A MECHANISM OF INTRACELLULAR P2X RECEPTOR ACTIVATION

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Background: P2X receptors are localised both to the cell surface and within intracellular vacuoles. Mechanisms activating intracellular receptors are unclear.

Results: P2X receptor ATP binding site faces into the vacuole lumen. ATP translocation triggers P2X receptor dependent release of calcium.

Conclusion: Vacuolar P2X receptors are luminal ATP sensors releasing stored calcium in response to luminal ATP accumulation.

Significance: Intracellular P2X receptors are calcium release channels

Summary

P2X receptors (P2XRs) are ATP-activated calcium permeable cation channels directly activated by adenosine 5'-triphosphate (ATP). P2XRs are evolutionarily conserved between mammals, trematode, amoeba and single celled algae, yet absent in higher plants, yeast, fruit flies and nematodes. A traditional view of P2XRs is that of cell surface sensors for extracellular ATP, sensing ATP released in physiological processes including pain, inflammation, taste, mechanical stress and tissue necrosis. However, P2XRs also populate membranes of intracellular compartments in mammalian and other eukaryotic cells including lysosomes, phagosomes and the contractile vacuole (CV) of the amoeba Dictyostelium. The function of intracellular P2XRs is unclear and represents a major gap in our understanding of ATP signalling. Here, we exploit the genetic versatility of Dictyostelium to investigate the effects of physiological concentrations of ATP on calcium signalling in isolated CVs. Within the CV, an acidic calcium store, P2XRs are orientated to sense luminal ATP. Application of ATP to isolated vacuoles leads to luminal translocation of ATP and release of calcium. Mechanisms of luminal ATP translocation and ATP-evoked calcium release share common pharmacology suggesting they are linked processes. The ability of ATP to mobilise stored calcium is reduced in vacuoles isolated from P2XAR knockout amoeba and ablated in cells devoid of P2XRs. Pharmacological inhibition of luminal ATP translocation or depletion of CV calcium attenuates CV function in vivo, manifesting as a loss of regulatory cell volume decrease following osmotic swelling. We propose that intracellular P2XRs regulate vacuole activity by acting as calcium release channels, activated by translocation of ATP into the vacuole lumen.

P2X receptors (P2XRs) comprise a family of calcium permeable cation channels directly activated by adenosine 5'-triphosphate (ATP). P2XRs are evolutionarily conserved between mammals, trematode, amoeba and single celled algae, yet absent in higher plants, yeast, fruit flies and nematodes. A traditional view of P2XRs is that of cell surface sensors for extracellular ATP, sensing ATP released in physiological processes including pain, inflammation, taste, mechanical stress and tissue necrosis. However, P2XRs also populate membranes of intracellular compartments in mammalian and other eukaryotic cells including lysosomes, phagosomes and the contractile vacuole (CV) of the amoeba Dictyostelium. The function of intracellular P2XRs is unclear and represents a major gap in our understanding of ATP signalling. Here, we exploit the genetic versatility of Dictyostelium to investigate the effects of physiological concentrations of ATP on calcium signalling in isolated CVs. Within the CV, an acidic calcium store, P2XRs are orientated to sense luminal ATP. Application of ATP to isolated vacuoles leads to luminal translocation of ATP and release of calcium. Mechanisms of luminal ATP translocation and ATP-evoked calcium release share common pharmacology suggesting they are linked processes. The ability of ATP to mobilise stored calcium is reduced in vacuoles isolated from P2XAR knockout amoeba and ablated in cells devoid of P2XRs. Pharmacological inhibition of luminal ATP translocation or depletion of CV calcium attenuates CV function in vivo, manifesting as a loss of regulatory cell volume decrease following osmotic swelling. We propose that intracellular P2XRs regulate vacuole activity by acting as calcium release channels, activated by translocation of ATP into the vacuole lumen.
osmoregulatory organelle in protists (3,11). Genetic disruption of P2XRs in *Dictyostelium* causes an aberration in regulatory cell volume decrease (RCVD) following osmotic swelling (3,11), though the severity of this phenotype shows some strain variance. Genetic disruption of P2X_A in AX4 strain amoeba causes a loss of RCVD, described previously in our own study (3). However, disruption of all five receptors in AX2 strain amoeba causes a significant delay in RCVD but not a loss (11). The reason for this difference in phenotype between strains is unknown, though aberration in RCVD observed in both strains suggests P2XR knockout does impair CV function though the underlying mechanism is not described (3,11). Genetic variation in laboratory strains of *Dictyostelium* is widespread (12) and phenotypic differences between AX2 and AX4 strains are apparent from previous studies (13-15). Despite this difference, AX4 *Dictyostelium* remains the best genetically amenable model organism with which to investigate intracellular P2XR function and their mechanism of activation.

The CV is an acidic calcium store closely related to acidocalcisomes found in animal cells, possessing both bafilomycin-sensitive vacuolar proton pumps (V-H^+^-ATPase) and vanadate-sensitive Ca^{2+} pumps (Ca^{2+}-ATPase) (16). The CV is decorated with calcium-sensitive signalling proteins, including calmodulin (3,17), and there is some evidence that the CV participates in receptor-mediated calcium influx (18). Voiding of the CV is not via a conventional exocytosis, rather the CV membranes are recycled after emptying (19). During voiding the CV and plasma membrane are transiently connected by a channel permitting ejection of the CV content without exchange of plasma and CV membranes (19-21). This has important implications for potential mechanisms of intracellular P2XR activation in *Dictyostelium*, suggesting receptors are not exposed to the extracellular face of the cell and extracellular ATP is not a source of agonist. In addition, Ludlow et al (2009) provide some evidence that the postulated ATP binding site is orientated within the CV lumen and not the cytoplasm. We here use *Dictyostelium* as a model to test the hypothesis that intracellular P2XRs operate as calcium release channels and investigate how intracellular P2X receptors are activated.

