Calcium-dependent regulation of Rab activation and vesicle fusion by an intracellular P2X ion channel

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Rab GTPases play key roles in the delivery, docking and fusion of intracellular vesicles. However, the mechanism by which spatial and temporal regulation of Rab GTPase activity is controlled is poorly understood. Here we describe a mechanism by which localized calcium release through a vesicular ion channel controls Rab GTPase activity. We show that activation of P2XA, an intracellular ion channel localized to the Dictyostelium discoideum contractile vacuole system, results in calcium efflux required for downregulation of Rab11a activity and efficient vacuole fusion. Vacuole fusion and Rab11a downregulation require the activity of CnrF, an EF-hand-containing Rab GAP found in a complex with Rab11a and P2XA. CnrF Rab GAP activity for Rab11a is enhanced by the presence of calcium and the EF-hand domain. These findings suggest that P2XA activation results in vacuolar calcium release, which triggers activation of CnrF Rab GAP activity and subsequent downregulation of Rab11a to allow vacuole fusion.

Regulation of intracellular vesicle traffic is fundamental for normal cell function and its mis-regulation is associated with congenital developmental disorders, cancer and neurological dysfunction1. Studies of vesicle traffic in different systems have revealed the evolutionarily conserved role played by Rab GTPases. Every organelle of both the endocytic and exocytic pathways expresses several Rab GTPases, which must be sequentially activated to allow precise delivery, docking and fusion of different membrane compartments2-6. Another regulator of vesicle fusion events is intracellular calcium. Transient and localized increases in calcium have been shown to facilitate some, but not all, vesicle fusion events7-19. However, it is unknown whether interplay between calcium and Rab GTPases could coordinately regulate vesicle fusion.

The Dictyostelium discoideum contractile vacuole (CV) system is an intracellular vesicle required for osmoregulation. The CV cycle is a highly regulated process, orchestrated by Rab proteins, their regulators and their effectors20-27. The CV is also an acidic calcium store (acidocalcisome)28, and therefore provides an excellent model system to study the coordinated regulation of vesicle trafficking by Rab proteins and calcium. On hypo-osmotic shock, water enters tubules of the CV system, a process accompanied by activation of Rab11a that is localized to CV membranes22,25. Drainin, a putative volume-sensing Rab11a-GTP-binding protein, is subsequently recruited to maturing vacuoles20,22,29,30. Next, vacuoles are prepared for fusion with the plasma membrane through the recruitment of the Rab GAP disgorgin and Rab8a (refs 22,23). Once tethered to the plasma membrane, the 'non-polarized' CV becomes 'polarized' and committed to pore formation23. This process is defined by a 'ring to patch transition', in which different proteins become concentrated at the front or back of the CV (ref. 23). How the correct spatial and temporal regulation of these Rab proteins is achieved is poorly understood.

Recently, we discovered that a homologue of mammalian P2X receptors, P2XA, is exclusively localized to the Dictyostelium CV system31. P2X receptors are calcium-permeable ion channels gated by ATP that function in diverse physiological processes32,33. However, the intracellular localization of P2XA in Dictyostelium cells is inconsistent with a role in regulating responses to extracellular ATP (ref. 31). Instead, P2XA knockout cells exhibit defects in responses to hypo-osmotic shock31,34 (Supplementary Fig. 1). Under hypo-osmotic shock, knockout cells become rounded and the rate of CV discharge is much reduced31,34. This raises the possibility that P2XA may be an important conduit for vacuolar calcium release, and that this calcium is required for the correct regulation of vacuolar cycling35. However, major questions remain unanswered. First, it is unknown whether the intracellular function of P2XA indeed requires ion channel activity and calcium flux. Second, it is unknown whether

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disruption of P2XA activity, and therefore calcium flux, affects vesicle fusion or some other event in the CV cycle, such as maturation or delivery. Finally, it is unknown how an ATP-gated ion channel could regulate a Rab–GTP-dependent process in a calcium-dependent manner at the molecular level.

