseline values when NAADP was added were $35 \pm 5\%$ (20 μ M FCCP) and $45 \pm 4\%$ (Val + FCCP), when expressed as a percentage of the maximal NH_4Cl . The relative baseline was not determined for all three agents simultaneously as preincubation was carried out 'offline' (but see Figure 5).

Preincubation	NAADP response (fold control)
FCCP	0.71 ± 0.09 (9)***
Baf	1.52 ± 0.09 (16)***
Val	1.03 ± 0.19 (8)
Val + FCCP	0.69 ± 0.07 (13)*
Val + FCCP + Baf	1.92 ± 0.10 (11)***

compared with ionomycin; Figure 7C). Secondly, the SERCA inhibitor, thapsigargin, did not significantly alter Acridine Orange fluorescence over 5–10 min ($P > 0.05$; Figures 7D and 7E), nor did it affect a subsequent NAADP-induced response (NAADP response, % NH_4Cl : control, 16 ± 1 ; plus thapsigargin, 18 ± 2 ; $P > 0.4$). That is, Ca^{2+} released from the ER is unlikely to mimic or substantially contribute to the pH_L changes evoked by NAADP.

Secondly, we increased extravesicular $[Ca^{2+}]$ directly, independently of release from stores, by bolus addition of Ca^{2+} to the cuvette and again measured $[Ca^{2+}]$ and pH_L changes in parallel (cf. Ca^{2+} addition to mitochondria [32]). As expected, substantial buffering by the homogenate occurred since addition of micromolar concentrations of Ca^{2+} corresponded to an increase in the free, extra-vesicular $[Ca^{2+}]$ which was several orders of magnitude lower (Figure 8A). Equivalent cumulative additions of Ca^{2+} produced a step-wise increase in pH_L that was at least partially ($> 40\%$) reversed by chelating Ca^{2+} with EGTA (Figure 8B); note that the medium pH was demonstrably unaffected by EGTA addition under these conditions (results not shown). However, when the relationship was plotted between the empirically-determined $\Delta[Ca^{2+}]_{free}$ and the ΔF of Acridine Orange, the NAADP response did not lie on this curve (Figure 8C). In other words, although increases in extravesicular Ca^{2+} are capable of driving alkalinization, they are unlikely to underlie the NAADP pH_L response as its Ca^{2+} response is too small. We proved this directly by testing whether NAADP-induced pH_L responses could occur when extravesicular Ca^{2+} was clamped with a Ca^{2+} chelator. The addition of 5 mM EGTA produced a prompt acidification (consistent with inhibition of Ca^{2+}/H^+ exchange) and subsequent addition of NAADP induced a similar alkalinization as controls without EGTA (Figure 8D; % control, $108 \pm 7\%$; $n = 13$, $P > 0.5$). It should be noted that EGTA did not actually affect the kinetics (results not shown). Taken together, the results suggest that NAADP alters pH_L independent of an increase in cytosolic

Figure 7 Ca^{2+} release from the ER does not mimic NAADP-induced pH_L responses

pH_L was measured in Acridine Orange-labelled homogenates in response to application of 10 μM cADPR (A), 4 μM IP₃ (B), 5 μM ionomycin (Iono) (C) or 1 μM thapsigargin (Tg) (D). NAADP was subsequently applied at 1 μM (C and D). (E) Shows the means $\pm$ S.E.M. of responses to the agents alone as indicated; all observations were normalized to 10 mM NH₄Cl for 5–28 experiments.

Ca^{2+} and is due to a direct effect of NAADP upon the vesicle itself (e.g. via a fall in the luminal $[\text{Ca}^{2+}]$).

We next verified that the changes we were seeing in homogenate were relevant for the intact egg. To that end, we labelled eggs with Acridine Orange in order to measure pH_L changes as reported previously [21] and then microinjected the centre of the egg with or without NAADP (approx. 1 μM intracellular concentration). Whilst control injections failed to alter Acridine Orange fluorescence ($\Delta F/F_0$, 0.00 ± 0.01 ; $n = 13$; $P > 0.6$), NAADP injection elicited a rapid, robust alkalinization ($\Delta F/F_0$, 0.25 ± 0.03 ; $n = 16$; $P < 0.001$; $P < 0.001$ compared with the control). However, it should be noted that the NAADP response was not homogeneous, being greater in the egg periphery, in contrast with 10 mM NH₄Cl, which enhanced fluorescence globally (results not shown; cf. [21]). Nonetheless, it confirms that the NAADP responses we observe in homogenates are present in the intact egg.

