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The luminal Ca^{2+} chelator, TPEN, inhibits NAADP-induced Ca^{2+} release

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ABSTRACT

The regulation of Ca^{2+} release by luminal Ca^{2+} has been well studied for the ryanodine and IP_3 receptors but has been less clear for the NAADP-regulated channel. In view of conflicting reports, we have re-examined the issue by manipulating luminal Ca^{2+} with the membrane-permeant, low affinity Ca^{2+} buffer, TPEN, and monitoring NAADP-induced Ca^{2+} release in sea urchin egg homogenate. NAADP-induced Ca^{2+} release was almost entirely blocked by TPEN (IC_{50} 17–25 μM) which suppressed the maximal extent of Ca^{2+} release without altering NAADP sensitivity. In contrast, Ca^{2+} release via IP_3 receptors was 3- to 30-fold less sensitive to TPEN whereas that evoked by ionomycin was essentially unaffected. The effect of TPEN on NAADP-induced Ca^{2+} release was not due to an increase in the luminal pH or chelation of trace metals since it could not be mimicked by NH_4Cl or phenanthroline. The fact that TPEN had no effect upon ionophore-induced Ca^{2+} release also argued against a substantial reduction in the driving force for Ca^{2+} efflux. We propose that, in the sea urchin egg, luminal Ca^{2+} is important for gating native NAADP-regulated two-pore channels.

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1. Introduction

Ca^{2+} release channels on intracellular stores are not only subject to regulation by second messengers but also by additional factors that include accessory proteins, pH and phosphorylation [1–5]. Of primary importance is the exquisite regulation of the channels by Ca^{2+} itself, a feedback that is essential for generating the hierarchy of Ca^{2+} signals such as local release events, Ca^{2+} oscillations or Ca^{2+} waves [6]. This feedback is multifaceted and is not restricted to one site on a given channel: Ca^{2+} can stimulate or inhibit channel activity since there can be multiple Ca^{2+}-binding sites on the channel complex, some on the cytosolic face, others on the luminal face.

Cytosolic Ca^{2+} is well accepted to stimulate or inhibit inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs) and ryanodine receptors (RyR) as it follows a bell-shaped concentration response curve [5,6]. By contrast, the NAADP (nicotinic acid adenine dinucleotide phosphate) receptor has hitherto been reported to be insensitive to cytosolic Ca^{2+} (or surrogate ions) [1,7–9] and therefore local, "trigger" Ca^{2+} released by NAADP is necessarily amplified by proximal IP_3Rs or RyRs which are Ca^{2+}-sensitive [10,11].

Whether these Ca^{2+}-release channel families are regulated by Ca^{2+} within the lumen of the stores themselves is more controversial [12]. For IP_3Rs and RyRs, higher luminal Ca^{2+} concentrations promote channel opening, possibly via intermediate luminal Ca^{2+}-binding proteins [5,6,12,13]. However, NAADP-regulated channels were initially reported to be insensitive to luminal Ca^{2+} [9]. More recently, mammalian members of the TPC (two-pore channel) family – the newly discovered target of NAADP [14–16] – exhibited sensitivity to luminal Ca^{2+} whereby increasing luminal Ca^{2+} concentrations enhanced channel activity in lipid bilayers [17,18]. Although plant TPC has not yet been shown to be modulated by NAADP, the channel is also influenced by luminal Ca^{2+}, albeit in an inhibitory manner [19].

In view of the potential confusion surrounding these disparate results, we have re-examined the role of luminal Ca^{2+} in regulating NAADP responses in sea urchin egg, a system in which TPCs are channels regulated by NAADP [20,21] possibly via smaller accessory proteins that are the NAADP-binding moieties [22,23]. By using a membrane-permeant Ca^{2+} chelator, TPEN (N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine), we manipulated the luminal Ca^{2+}, an approach taken previously in other systems for IP_3Rs [13] and RyRs [24], and our data are consistent with a role for luminal Ca^{2+} in NAADP-regulated channel gating.