RESULTS

Intracellular P2XA function requires ion channel activity

To determine whether P2XA ion channel activity is required for osmoregulation, mutations were generated that resulted in inactive (K67A/K289A), less active (R285K) or hyperactive (R63A) versions of P2XA when tested in HEK293 cells (Fig. 1a,b). Next, gene-replacement Dictyostelium strains were generated in which the endogenous gene was replaced with wild-type or point-mutated versions and tested for osmotic shock defects. In each case, mutated receptors still localized to the CV (Supplementary Fig. 2). However, the gene replacement strains exhibited clear differences in their ability to respond to osmotic shock, with responsiveness correlating very well with ion channel activity (Fig. 1c,d).

P2XA mutant cells exhibit aberrant CV system morphology

Previous studies have suggested that P2XA disruption results in a prolonged CV cycle with fewer fusion events37. We next sought to determine whether these defects were due to a failure in CV maturation, trafficking, tethering, pore formation or fusion. To address this, we first examined the morphology and dynamics of the CV system following hypo-osmotic shock in cells expressing dajumin–GFP, which labels the CV system throughout the cycle. These studies showed that the CV system was strikingly aberrant in P2XA mutant cells (Fig. 2a and Supplementary Videos 1 and 2). Mutant cells contained many more vacuoles (Fig. 2b), which were found not only at the bottom of the cell where it contacts the substrate, but unlike wild-type cells, also throughout the cell (Fig. 2c). Although many of these vacuoles seemed to be statically tethered to the plasma membrane, few fusion events were observed (Fig. 2d,e). Finally, the P2XA mutant vacuoles were typically smaller than wild-type vacuoles (Fig. 2f). Live-cell imaging revealed that this was not due to a simple failure to mature to the correct size. Instead, normal-sized vacuoles did form but tended to split into multiple smaller vacuoles after prolonged periods of tethering (Fig. 2g).

P2XA is required for vacuoles to transition from tethered to fused states

As the P2XA mutant contains large numbers of vacuoles that are tethered but do not fuse, we next examined whether this might be explained by a failure to recruit, or correctly localize, factors required for vesicle maturation or fusion. First, we found that drainin (a marker for vesicle maturation and tethering) localized to all mutant vesicles even when very small (Fig. 3a and Supplementary Videos 3 and 4). Second, Rab8–GFP, a marker normally only transiently recruited to mature vesicles just before fusion, localized to most mutant vesicles (Fig. 3b and Supplementary Videos 5 and 6). In wild-type cells, tethered vesicles expressing drainin and Rab8 quickly undergo a dynamic rearrangement (‘ring to patch’ transition), which is required for recruitment of the pore formation complex and thus efficient fusion. Most notably, in wild-type cells, seconds after Rab8 was recruited to the vacuole, drainin disappeared from the site of contact with the plasma membrane, followed by a reciprocal enrichment of Rab8 at the point of plasma membrane contact. In contrast, in P2XA mutant cells, this ‘ring to patch’ rearrangement of drainin and Rab8 did not occur, thus providing an explanation for inefficient fusion (Fig. 3a,b). From these observations, many P2XA mutant vacuoles therefore seem to be ‘trapped’ in a mature, membrane-tethered and fusion-competent state, yet are unable to undergo fusion.

P2XA activity is dynamically regulated during the CV cycle

The above genetic studies suggest that P2XA receptors function to promote vacuole fusion. As P2XA is present throughout the entire CV cycle (Supplementary Fig. 4), we next investigated whether the temporal regulation of P2XA activity was consistent with its apparent function. For this, we developed a sensor for P2XA ion channel activity in which the calcium-sensitive GCaMP2 reporter is fused to the carboxy terminus of the P2XA receptor. As P2XA encodes a calcium-permeable ion channel, we reasoned that channel activation would result in a transient fluorescence increase. Indeed, an analogous system based on fluorescence resonance energy transfer has been successfully employed to monitor mammalian neuronal P2X receptor activation36. We found that when P2XA–GCaMP2 was expressed in HEK293 cells, the presence of the GCaMP2 tag did not affect the concentration–response curves for ATP (Fig. 4a,b). Furthermore, P2XA–GCaMP2 acted as a reporter for P2XA activity, because when changes in fluorescence were used to measure dose responses, measurements were similar to those achieved with electrophysiological recordings, and detectable at concentrations as low as 30 μM ATP (Fig. 4c and Supplementary Fig. 4). Fluorescence imaging also revealed that these transient increases on stimulation with ATP fluorescence initially took place at the plasma membrane (Fig. 4d, Supplementary Fig. 4 and Videos 7 and 8). Finally, fluorescence increases were dependent on ion channel activity as P2XA(K67A/K289A)–GCaMP2 showed no change on ATP stimulation (Fig. 4e and Supplementary Fig. 4).