DISCUSSION

Properties of pH_L -sensitive dyes

Both Acridine Orange and LysosensorTM Green DND-189 were used to monitor pH_L changes in sea urchin egg homogenates, and both gave increases in fluorescence upon alkalinization. While this was to be expected for Acridine Orange [21], we anticipated that LysosensorTM Green DND-189 would undergo a reduction in signal as its fluorescence is weaker at alkaline pH. This paradox can be explained by the hitherto unknown

fact that LysosensorTM Green DND-189 also self-quenches at micromolar concentrations *in vitro*, levels which could easily be exceeded *in situ* with net accumulation (although we have no firm idea of the luminal concentration of LysosensorTM Green DND-189 or Acridine Orange). Hence, we propose that, as with Acridine Orange, the alkalinization of acidic organelles reduces the accumulation of LysosensorTM Green DND-189, and the loss of some of the dye from the lumen results in the partial relief of self-quenching from that remaining, and an increase in fluorescence ensues under our experimental conditions. We estimated that bafilomycin A1-sensitive vesicles account for at least 80 % of the Acridine Orange labelling, although this is likely to be a low estimate owing to the incomplete alkalinization even over long time periods, as well as the inevitable non-linearity of the Acridine Orange signal. Overall, we confirm that the dyes are a reliable index of pH_L in sea urchin egg acidic stores [21].

NAADP-induced changes in pH_L

NAADP-sensitive stores are physically separated from those responding to other messengers [8] and are acidic organelles in a number of systems [5,7,12,18] including sea urchin egg, where they probably correspond to the yolk platelets [6]. However, the notion of an acidic NAADP-sensitive store has been challenged [9–11], although subsequently partially resolved in exocrine pancreas [12]. Since Ca^{2+} release from acidic vesicles (e.g. secretory granules [33,34]) has repercussions for pH_L [29], we reasoned that NAADP might affect pH_L of acidic Ca^{2+} stores in sea urchin egg homogenates. This preparation has not only been instrumental in the discovery and characterization of NAADP

Figure 8 Relationship between extravesicular [Ca²⁺] and pH_L

(A) Sequential boluses of 1–100 μM Ca²⁺ were applied directly to homogenates in the cuvette and extravesicular [Ca²⁺] changes were measured with Fluo-3. (B) Corresponding pH_L trace measured in homogenates labelled with Acridine Orange in response to incremental Ca²⁺, 5 mM EGTA and 10 mM NH₄Cl. (C) Relationship between [Ca²⁺] changes determined with Fluo-3 in (A) and corresponding pH_L changes measured with Acridine Orange in (B). Open circles (○) represent bolus additions of Ca²⁺, the grey triangle those associated with 1 μM NAADP in the same preparation. Results are means ± S.E.M. for 8 (NAADP) and 31 experiments (Ca²⁺). (D) clamping cytosolic Ca²⁺ with 5 mM EGTA produces a fall in pH_L, but does not block the alkalinization induced by 1 μM NAADP (*n* = 13). (B and D) Inset panels show hypothetical Ca²⁺ and H⁺ movements for orientation.

itself [35], but also proven to be an experimentally ‘cleaner’ system, since the normal coupling between Ca²⁺-releasing second messengers [36] i.e. ‘channel chatter’ [37], is disrupted upon homogenization.

Using two fluorescent dyes in egg homogenates, we have revealed that the luminal pH of NAADP-sensitive acidic Ca²⁺-storing organelles is coupled to Ca²⁺ release as a prompt alkalinization. The pH_L response bore all the hallmarks of activation of the NAADP receptor as judged by a number of criteria: the nucleotide selectivity, the EC₅₀ for NAADP (10–30 nM) and the desensitization protocol, which inhibited both Ca²⁺ release and the pH_L increase. This is the first demonstration of NAADP altering pH_L and also reveals that desensitization is not driven by pH_L changes as 5 nM NAADP barely altered pH_L. The fact that an increase in Acridine Orange fluorescence is observed in intact eggs upon NAADP microinjection confirms that the alkalinization is not an artefact of homogenization.