2. Methods

2.1. Homogenate preparation

Sea urchin egg homogenate was prepared as detailed [25]. Eggs from *Lytechinus pictus* were harvested by intracoelomic

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injection of 0.5 M KCl, collected in artificial sea water (ASW (mM): 435 NaCl; 40 $MgCl_2$; 15 $MgSO_4$; 11 $CaCl_2$; 10 KCl; 2.5 $NaHCO_3$; 20 Tris base, pH 8.0) and de-jellied by passage through 100-μm nylon mesh (Millipore). Eggs were then washed four times in Ca^{2+}-free ASW (the first two washes containing 1 mM EGTA) and then washed in intracellular-like medium (GluIM (mM): 250 potassium gluconate; 250 N-methylglucamine (NMDG); 20 HEPES and 1 $MgCl_2$, pH 7.2). Homogenization was effected with a glass Dounce tissue homogenizer in ice-cold GluIM supplemented with 2 mM MgATP; 20 U/ml creatine phosphokinase; 20 mM phosphocreatine; Complete™ EDTA-free Protease Inhibitor tablets (Roche). Homogenate (50%, v/v) was centrifuged at 13,000 × *g*, 4 °C for 10 s and the supernatant stored at −80 °C. On the day of use, an aliquot of homogenate was sequentially diluted in equal volumes of GluIM containing the ATP regenerating system over a period of 3 h at 17 °C to give a 2.5% (v/v) final concentration.

2.2. Fluorimetry

2.2.1. Ca^{2+} release

All fluorimetry was conducted at 17 °C in a microcuvette containing a magnetic stir bar mounted in a Perkin Elmer LS-50B fluorimeter. Ca^{2+} release was measured in homogenate with 3 μM fluo-3 (excitation/emission: 506/526 nm) which was calibrated using the standard equation $[Ca^{2+}] = K_d \times (F - F_{min})/(F_{max} - F)$, using a K_d of 0.4 μM; F_{min} and F_{max} were determined by addition of 0.5 mM EGTA and 10 mM Ca^{2+} respectively at the end of each run. TPEN (dissolved in ethanol) had no effect upon dye calibration (F_{min} and F_{max} values were 98 ± 5% and 101 ± 3% of ethanol controls respectively; $n = 9$, $P > 0.5$ paired *t* test). The upstroke kinetics were determined by linear regression of the raw fluorescence (in units (U)/s) normalized to the resting fluorescence (F_0) to account for machine variability and therefore expressed as units•F_0/s (U•F_0/s).

2.2.2. Acidic vesicle pH

We monitored luminal pH (pH_L) as before [26]. 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 (acquisition wavelengths were the same as for fluo-3). An increase in fluorescence represents an increase in pH_L. Data were expressed as a percentage of the maximum minus minimum fluorescence (the maximum was defined as the fluorescence after addition of 10 mM NH_4Cl at the end of the run; this was equivalent to the pre-quench acridine orange fluorescence at the beginning of the recording [26]).

2.3. Data analysis and source of reagents

Representative traces are plotted as raw fluorescence (relative fluorescence units, RFU) against time. Data are expressed as the mean ± SEM. Two data sets were compared using Student's *t* test, whereas multiple groups were analysed using ANOVA and a Tukey–Kramer or Dunnett's post-test. Data were paired where appropriate and significance assumed at $P < 0.05$. Graphs were annotated using the following conventions: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***). Curve fitting was conducted using Graphpad Prism.

NAADP was enzymatically synthesised [25] or purchased from Sigma–Aldrich (Poole, Dorset, UK). IP_3 was from LC Laboratories (Woburn, MA, USA). Acridine orange and fluo-3 (K^+ salt) were from Invitrogen (Paisley, UK). Nigericin, TPEN, phenanthroline and potassium oxalate were from Sigma–Aldrich whilst ionomycin (free acid) was from Calbiochem-Novabiochem (Merck Biosciences, Nottingham, UK). All other reagents were of analytical grade.