### Experimental Procedures

**Cell culture and transformation** – AX4 strain amoebae were cultures in shaking flasks containing HL5 medium with glucose at 22°C. Amoeba were transformed with plasmids encoding P2X_A-GFP, dajumin-GFP (Professor Gunther Gerisch, Max-Plank Institute for Biochemistry) or calreticulin-GFP (Dictybase depository), and selected with G418. P2X_A knockout amoeba were generated as previously described (3) using a targeting vector conferring blasticidin resistance (Dr Steve Ennion, University of Leicester). Quintuple P2X receptor knockout AX2 strain was generously provided by Dr Steve Ennion.

**Fluorescence microscopy and protease protection assay** – Amoeba expressing a P2X_A C-terminal GFP fusion protein were seeded on glass and washed with phosphate buffered saline (PBS). Endosomes were labelled with TRITC-dextran (1mg/mL, 30mins; Invitrogen) and lysosomes labelled with Lysotracker Red DND-99 (500nM, 45mins; Invitrogen). When performing the protease protection assay, amoeba expressing either P2X_A-GFP or calreticulin-GFP were seeded on glass and washed with PBS containing 2mM MgCl_2. Selective permeabilisation of the plasma membrane was achieved by incubating cells with 20µM digitonin for 2mins at room temperature. Cells were washed with PBS then exposed to 5mM trypsin (22). Fluorescence intensity was captured at 15s intervals using a time-lapse IX71 Olympus microscope equipped with a Hamamatsu digital camera. Images were captured using Simple PCI software (Digital Pixel, UK).

**Isolation of vacuoles** – For isolation of calcium loaded vacuoles, 2.5x10^9 amoeba were sedimented, washed with PBS and resuspended in ice cold lysis buffer (mM): 125 sucrose, 50 KCl, 0.5 EDTA, 5 dithiothreitol and 20 HEPES, pH 7.2 and protease inhibitor cocktail. For isolation of calcium depleted vacuoles, amoebae were shaken in Sorenson’s phosphate buffer containing 5mM EGTA for 5hrs prior to sedimentation, washing and lysis. All cells were lysed by repetitive vortexing with glass beads (Sigma) followed by clarification of lysate at 1,500xg for 10mins at 4°C. Pellets were resuspended in lysis buffer and homogenised using a 22 gauge needle. The homogenate was
fractionated on a discontinuous iodixanol gradient (Optiprep, Sigma). The discontinuous gradient was centrifuged at 50,000xg for 1 hour at 4°C, followed by collection of 40 1ml fractions. Estimations of protein concentration in fractions and whole cell lysates were by Bradford assay.

**Estimation of fraction purity** - The enzymatic activity of marker enzymes and the distribution of GFP-tagged organelle markers were used to establish successful purification of intact contractile vacuole. Marker enzyme assays for acid phosphatase (AcP, lysosome), alkaline phosphatase (ALP, contractile vacuole) and succinate dehydrogenase (SDH, mitochondria) were carried out on all fractions. Reaction volumes were 250µl and initiated by addition of 12.5µl fraction. AcP and ALP reactions contained 5mM p-nitrophenol phosphate in 250mM glycine-HCl, pH3.0 for AcP assay or in (mM) 100 Tris, 100 NaCl, 1 MgCl₂, pH9.5 for ALP assay. Reactions were incubated at 25°C for 1 hour or 15 min for ALP and AcP assay, respectively, and terminated by addition of 1mL 1M NaOH. Liberation of p-nitrophenol was measured at 405nm. SDH assay reaction buffer contained (mM) 100 Na-phosphate, pH 9.5 and 20 Na-succinate and 0.6 nitro blue tetrazolium (NBT). Reactions were incubated at 25°C for 30mins and terminated by addition of 1mL 2% Triton X-100, was calculated in an effort to demonstrate the intactness of the contractile vacuole and other organelles.

**ATP translocation assay** - Fractions 1-3 were pooled and diluted 1:3 with transport buffer (50mM KCl, 2mM MgCl₂, 3% sucrose, 6µg/mL antimycin A, 6µg/ml oligomycin, 100µg/mL NaN₃ and 10mM HEPES, pH 7.2), centrifuged at 18,500xg for 30mins at 4°C and pellet resuspended in transport buffer. 1mL reactions contained 200µg/mL protein fraction and were incubated at 23°C for 15mins. Reactions were initiated by addition of 4mM ATP and quenched at stipulated time intervals by the addition of 1mL ice cold transport buffer. 100µl supernatant from the final spin was collected to estimate background ATP and pellet resuspended in 100µl transport buffer. Samples were briefly heated at 105°C for 2 mins immediately prior to assay. ATP was quantified using the luciferase-luciferin assay (Roche) and protein estimated by Bradford assay. Background ATP readings were subtracted from pellets and measurements at time zero subtracted from all time points. For experiments including ATP regeneration, 4µg/mL creatine kinase (CK) and 5mM creatine phosphate (CP) (Roche) was added and reactions initiated by the addition of ATP.