We next examined whether the P2XA–GCaMP2 fusion protein was fully functional in Dictyostelium cells. P2XA–GCaMP2 was found to specifically localize to the CV system (Fig. 4g and Supplementary Video 9) and overexpression effectively rescued the P2XA-null osmoregulation defects (Supplementary Fig. 5). Furthermore, in control experiments, GCaMP2 alone acted as an efficient calcium sensor in Dictyostelium cells (Supplementary Fig. 5). On the basis of these findings, we used CV P2XA–GCaMP2 fluorescence to measure channel activity in vivo. P2XA–GCaMP2 fluorescence was measured at different CV stages: early, docked and during fusion. We found that fluorescence, and thus P2XA activity, was highest when CVs were docked or underwent fusion (Fig. 4f,g and Supplementary Videos 9 and 10). These changes in fluorescence were dependent on P2XA ion channel activity, because the inactive P2XA(K67A/K289A)–GCaMP2 mutant showed no increase in fluorescence (Fig. 4f,g and Supplementary Video 11). This also correlated with a failure of this strain to undergo the ‘ring to patch’ transition (Fig. 4g and Supplementary Video 11). These findings provide further support for the idea that increases in calcium levels are required for CV fusion. Finally, they suggest that the changes in calcium levels that result in increased GCaMP2 fluorescence are due to calcium efflux from the vacuole through the P2X ion channel, rather than coincident increases in calcium levels in or around the CV at this stage.
Figure 1 P2XA mutants with altered ATP sensitivity show defects in osmoregulation. (a) Currents evoked by ATP (10 μM–3 mM) in HEK cells expressing wild-type (WT) or mutated P2XA receptors. Each panel shows superimposed current traces for the concentrations indicated. ATP application was 2 s (black bar). (b) Concentration–response curves for wild-type (black), R285K (blue), R63A (purple) and K67A/K289A (green) receptors. Error bars represent s.e.m. of responses from wild-type (8 cells), R285K (4 cells), R63A (4 cells) and K67A/K289A (3 cells) cells. Compared with wild-type P2XA (black), concentration–response curves for R63A (purple) are shifted left (P < 0.001 in a Tukey test), and R285K (blue) is shifted right (P < 0.001 in a Tukey test). K67A/K289A (green) is non-functional (P < 0.0001 in a Tukey test). (c) Bright-field images of Dictyostelium AX4 cells in KK2 (0 min), and at 10 and 60 min after changing the solution to water. Scale bar, 5 μm. (d) Time course of cell increase in circularity, and recovery. Wild-type cells (black) round up for 10–15 min and then regain their normal shape by 40 min. P2XA− cells (red) stay round throughout the entire time course. A paired t-test between P2XA+ and wild-type cells revealed no significant differences at 0 and 10 min (P = 0.72) but showed significantly different (P < 0.0001) behaviour throughout the rest of the cycle. P2XA(R63A) cells (purple) initially round up; however, they fully recover their shape significantly more quickly than wild-type cells (P < 0.001 at 20 min). Both P2XA(R285K) (blue) and P2XA(K67A/K289A) (green) round up within 10 min, P2XA(K67A/K289A) remains round and is not significantly different from P2XA+ cells throughout the cycle (P > 0.84). P2XA(R285K) is able to partially recover to an intermediate phenotype that is significantly different from both wild-type (P < 0.001 at 60 min) and P2XA− cells (P < 0.001 at 60 min). Error bars represent s.e.m. and results are means of n = 4 independent experiments, each with 70 cells. Statistical source data for b and d can be found in Supplementary Table 2.
**P2XA is required to inhibit Rab11a activity**