H⁺ pumps and leaks

The pH_L of vesicles is strongly influenced by the balance between H⁺ pumping into and H⁺ efflux (leaks) from them [23]. Experiments with ionophores were instructive because they revealed that acidic vesicles in our preparation were not very leaky with respect to H⁺ owing to both an inhibitory membrane potential as well as a negligible H⁺ leak pathway (known to decrease during secretory/acidic vesicle maturation [23]). Indeed, the very fact that FCCP by itself causes little change in pH_L also implies that

the movement of other possible counter ions is negligible in these ‘tight’ vesicles.

Because passive H⁺ efflux contributed little to the resting pH_L, bafilomycin A1 treatment alone did not substantially alter pH_L. The same cannot be said under stimulated conditions when V-ATPase inhibition actually enhanced the NAADP pH_L response, from which we can draw several conclusions: first, this offers further support for V-ATPase expression on NAADP-sensitive stores [6]; secondly, it eliminates the V-ATPase as a target for NAADP action; thirdly, it is consistent with the V-ATPase acting as a modulator of the NAADP-induced response such that alleviating the acidifying brake increases the alkalinization response.

Our previous results in sea urchin eggs [6] revealed that NAADP-sensitive stores did not readily leak Ca²⁺ upon bafilomycin A1 application, which apparently differs from mammalian cells [5,7,18]. In the light of the results of the present paper, we would suggest that the primary reason for this discrepancy is that the pH_L gradient in sea urchin egg acidic stores does not readily collapse upon bafilomycin A1 treatment (although this clearly does not exclude an additional Ca²⁺ leak).

NAADP and transmembrane ion movements

In view of the tightness of these vesicles, it is interesting that NAADP induces an alkalinization and raises the question of whether NAADP alters H⁺ leakiness and/or the vesicle membrane potential (as it does in the starfish oolemma [38]). The majority

of our results are not easily reconciled with NAADP increasing a H^+ leak: (a) the NAADP response largely persists in the presence of 20 μ M FCCP, which already confers a substantial H^+ leak; and (b) even when the H^+ leak is facilitated by valinomycin, the magnitude of the NAADP response is not changed considerably, even when additionally combined with FCCP. The only piece of data consistent with an H^+ efflux is the enhancement of the NAADP response kinetics by valinomycin alone. In summary, we suggest that most of our results do not support H^+ efflux as a major mechanism, but any that does occur is likely to be secondary to other transport processes.

If NAADP was only stimulating a Ca^{2+} efflux from the acidic stores, this cation efflux would serve to dissipate the membrane potential that inhibits the V-ATPase and promote acidification (cf. valinomycin), which is the opposite of what we observe. By the same token, the NAADP-induced efflux of cations other than Ca^{2+} (e.g. K^+) would also be expected to acidify and so can also be discounted. Therefore, NAADP is working via some other additional (or adjunct) mechanism to alter pH_L .

Overall, our data are most consistent with the electrogenic V-ATPase establishing a membrane potential in the NAADP-sensitive acidic Ca^{2+} store, inside positive as observed in other acidic vesicles [39,40]. However, until we have a better idea of ion transporters and the ion concentrations on either side of the acidic store membrane, this remains speculative. In the context of NAADP, it is interesting that K^+ channel blockers inhibit NICR [22], either as a result of their effect upon membrane potential or, indeed, as a consequence of their being ammonium analogues, which could directly affect pH_L .

Cytosolic Ca^{2+} and pH_L

We then undertook a basic characterization of how Ca^{2+} movements interact with the NAADP-induced pH_L response. Our understanding of how pH_L is regulated by Ca^{2+} in egg acidic vesicles is minimal compared with that of secretory vesicles, which are prototypic acidic Ca^{2+} stores [5,12,29,30], and were first characterised in terms of their IP_3 -dependent responses [29,41]. The influence of counterions and membrane potential [23,42,43] adds to the complexity, but we chose to focus on how Ca^{2+} movements affect pH_L .

As already alluded to, one direct model describes how messenger-induced Ca^{2+} release results in a fall in luminal $[Ca^{2+}]$, thereby producing a decrease in the free $[H^+]$ (alkalinization) as a consequence of their competitive binding to a sequestering, polyanionic matrix [29,41]. In an alternative indirect model, increases in cytosolic (extravesicular) $[Ca^{2+}]$ lead to alkalinization because of Ca^{2+} uptake via a putative Ca^{2+}/H^+ exchanger in the vesicle membrane [6,30]. We tested which of these models better applies to NAADP-induced pH_L changes.