**Identification of vacuolar nucleotides** - Nucleotide extraction was performed following the methodology of Nedden et al (2009) (23) using fractions 1-3. 200µl fractions were diluted 1:3 with ice cold transport buffer and centrifuged at 18,500xg for 30mins at 4°C. Pellets were resuspended in either luciferase buffer for quantitation by the luciferase-luciferin assay (Roche) or in homogenisation buffer for HPLC analysis. Perchloric acid (5% final concentration) was added and thoroughly mixed, followed by centrifugation at 16,060xg for 2mins at 4°C. Supernatants were precipitated by addition of 400µl tri-n-octylamine in 1,1,2-trichlorotrifluoroethane (1:1 v/v) and incubating on ice for 10mins. After centrifugation at 12,100xg for 2mins at 4°C, organic extraction was repeated twice with the upper aqueous phase. Aqueous phase was mixed 1:1 with Buffer A and incubated on ice for 15 mins. For quantification of ATP, 10µl of nucleotide extract was added to 90µl luciferase assay buffer and 100µl luciferase reagent (Roche). Bioluminescence for measured in a Modulus luminometer (Turner Biosystems). For ion-pair reverse phase HPLC, the mobile phase consisted of Buffer A (mM: 65 K-phosphate, 39 K₃H-phosphate, 26 KH₂-phosphate and 4 tetrabutyllumonium hydrogen sulphate, pH6) and Buffer B (mM: 65 K-phosphate, 39 K₃H-phosphate, 26 KH₂-phosphate and 25% methanol, pH6). Buffers were prepared in deionised water and filtered through a 0.4µm filter. A Supercosil LC-18-T column (Sigma) was equilibrated with 10 column volumes of Buffer B and 30 column volumes of Buffer A at a flow rate of 1mL/min. Analytical samples were injected after two blank injections and compared to nucleotide standards.
Measurement of vacuole calcium release - For preparation of calcium depleted vacuoles, amoeba where cultured in PBS containing 5mM EGTA for 4 hours prior to fractionation. Fractions were diluted 1:3 with transport buffer, centrifuged at 18,500g for 30mins at 4°C and pellets resuspended in transport buffer. 200µg/mL protein fractions were assayed for calcium release in transport buffer contained 0.5µM fluo-3 (Invitrogen). 1.6mL reactions were incubated for 15 mins at 23°C in a quartz cuvette with constant mixing in a fluorescence spectrophotometer (Hitachi F-2000), 505nm excitation and 526nm emission. Reactions were initiated by injection of 2mM ATP. Competing nucleotides were added at 1mM.

Real-time measurement of cell size - Changes in cell size were monitored by right-angled light scattering using a Hitachi F-2000 spectrophotometer. 1x10⁶ cells/mL were exposed to hypotonic stress by complete replacement of HL5 medium with distilled water. Scattered light was collected at 600nm. For depletion of CV calcium, cells were preincubated in Sorenson’s medium containing 10mM EGTA for 4 hours prior to experimentation.

Statistical Analysis - Average results are expressed as the mean ± s.e.m from the number experiments indicated (minimum of 3 for all). Data are analysed by an unpaired two-tailed Student’s t-test to determine significant differences between data groups.

RESULTS

Intracellular P2XRs are orientated to sense changes in luminal ATP. We previously reported that P2Xₐ localises to the CV in Dictyostelium. However, whether this is an exclusive localisation or a dynamic association remains unclear. The nature of P2Xₐ localisation is important when considering its involvement in regulating CV function, as some proteins associate with both CV and endosomal compartments in Dictyostelium including V-H⁺-ATPase (20) and rab4-like GTPase (24). To this end, we examined the association of P2Xₐ-GFP with lysosomes and endosomes using fluorescent marker dyes. The use of GFP as a tag has been used extensively to localise proteins in Dictyostelium (3,11,19,21). P2Xₐ-GFP labelled both the bladder and tubular network of the CV, showing no overlap with lysotracker dye (lysosomes) or TRITC-dextran (endosome) labelled compartments (Fig. 1A). P2Xₐ remained associated with the CV network during both filling and voiding of the vacuole (Fig. 1B). A video of P2Xₐ-GFP movement is given as supplementary data. Cell surface P2XRs are orientated to sense extracellular ATP as the ATP-binding ectodomain faces outward (25), with the receptor N- and C-termini residing within the cytoplasm (Fig. 2A). We used two independent techniques to determine whether P2Xₐ is orientated to sense cytoplasmic or luminal ATP. First, we sought to determine the localisation of the receptor C-terminus by performing a fluorescence protease protection assay (22) with amoeba expressing a C-terminal GFP fusion of P2Xₐ (3). P2Xₐ-GFP or calreticulin-GFP (a membrane bound ER marker) expressing cells were briefly exposed to digitonin to enable selective permeabilisation of the plasma membrane, followed by addition of trypsin. Calreticulin-GFP fluorescence showed no significant depreciation upon trypsin addition (Fig. 2B). In stark contrast P2Xₐ-GFP fluorescence decayed rapidly (Fig. 2C). Calreticulin-GFP became trypsin sensitive only after prolonged permeabilisation (data not shown). The susceptibility of the P2Xₐ receptor C-terminus to proteolytic degradation by trypsin demonstrates that the C-terminus resides within the cytoplasm and is not membrane bound within the cell. Second, the P2Xₐ receptor has 6 predicted glycan acceptor sites (5 N-linked and 1 O-linked) all of which are within the ectodomain (Fig. 2D). Immunodetection of P2Xₐ-GFP from enriched vacuole fractions revealed 2 distinct bands. A lighter band (~70KDa) corresponding to the predicted mass of unmodified P2Xₐ-GFP, and a heavier more diffuse band sensitive to degradation by PNGase F endoglycosidase (Fig. 2E). Anti-GFP immunoreactivity was not detected in parental AX4 amoeba (Fig. 2E). These data suggest that the P2Xₐ receptor is modified by N-linked glycosylation. As the only N-linked glycan sites are predicted to be within the ectodomain (Fig. 2A) this indirectly suggests that the receptor ectodomain is exposed to the golgi lumen during maturation, placing the ectodomain with the CV lumen by definition of the biosynthetic pathway of membrane proteins in eukaryotes. These data are in good agreement with the receptor orientation as determined by Ludlow et al (2009) (11) and suggest the receptor may served to sense vacuolar ATP.