3. Results

3.1. NAADP-induced Ca^{2+} release

NAADP-induced Ca^{2+} release (NICR) in sea urchin egg homogenate was monitored fluorescently in the presence of ethanol vehicle or the membrane-permeant, low affinity (40–130 μM [27,28]) Ca^{2+} chelator, TPEN. TPEN crosses membranes rapidly to lower luminal free Ca^{2+} [28] and a 2-min preincubation with TPEN produced a substantial concentration-dependent inhibition of NICR, both in terms of amplitude and kinetics (IC_{50} of 17 and 27 μM respectively; Fig. 1A–C). This did not reflect a general perturbation by TPEN of Ca^{2+} release (or the assay) because the response to the Ca^{2+} ionophore, ionomycin, was essentially unaffected (Fig. 1A–C), as was the Ca^{2+} dye calibration (see Section 2). By varying the NAADP concentration (Fig. 1D–F), we found that the major effect of TPEN was to reduce the maximal extent of NICR without substantially altering the affinity of the receptors for NAADP (EC_{50} (95% confidence intervals) – amplitude: ethanol 32 nM (9–113 nM), TPEN 56 nM (7–47 nM); kinetics: ethanol 79 nM (17–370 nM), TPEN 218 nM (33–1460 nM)).

3.2. IP_3-induced Ca^{2+} release

To ascertain whether the effect of TPEN was unique to NICR, we examined the effect of TPEN upon another channel regulated by luminal Ca^{2+}, the IP_3 receptor. Similar to NICR, IP_3-induced Ca^{2+} release was also inhibited by TPEN but a major difference was that it required 3- to 30-fold higher concentrations of TPEN (estimated IC_{50} of 79 μM and 536 μM for kinetics and amplitudes respectively; Fig. 2A–C). Once again, these higher TPEN concentrations were essentially without effect upon ionomycin-induced Ca^{2+} release (Fig. 2D–F). As with NAADP, a sub-maximal concentration of TPEN predominantly affected the IP_3 maximum and not the affinity, although the TPEN effect was more evident upon the kinetics than the amplitude (Fig. 2G–I): IP_3 (EC_{50} (95% confidence intervals) – amplitude: ethanol 217 nM (46–1028 nM), TPEN 143 nM (50–413 nM); kinetics: ethanol 1303 nM (450–3774 nM), TPEN 953 nM (11–8106 nM)). The data are consistent with TPEN altering ER channel gating by chelating luminal Ca^{2+} as it does in other systems [13,24] and verify that the inhibition by TPEN is not peculiar to the NAADP-regulated channel.

3.3. TPEN and other ions

Whilst IP_3 releases Ca^{2+} from the neutral ER, NAADP mobilizes Ca^{2+} from acidic Ca^{2+} stores which, in the sea urchin egg, appear to be the lysosome-related organelles, yolk platelets [29,30]. Since acidic Ca^{2+} store loading [11] and TPC channels [11,17,18,31] may be sensitive to luminal pH (pH_L), we investigated whether the inhibition by TPEN was due to changes in pH_L rather than Ca^{2+}. First, we monitored pH_L in NAADP-sensitive vesicles using acridine orange as reported previously [26]. TPEN did indeed raise pH_L slightly as judged by the increase in acridine orange fluorescence but with a lower potency than its effect upon NICR (estimated EC_{50} 222 μM; Fig. 3A and C). Although this 10-fold lower potency argued against pH_L as the factor underlying NICR inhibition, we directly tested whether an acute change in pH_L could modulate Ca^{2+} release by applying the base, NH_4Cl. As expected, NH_4Cl profoundly increased acridine orange fluorescence (pH_L) with an EC_{50} of ~1 mM (Fig. 3B and C) but, despite this, NH_4Cl had no major effect upon NICR (Fig. 3D and E) or subsequent ionomycin-induced Ca^{2+} release from neutral stores (Fig. 3D and F). This suggested that TPEN does not act

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