To mimic NICR, increases in extravesicular (cytosolic) Ca^{2+} were generated in two ways, either by release from non-acidic Ca^{2+} stores, or by direct addition of Ca^{2+} to the cuvette. Ca^{2+} release induced by the other second messengers, IP_3 or cADPR, failed to mimic NAADP despite the Ca^{2+} responses to all three being similar. This profile mirrors their organellar selectivity in sea urchin egg [6] and other systems ([5,7,18], but see [12]) with IP_3 and cADPR releasing from non-acidic ER. Similarly, ionomycin gave a larger Ca^{2+} signal than NAADP, but still had no effect upon pH_L . Under these conditions, ionomycin will only release Ca^{2+} from neutral stores such as the ER because the low pH_L of the acidic stores blocks the ionophore Ca^{2+}/H^+ exchange activity [31,44–47]. Finally, the SERCA inhibitor, thapsigargin, did not significantly alter pH_L or block the NAADP-induced pH_L response (which mirrors the thapsigargin-insensitivity of NICR [4]). We

conclude that Ca^{2+} release from the ER itself does not result in pH_L changes nor is it likely to be the driving force underlying the NAADP-induced alkalinization.

Manipulating cytosolic Ca^{2+} independently of release from the ER involved additions of Ca^{2+} or EGTA to the cuvette. Cumulative increases in the $[Ca^{2+}]$ corresponded to cumulative increases in pH_L , plausibly via a Ca^{2+}/H^+ exchanger. However, the affinity of this exchanger for Ca^{2+} was too low to mediate the NAADP pH_L response whose corresponding global Ca^{2+} signal was 8–10-fold too low. One criticism of this conclusion is that the local $[Ca^{2+}]$ induced by NAADP might reach levels high enough to be relevant, similar to the ER–mitochondrion interface [32]. This was not the case, however, since EGTA did not affect the NAADP-induced alkalinization, even though 5 mM would be more than sufficient to swamp the Ca^{2+} concentration (1–2 μ M) required to mimic the NAADP pH_L response. One puzzling result was that the addition of EGTA after the bolus addition of Ca^{2+} only resulted in an incomplete reversal to basal Acridine Orange fluorescence when the predicted $[Ca^{2+}]$ would be in the nanomolar range. We have no definitive explanation for this result, but it is conceivable that the previous Ca^{2+} exposure affected H^+ movements (or membrane potential), since acidic vesicle transporters are known to be affected by cytosolic Ca^{2+} [48], luminal Ca^{2+} loading [49] or pH_L [15,49]. Taken together, these data confirmed that the NAADP pH_L response was independent of cytosolic Ca^{2+} .

If an increase in cytosolic Ca^{2+} does not drive alkalinization, then we are left with some variant of the direct model where NAADP binds to, and modulates the pH_L of, the target organelle. One possibility is that the fall in the luminal $[Ca^{2+}]$ allows H^+ to bind to vacated sites on a luminal anionic matrix, in much the same way as has been suggested for IP_3 -dependent acidic stores [29,41]. We have attempted to test this directly by lowering the luminal $[Ca^{2+}]$ with membrane-permeant Ca^{2+} chelators such as TPEN and oxalate, and while we did observe a robust alkalinization consistent with our hypothesis, we could not unequivocally exclude an artefact of their own acid/base properties (results not shown).

Our data show that NAADP alters pH_L in its own acidic Ca^{2+} stores, independently of cytosolic Ca^{2+} changes. This provides further evidence in support of the somewhat controversial idea that acidic Ca^{2+} stores are unique targets for NAADP since IP_3 and cADPR could not mimic the alkalinization. The ramifications of an increased pH_L in acidic stores is unknown at present, but in terms of NAADP Ca^{2+} store function, an alkalinization might reduce Ca^{2+} reloading and prolong the refractory period to NAADP. Sea urchin eggs have proven an excellent model for higher organisms [4], and our observations may well have implications for mammalian cells which utilize NAADP-sensitive acidic vesicles, e.g. alkalinization of secretory vesicles in pancreatic acinar cells [12] could alter their exocytotic threshold [50], while lysosomal pH in smooth muscle cells [7] might affect proteolysis [25].

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