![Image](http://www.jbc.org/content/382/4/5302/F2.large.jpg)
Identification of receptor ligand within vacuole lumen. If intracellular P2XRs are activated by luminal ATP, then ATP must be present within the CV lumen. To test this we adopted a strategy to isolate intact CV from cultures, and ensure freedom from contaminated intact mitochondria, ER and lysosomes. Only the latent activity of biochemical markers was taken into account as to determine isolation of intact organelles. Importantly, as ATP is a major cytoplasmic component we opted to liberate the water soluble components of CVs by organic extraction (23) only after extensive washing to remove any trace of contaminating bound cytoplasmic ATP. The distribution of intact CVs was determined by assaying alkaline phosphatase (ALP) activity, which is present with the CV (26). Over 40 fractions peak latent ALP activity was detectable in the most buoyant fractions, with ~50% total cellular latent ALP activity recovered in fractions 1-3 (Fig. 3A). Peak succinate dehydrogenase activity peaked at fraction 21 and represented less than 1.5% total cellular activity in fractions 1-3 (Fig. 3B). No significant latent acid phosphatase activity (lysosomes) was detectable in fractions 1-16, though non-latent activity did show a non-uniform distribution across fractions (Fig. S1). Non-latent acid phosphatase activity was ~4% total activity cellular activity in fractions 1-3. These data reveals that lysosomes isolated using this protocol are not intact and therefore negated as a source of ATP in fractions 1-3.

As the purity of the CV preparation is critical to determination of luminal ATP, we further demonstrate the purity of isolated CVs by determining the distribution of several GFP-tagged organelle markers across isolated fractions. The distribution of CV markers P2X₄-GFP and dajumin-GFP (19) correlated well with the distribution of latent ALP activity (Fig. 3B). The peak ER fraction, as labelled by calreticulin-GFP, was distinct from that of the buoyant CV-enriched fractions peaking at fraction 18 (Fig. 3B). These data demonstrated that fractions 1-3 do not contain intact mitochondria, lysosomes or ER, and intact CVs are successfully purified. Following pooling, sedimentation and extensive washing of fractions 1-3, organic extraction liberated ATP detectable by both HPLC (Fig. 3C and 3D) and luciferase-luciferin assay (Fig. 2E). Importantly, nominal amounts of ATP were detectable in the supernatant following sedimentation of vacuoles (Fig. 3E), suggesting washing had successfully removed any cytoplasmic ATP, suggesting that any liberated ATP is derived from inside isolated vacuoles.

ATP translocation triggers ATP-evoked calcium release in isolated vacuoles. Millimolar quantities of free cytoplasmic ATP establish a large chemical gradient across the membranes of organelles, yet ATP is a strong anion at physiological pH and unable to passively to diffuse. The detection of ATP within the CV lumen suggests the presence of a mechanism to facilitate ATP transport (27) or a mechanism for ATP synthesis de novo (10). HPLC analysis of vacuole nucleotides failed to detect any significant amounts of AMP or ADP (Fig. 3D) suggesting that substrates necessary for ATP synthesis are not present, or present at levels below the detection limit of HPLC. To this end, we tested for the presence of an ATP translocating mechanism. Using 4mM ATP to mimic cytoplasmic conditions, purified vacuoles accumulated ATP in a time-dependent manner at an initial rate of 1.8±0.4 nmol/mg/min (N=3) (Fig. 4A). The translocation of ATP was initially very rapid reaching a plateau after 2 mins (Fig. 4A). ATP regeneration using a creatine kinase-creatine phosphate (CK-CP) system significantly increased both the initial rate of ATP translocation (3.2±0.1 nmol/mg protein/min; N=5, P<0.01) and the total amount of ATP transported at steady-state with respect to ATP alone (Fig. 4A). These data suggest that ATP may be hydrolysed to ADP upon addition to isolated vacuoles and that CK-CP increases the amount of free ATP for transport. Not surprisingly ATP is rapidly hydrolysed upon addition to vacuoles (Fig. S2). Translocation of ATP was attenuated by the addition of other nucleotides to varying degree, with ADP causing the greatest inhibition (Fig. 4B). HPLC analysis of luminal ATP revealed transport of ADP, though GTP and UTP were not transported (Fig. S3). These data are indicative of adenine nucleotide selective transport and suggest CK-CP addition may facilitate ATP transport (Fig. 4A) by depleting the pool of ADP, derived from ATP hydrolysis, available to attenuate ATP transport. ATP transport was inhibited by Evans blue, quercetin and vanadate, though insensitive to the mitochondrial ATP/ADP exchanger antagonist atracyloside (Fig. 4B).

The Dictyostelium P2X₄ receptor, like other P2XRs, is highly permeable to calcium (3).
Moreover, the combination of the receptor orientation and the presence of an ATP translocating mechanism suggest ATP translocation into the vacuole lumen may be a means to activate vacuolar P2XRs and trigger release of stored calcium. To test this hypothesis we mimicked the experimental conditions used to observe ATP translocation (Fig. 4A) but instead measured extra-vacuolar calcium using the membrane impermeable indicator Fluo-3. Strikingly addition of ATP to vacuoles evoked a release in calcium which progressed over several minutes (Fig. 4C). Subsequent addition of CK-CP caused a rapid rise in calcium release which slowed but progressed over several minutes (Fig. 4C). Addition of ATP or CK-CP caused no rise in Fluo-3 fluorescence in the absence of vacuoles, nor could creatine or inorganic phosphate substitute for CP in evoking a calcium release in the presence of vacuoles (data not shown). ATP-evoked calcium release was inhibited by ADP (76.4±2.4%; N=4), GTP (44.2±1.2%; N=3) and UTP (15.4±1.2%; N=3) (Fig. 4D). Vanadate ablated ATP-evoked calcium release (95±5.2%; N=4) but atractyloside was ineffective (1.2±3.2%; N=3) (Fig. 4D). These data demonstrate common pharmacology between the ATP translocation and ATP stimulated calcium release. We found that Evans blue and quercetin directly quenched Fluo-3 fluorescence at concentrations that inhibit ATP translocation (Fig 4B) and therefore could not be used to probe calcium release. ADP, AMP nor GTP, which do not activate recombinant *Dictyostelium* P2XRs (3,11), did not evoke calcium release suggesting an effect specific to ATP (Fig. 4E). β,γ-imido-ATP also could not evoke calcium release (Fig 4E). To rule out the possibility that ATP-evoked calcium release is not specific to CV we tested the ability of ATP to mobilise calcium in fractions enriched with other organelles. ATP evoked a calcium release in crude whole cell lysates (Fig. 4F) though the magnitude of the response was greater in CV-enriched fractions (Fig 4F; Fig. 3A & Fig. 3B). ATP did not mobilise calcium in fractions enriched in ER and mitochondria (Fraction 21) or denser fractions (Fraction 38) (Fig. 4F), indeed addition of ATP caused a decrease in Fluo-3 fluorescence in these fractions indicative of ATPase-dependent calcium loading (16).