To gain insight into how P2XA activation affects CV fusion at the molecular level, we took a proteomic approach to identify candidate proteins regulated by P2XA. For this, tagged versions of P2XA were expressed in *Dictyostelium* cells and used to pull down interacting proteins, which were identified by mass spectrometry (Fig. 5a and Supplementary Table 1). One major interacting protein was Rab11a (Fig. 5b). Consistent with previous studies\(^5\), Rab11a–RFP was localized to the CV system where it co-localized extensively with P2XA (Fig. 5c).

Moreover, we found that clones that expressed high levels of Rab11a exhibited CV system defects similar to those observed when P2XA function was disrupted, with large numbers of small vacuoles adjacent to the plasma membrane (Fig. 5d). Furthermore, these clones showed severe defects in osmotic shock responses (Fig. 5d). Importantly, it is likely that these defects are due to changes in the level of Rab11a activation, because pulldowns on cell extracts with an antibody that is highly specific for activated Rab11a\(^{GTP}\) revealed that even though the ratio of active Rab11a\(^{GTP}/ inactive\) Rab11a–GDP was the same in each clone, the absolute levels of activated Rab11a–GTP correlated well with the observed defects (Fig. 5e). On the basis of these observations, we reasoned that a simple explanation for the similarity in phenotype between the P2XA mutant and Rab11a-overexpressing clones is that P2XA is required to inhibit Rab11a activity. Consistent with this idea, pulldown assays using fusion/expulsion events than wild-type vacuoles during the time course. Error bars represent s.e.m. and results are means of \(n = 3\) independent experiments, each with 50 cells. (e) The time taken for a CV to complete a cycle was twice as long for a P2XA\(^–\) vacuole compared with wild-type vacuoles. Error bars represent s.e.m. and results are means of \(n = 3\) independent experiments, each with 50 vacuoles. (f) P2XA\(^–\) cells have larger numbers of small vacuoles than wild-type cells, and very few bigger vacuoles. P2XA\(^–\) cells overexpressing P2XA–GFP have more small vacuoles than wild-type cells, but the number of bigger vacuoles is similar to wild-type cells. Error bars represent s.e.m. and results are means of \(n = 3\) independent experiments, each with 50 cells. Statistical source data for b and d–f can be found in Supplementary Table 2. (g) Zoom of fluorescence images of P2XA\(^–\) cell from a expressing daumin–GFP. White arrows indicate a ‘budding’ vacuole. Scale bar, 5 \(\mu\)m.

**Figure 2** P2XA\(^–\) cells exhibit aberrant CV number, dynamics and morphology. (a) Fluorescence images of wild-type and P2XA\(^–\) cells expressing daumin–GFP during CV cycling. Closed arrows indicate points in the CV cycle where a CV fuses to the plasma membrane and water is expelled. Numbers represent the time in seconds after changing the media from KK2 to water. Scale bar, 5 \(\mu\)m. (b) P2XA\(^–\) cells have more vacuoles per cell than wild-type cells. P2XA\(^–\) cells overexpressing P2XA–GFP have slightly more vacuoles per cell than wild-type cells, but fewer than P2XA\(^–\) cells. Error bars represent s.e.m. and results are means of \(n = 3\) independent experiments, each with 50 cells. (c) Fluorescence images of wild-type and P2XA\(^–\) cells expressing daumin–GFP taken within the plane of the middle of the cell. Numbers represent the time in seconds after changing the media from KK2 to water. Scale bar, 5 \(\mu\)m. (d) The vacuoles in P2XA\(^–\) cells undergo far fewer...
Figure 3  
P2XA<sup>−</sup> cells fail to undergo the ring-to-patch transition stage of the CV cycle. (a) Fluorescence images of a wild-type and P2XA<sup>−</sup> cells expressing drainin-GFP. Arrows indicate vacuoles at the 'ring to patch' transition stage. In P2XA<sup>−</sup> cells, drainin-GFP becomes recruited to CVs as they mature. However, drainin-GFP localization does not change and 'ring to patch' transition is not observed. Arrows indicate vacuoles that have reached the stage where 'ring to patch' transition should occur. (b) Fluorescence images of wild-type and P2XA<sup>−</sup> cells expressing Rab8a-GFP. Arrows indicate a vacuole at the 'ring to patch' transition stage. In P2XA<sup>−</sup> cells, Rab8a-GFP becomes recruited to mature CVs. However, Rab8a-GFP localization does not change and 'ring to patch' transition is not observed. Numbers represent the time in seconds after changing the media from KK2 to water. Scale bars, 5 μm.