The ability of ATP to mobilise calcium is dependent on the calcium state of the store.

Detergent solubilisation of vacuoles used in this study releases calcium ~30-fold that of the magnitude of the ATP stimulated release, demonstrating that the vacuoles are pre-loaded with calcium (Fig. 5A). Previous studies examining movement of calcium in isolated CVs from *Dictyostelium* have used CVs isolated following depletion of cellular calcium for several hours with EGTA (18,28) to allow Ca$^{2+}$-ATPase dependent loading stimulated by ATP addition. We confirm that the effect of ATP addition on vacuolar calcium is opposite in CVs from cultures following EGTA-buffering. Similar to previous studies (16,18) robust vanadate-sensitive uptake of calcium is observed upon ATP addition in calcium depleted vacuoles (Fig. 5B). Interestingly these date suggest the net movement of calcium across the vacuole membrane evoked by ATP is dependent upon the calcium state of the vacuole. Although ADP alone could not evoke calcium (Fig. 4E), ADP in the presence of CK-CP could (Fig. 5C). In these experiments creatine could not substitute CP (data not shown) suggesting it is the conversion of ADP to ATP that stimulates release (Fig. 5C). The initial rapid calcium release evoked by ADP plus CK-CP was significantly less than that evoked by ATP plus CK-CP (Fig. 5C) but the magnitude of calcium where similar after 10 mins (Fig. 5C). In good correlation, the magnitude of luminal ATP translocation at steady state was not significantly different if ATP or ADP were the ATP source, yet the initial rate of ATP translocation were significantly less if ADP was the source and not ATP (Fig. 5D). An explanation for this data is that the processes of luminal ATP translocation and ATP-evoked calcium release are coupled. A direct comparison of ATP translocation in vacuoles isolated from wild-type and P2X$_{\lambda}$ knockout cells revealed no significant difference (Fig. 5E).

**ATP-evoked calcium release is dependent upon P2XRs.** To test for an involvement of P2XRs in mediating ATP-evoked calcium release, we compared the magnitude of release in wild-type AX4 and P2X$_{\lambda}$ knockout amoeba (3). The magnitude of calcium release stimulated by ATP was reduced by 72% (F/F$_{0}$ 0.17±0.02 vs 0.05±0.01 AX4 wild-type vs P2X$_{\lambda}$ KO cells; N=6-7, P<0.01) in knockout cells (Fig. 6A & Fig. 6B). ATP plus CK-CP could also evoke a calcium release in P2X$_{\lambda}$ knockout cells but with a 42% reduced response (F/F$_{0}$ 2.76±0.18 vs 1.5±0.12 wildtype vs KO; N=7, P<0.01) (Fig. 6A).
The latent activity of ALP in CV enriched fractions (pooled fraction 1-3) was not significantly different in wild-type versus P2X_A knockout cells (Fig. S4A). Moreover, the specific activity of ALP was not significantly different between wild-type and knockout cells (Fig. S4B), demonstrating that the amount of ALP in CV was not significantly different between the two cell types and cannot account for the decreased magnitude in P2X_A knockout cells. One possible reason underlying the reduced ATP stimulated calcium release in P2X_A knockout cells is that ATP translocation is also reduced in these cells versus parental cells. These data can be explained if the P2X_A receptor mediates some of the ATP stimulated calcium release. To test whether the residual ATP-evoked calcium release was dependent upon other P2X receptor subtypes present in the vacuole (Ludlow et al 2009), we used a quintuple knockout strain devoid of P2X_A-E. Strikingly, ATP mobilisation of CV calcium was ablated in the quintuple knockout strain (F/F_5_0.06±0.001 vs -0.02±0.01 wild-type vs KO cells; N=5, P<0.01) (Fig. 6A). Indeed, a significant decrease in extravesicular calcium is observed upon addition of ATP in P2XR null cells, indicative of calcium loading (Fig. 6A). Release of calcium evoked by addition of ATP plus CK/CP was inhibited by 78% (F/F_0 0.49±0.08 vs 0.107±0.05 wildtype vs KO; N=5, P<0.01) in P2XR null cells (Fig. 6B). ATP evoked calcium mobilisation was significantly smaller in AX2 versus AX4 strains.

**Antagonists of luminal ATP translocation and ATP-evoked calcium release impair osmoregulation.** In *Dictyostelium* RCVD following osmotic swelling is achieved by the expulsion of water from the CV. In an effort to link the information gained from purified vacuoles with cellular function, we sought to test the requirement of normal osmoregulation for ATP-evoked calcium release from the CV using antagonists that block luminal ATP translocation and ATP-evoked calcium release. Exposure of *Dictyostelium* to hypotonic stress caused a robust cell swelling followed by RCVD of ~50% in control experiments (Fig. 7A & 7C). Culturing cells in the presence of EGTA in order to deplete cellular CV calcium (Fig. 5B) abolished RCVD (Fig. 7A & 7C). The effect of EGTA was not likely to be due to a perturbation of general intracellular calcium signalling as inhibition of the ER by thapsigargin had no effect on RCVD (Fig. 7A). Moreover, cells treated with Evans blue, quercetin or vanadate all attenuated RCVD in response to osmotic swelling (Fig. 7B & 7C). RCVD was insensitive to atractyloside (Fig. 7B). These data suggest common antagonist pharmacology is shared by luminal ATP translocation, P2X receptor-dependent CV calcium release and RCVD.

**DISCUSSION**

In this study we demonstrate that translocation of ATP into the vacuole lumen is a mechanism by which intracellular P2XRs can be activated. This mode of activation is atypical amongst other calcium release channels which are activated by ligands including nucleotides. Activation of the inositol 1,4,5-triphosphate receptor, ADP-ribose activated TRPM2 channels and presumably NAADP-activation TPC channels is via interaction with ligand on the cytoplasmic face of calcium stores (29-31). Our evidence for the orientation of intracellular P2XRs in *Dictyostelium* with the ATP-binding site facing in the CV lumen is consistent with previous work (11), and also consistent with the postulated orientation of the P2X4 receptor in mammalian lysosomes (7). Unlike the ATP-binding site of P2X4 which can become exposed to the extracellular environment following lysosome exocytosis (7-8), this study and the study of others (19,32) argues that the membranes of CV and plasma membrane do not mix upon exocytosis and hence that intracellular P2XRs in *Dictyostelium* would never be exposed to the extracellular space. The P2X_A receptor would be a rather ineffective sensor of cytoplasmic ATP as one would predict the receptor to exist in a permanently desensitised state owing to the millimolar amounts of ATP in the cytoplasm (3). Despite the detection of ATP we have not determined the absolute concentration of ATP within the lumen of a single vacuole, nor can we estimate such from our assays carried out on populations of vacuoles. This is for a number of reasons (i) the number of vacuoles varies per cell and the absolute number of vacuoles assayed is not known and (ii) the volume of CV is dynamic and changes with time due to swelling by water...
accumulation. Whatever the absolute concentration of ATP, translocation of ATP in isolated vacuoles is capable of raising the luminal ATP concentration high enough to activate P2X\(_A\) (EC\(_{50}\) \ (~170 \mu M) (3). The observation that ATP stimulated calcium release is significantly attenuated but not abolished in P2X\(_A\) knockout cells is suggestive of a level of redundancy between vacuolar P2XRs. Indeed, P2X\(_B\) and P2X\(_E\) have recently been shown to form functional receptors, though ATP is 3-5 fold less potent at activating B and E receptor subtypes compared to P2X\(_A\) (3,11). It is therefore interesting to observe that the calcium release evoked during ATP regeneration is only reduced by 42\% in P2X\(_A\) KO cells compared to the 72\% reduced in calcium released by ATP alone. Our data suggests that the rate and steady-state amount of ATP translocated is significantly greater with ATP regeneration than without, and that during these conditions there is less dependency on P2X\(_A\) for calcium release. One possible explanation for this observation is that different receptor subtypes, with higher ATP EC\(_{50}\) e.g. P2X\(_B\) and P2X\(_E\) (11), contribute to calcium release at higher initial and steady-state rates of luminal ATP translocation. This may allow the vacuole to fine-tune a response to ATP, as observed for the complex repertoire of cell surface P2XRs in mammalian cells (1). Experiments with P2XR null cells clearly demonstrates that ATP evoked calcium mobilisation is entirely dependent upon P2X\(_A\).

In combination with experiments with P2X\(_A\), these data supports a role for multiple receptor subtypes in releasing calcium in response to ATP. Intriguingly, the response to ATP plus CK/CP is heavily suppressed (78\%) though not abolished in P2XR null cells. It should be noted that the quasi-instantaneous release observed upon addition of ATP plus CK/CP is only observed in the presence of vacuoles, and therefore not an artifactual effect of CK/CP on Fluo-3 fluorescence. The molecular basis for the residual rapid component is unclear at present though P2XR activation represents the major component.

Our data shows that luminal ATP translocation increases \(~25\%\) in the presence of an ATP regenerating system than without, yet ATP regeneration triggers an immediate calcium release of some \(~500\%\), though this data does demonstrate that increased ATP translocation increases the magnitude of calcium release. It is more difficult to explain the differences observed in the magnitudes of responses without knowing the absolute change in luminal ATP concentration which results from a \(~25\%\) enhancement of ATP translocation. The relationship between ATP concentration and receptor activation is not linear and steep for *Dictyostelium* P2XRs (3,11) and it is therefore possible that small changes in luminal ATP could result in large real-term changes in receptor activity. The rapid release of calcium stimulated by ATP regeneration is not observed in the absence of vacuoles or if creatine phosphate is substituted for creatine, suggest vacuoles and ATP regeneration are required. More importantly the magnitude of calcium release stimulated by ATP regeneration is dependent upon P2X\(_A\).

One outstanding issue is the molecular correlate of the vacuolar ATP translocator. Vesicular adenine nucleotide transporters have been identified in diverse organisms (33-34), and are found in secretory vesicles of neurons (35), chromaffin cells (27) and pancreas (36). The ATP translocator identified in this study was inhibited by micromolar Evans blue in common with some mammalian ATP transporters (27,35,36). One interesting observation of this current study is that the translocation of ATP may be dependent upon ATP hydrolysis. Vanadate sensitivity suggests ATP hydrolysis may be required. In addition, the non-hydrolysable ATP analogue β,γ-imido-ATP is 10-fold more potent that ATP at activating P2X\(_A\) (3) and \(~3\)-fold more potent at activating P2X\(_B\) (11). Despite this β,γ-imido-ATP does not evoke calcium release. Our data demonstrates that this transporter is capable of transporting ATP and ADP, though not GTP or UTP. Recently, putative ABC transporters have been identified in a proteomic analysis of CV from *Trypansoma Cruzi* (37). Indeed MRP-type ABC transporters are responsible for the accumulation of ADP in secretory granules of platelets (38). The *Dictyostelium* genome encodes a plethora of ABC transporters including some homologous to mammalian MRP-type transporters (39). The
subcellular localisation of ABC transporters in *Dictyostelium* is not well defined but they remain potential candidates underlying the vacuolar transportation of ATP described here. We have revealed that ATP controls the net movement of calcium across the vacuole membrane by two counteracting mechanisms (i) ATP hydrolysis to drive calcium uptake and (ii) ATP activation of P2XRs to release calcium. Although this may initially appear counterintuitive, it should be noted we have studied this phenomenon on a macroscopic scale. Signalling events on a local level may be different and indeed Ca\(^{2+}\)-ATPases and the ATP translocator may utilise different pools of ATP (40). A more refined view will come from further investigation into the modulation of this signalling system.

In this study we tested the hypothesis that intracellular P2XRs can act as vacuolar calcium release channels *in vitro* in an attempt to understand why genetic disruption of P2X\(_{\alpha}\) impairs CV activity in AX4 *Dictyostelium in vivo* (3). Although we have not presented direct evidence that hypotonic stress triggers a calcium release from the CV *in vivo* there are some excellent examples from other studies which hypothesise that calcium signalling is important for normal CV function. These include (i) the CV is an acidic calcium store (16,18), (ii) the CV is decorated with calcium responsive signalling proteins (3,17), (iii) calcium permeable ion channels are exclusive CV residents (3,11) and (iv) CV calcium release and signalling necessitates osmoprotection and CV function in other protists (41-42). In this study we suggest that P2XR-dependent release of calcium triggered by vacuolar ATP translocation is necessary for normal CV function. These conditions that deplete CV calcium lead to loss of RCVD, which is not mimicked by depletion of ER calcium by thapsigargin. We have provided evidence that the mechanisms we have described in purified vacuoles are important for cellular function, and that pharmacological inhibitors of vacuolar ATP translocation and ATP-evoked release phenocopy the osmotic swelling observed in P2X\(_{\alpha}\) knockout cells (3). It is difficult to ascertain whether the compounds that we have used to inhibit RCVD are selective for ATP transport and ATP-evoked release, for example vanadate is a generic inhibitor of ATPase function. However, the observation that thapsigargin and atracyloside, inhibitors of other major ER and mitochondrial calcium pools, respectively, do not impair RCVD gives some degree of confidence of specificity. A pharmacological approach remains the only option until the molecular correlate of the CV ATP transporter is elucidated, allowing a genetic approach to be taken in future studies.

The identification of intracellular P2XRs as calcium release channels in the model eukaryote *Dictyostelium* it is tempting to speculate that a similar role exists for P2XRs that reside in intracellular compartments of mammalian cells. A potential candidate for this is the mammalian P2X\(_4\) receptor which targets to lysosomes (7-9). The receptor is protected from degradation in this compartment which is suggestive of a requirement for function. P2X\(_4\) receptors are regulated by pH (43) and lysosomes can accumulate ATP (44). Our observations further suggest that the compartmentalisation of ATP by translocation allows ATP to act as a discrete signalling molecule, either by its release into the extracellular space or shuttling into an intracellular compartment as described here. The evolutionary conservation of the intracellular residency of P2XRs between amoeba and mammals is highly suggestive of a conserved function. This study also suggests that cell signalling by the compartmentalisation of ATP has early evolutionary beginnings (5), and may have evolved to include plasma membrane events. Further work is required to elucidate any functional role for intracellular P2X receptors in mammalian cells.

**FOOTNOTES**

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FIGURE LEGENDS

Fig. 1. P2X₈ is a permanent contractile vacuole resident. (A) Absence of P2X₈ from lysosomes and endosomes. Live P2X₈-GFP expressing AX4 amoeba labelled with TRITC-dextran (endosomes; upper panels) or Lysotracker (lysosomes; lower panels). Arrow indicates contractile vacuole localisation. Scale 5µm. (B) Association of P2X₈ with the contractile vacuole during voiding and filling. Overlapping differential inference contrast (DIC) and fluorescence (GFP) images taken from a time-lapse movie showing P2X₈-GFP association with the contractile vacuole during both voiding (top panels) and filling (bottom panels) phases. Time in seconds (top right). Scale 5 µm. P2X₈-GFP localisation is shown in exploded view (inset). Arrow indicates contractile vacuole.

Fig. 2. P2X receptor is orientated to sense luminal ATP. (A) Schematic representation of P2XR topology. (B, C) Fluorescence protease protection performed using cells expressing calreticulin or P2X₈ both with C-terminal GFP tags. Relative fluorescence from representative experiments captured following trypsin addition to permeabilised cells. Trypsin added a time 0. *Inset*, representative images captured at time points 1 and 2. Scale 10µm. (D) Peptide sequence of Dictyostelium P2X₈ showing predicted N-linked (yellow) and O-linked (blue) glycan acceptor sites. Transmembrane are highlighted in grey. (E) Immunoblot for P2X₈-GFP showing anti-GFP immunoreactivity probed in lysates treated with PNGase F (+) or control lysates (-). No anti-GFP immunoreactivity is detected in parental AX4 cells (AX4). Molecular weights are kDa.

Fig. 3. Detection of P2X receptor ligand in vacuole lumen. (A) purification of intact contractile vacuoles by subcellular fractionation. Latent activity of alkaline phosphatase (contractile vacuole, red) and succinate dehydrogenase (mitochondria, green). N = 4 fractionations. Latent activity is expressed as % total cellular activity. (B) Distribution of GFP tagged organelle markers across different fractions. Markers are for contractile vacuole (P2X₈ and dajumin) and endoplasmic reticulum (calreticulin). GFP fluorescence is expressed as % peak fluorescence. Representative traces of 4 independent fractions. (C,D) Ion-pair reverse phase HPLC analysis of ATP in isolated vacuoles.
Separation of adenine nucleotide standards (1µM each) (C) or water soluble contents liberated from isolated vacuoles (D). ATP standard is given as reference. Representative traces of 4 independent experiments. (E) Detection of ATP liberated from vacuole pellet vs supernatant as determined by luciferase-luciferin assay. N=4; *P<0.01.

Fig. 4. Luminal ATP translocation triggers release of stored calcium. (A) Time-dependent luminal ATP translocation in the presence of 4mM ATP alone or ATP with creatine kinase-creatine phosphate (CK-CP). N=4; **P<0.01. (B) Effect of nucleotides (2mM each), vanadate, atractyloside (100µM), Evans blue (1µM) and quercetin (100µM) on luminal ATP translocation. N=3-4; *P<0.05, **P<0.01. (C) ATP evoked calcium release in isolated vacuoles. Representative traces of 6-7 independent experiments showing calcium release in response to ATP alone with subsequent (CK-CP) addition. (D) Effect of various inhibitors of luminal ATP translocation on ATP-evoked calcium release. Concentrations as in (B). (E) β,γ-imido-ATP, ADP, AMP or GTP cannot mimic ATP-evoked calcium release. N=4; *P<0.05, **P<0.01. (F) Specificity and enrichment of ATP-evoked calcium release in contractile vacuole fractions. Magnitude of ATP-evoked calcium release in crude lysate, CV-enriched fraction (fraction 2) or dense fraction (fraction 38).

Fig. 5. Net movement of vacuolar calcium evoked by ATP is dependent upon the calcium state of the store and the initial rate of luminal ATP translocation. (A) Vacuoles isolated from cultures without EGTA buffering a loaded with calcium. Release of calcium evoked by solubilisation of vacuoles with 0.1% triton-X100. (B) ATP causes vanadate-sensitive loading of vacuoles isolated from calcium depleted cultures. (C) Paired experiment showing ADP alone does not evoke calcium release. ATP synthesis from ADP using creatine kinase-creatine phosphate (CK-CP) can evoke a release, yet the initial rise is less but approaches that of ATP plus CK-CP after 10mins. (D) Comparison of initial rates and steady-state rates of luminal ATP translocation in the presence of ATP plus CK-CP or ADP plus CK-CP. N=4; **P<0.01. (E) Comparison of luminal ATP translocation in vacuoles isolated from WT (black) and P2Xₐ KO (red) amoeba (N=4).

Fig. 6. P2X receptors mediate ATP evoked calcium mobilisation. Representative traces showing the magnitude vacuolar calcium release evoked by ATP alone (A) or ATP plus creatine kinase-creatine phosphate (CK-CP) (B) in AX4 wild-type (WT) vs P2Xₐ receptor knockout (KO) cells and AX2 WT vs P2XR null (quintuple KO) cells. Right, average peak responses. N=4-5; *P<0.01 KO vs WT equivalent; #P<0.01 quintuple vs single KO.

Fig. 7. Inhibitors of luminal ATP translocation or depletion of CV calcium impairs recovery from osmotic swelling. (A and B) measurement of real-time changes in cell size by right-angled light scattering. (A) cell swelling and recovery in response to hypotonic stress in control cells or cells with depleted ER calcium (thapsigargin; 10µM, 30 mins) or depleted CV calcium (EGTA depletion). (B) effect of luminal ATP translocation inhibitors on cell swelling and recovery. Control traces are superimposed (grey) for comparison. (C) Images of control cells immediately after exposure to distilled water (top right time 0) and following 60 mins exposure to water with and without (control) inhibitors (bottom left). Average data on peak swelling given in (D). N=4; *P<0.05.
New Figure 1
Figure 2

MGFSFDWDDDFQYSTVKIVRIDRRLGILHLSFLVGIVAYIVYSAIIKKG
YLFTEVPIGSVRTLGNPEHNTCNYGVCNQRTYFTPLECNYWDEQQ
LALFPGEQDTFTCTTRLQKQEANCNTDPTCKFVDEGPSAKNIYIADI
ESFTILDHMYASSGSQFNAVLDHGYILNQDGDEVQIDANGTSIGVSGK
PDIMTIGQLLSFGVSDLQASPVSNSHRYVDGVALVVFITYSNTYTTST
DFKYYVSQQIANTTYPETEELIESIHSLLLKIRHGIHIFGTIGSF
HFQNLLTLEGSGGLLLAVATTVDQLAIRLLQRKSYSSKLVQTVESMSNPMKKRITTEDVEDVLYTRIEGL

PNGase F
AX4
- +

α-GFP

Figure 2
Figure 3
Figure 5

A

Triton

B

ATP

+ 100μM vanadate

ATP alone

ATP

0.25

1 min

F/F

0

Triton

1.0

1 min

F/F

0

A

B

C

D

initial rate

steady-state rate

ATP translocation

(nmol/mg protein/min)

**

ADP

ATP

0

1

2

3

4

5

D

0

1

2

3

4

5

6

ATP translocation (nmol/mg protein)

Time (min)

E

ATP translocation (nmol/mg protein)

Time (min)

Figure 5
Figure 6

A

WT

ATP

single KO

quintuple KO

response (% wildtype)

B

WT

CK/CP

ATP

single KO

quintuple KO

response (% wildtype)

* (#)
A mechanism of intracellular P2X receptor activation
Venketesh Sivaramakrishnan and Samuel J. Fountain

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