the Rab11a–GTP-specific antibody revealed that the ratio of bound active Rab11a–GTP/unbound inactive Rab11a–GDP was significantly increased in P2XA mutant cells (Fig. 5f,g). Consequently, P2XA mutant cells exhibited an almost 2.5-fold increase in the of levels Rab11a–GTP, which was comparable to increases in Rab11a overexpression sufficient to generate the same osmoregulation phenotype (Fig. 5d–g).

cnrF encodes a calcium-regulated Rab GAP required for Rab11a inactivation

As the P2XA ion channel is calcium-permeable, we reasoned that calcium passing through the activated channel could provide a regulatory mechanism for Rab activity regulation. We thus took a bioinformatic approach to identify putative calcium-sensitive regulators of Rab activity. Rab activity is controlled by the opposing actions of inactivating Rab GAPs and activating Rab GEFs. Whereas Rab GEFs remain poorly characterized, Rab GAPs can be identified by virtue of the conserved TBC domain. These studies resulted in the identification of a conserved family of proteins containing both a putative TBC Rab GAP domain and calcium-binding EF hands (Supplementary Fig. 7). As the Dictyostelium genome encodes three such proteins, to determine whether any were involved in Rab11a regulation, single-gene-disruption strains were generated. Consistent with this idea, we found that knockout of one such gene, cnrF, resulted in osmotic shock and CV defects identical to those observed in P2XA-null mutant or Rab11a-overexpressing cells (Fig. 6a–c). Furthermore, gene-replacement strains in which the wild-type cnrF locus was replaced by versions containing specific point mutations that disrupt either GAP activity (R270A; ref. 37) or calcium binding by the EF hands (E623Q/D659Q; ref. 38) were indistinguishable from the null mutant in their osmoregulation phenotype (Fig. 6a–c). Finally, disruption of cnrF activity, and point mutations that disrupt EF-hand function or Rab GAP activity, all led to a significant increase in the ratio of active Rab11a–GTP/inactive Rab11a–GDP (Fig. 6d,e). In contrast, cnrF overexpression led to a significant decrease in the ratio of Rab11a–GTP/Rab11a–GDP compared with wild-type cells (Fig. 6d,e).

The above results suggest that cnrF encodes a calcium-regulated Rab11a Rab GAP. To further investigate this idea we next measured the Rab GAP activity of bacterially expressed cnrF<sup>22,37</sup>. First, we found that
Figure 4 GCaMP2 sensor indicates that P2XA activity is increased in docked vesicles. (a) Currents evoked by ATP (black bar, concentrations indicated) in HEK cells expressing P2XA (left) or P2Xa–GCaMP2 (right) receptors. (b) Concentration–response curves for HEK cells expressing wild-type (black) or (green) P2Xa–GCaMP2 receptors show no significant difference in sensitivity to ATP. Error bars represent s.e.m. and results are means from \( n = 21 \) wild-type and \( n = 7 \) P2Xa–GCaMP-expressing cells. (c) Concentration–response curve for GCaMP2 fluorescence in HEK cells expressing P2Xa–GCaMP2. The ordinate is normalized to the maximal fluorescence observed with ATP. Effective concentrations of ATP are similar to those in b (error bars represent s.e.m. and results are means from \( n = 7 \) cells). (d) Line scans from HEK cells expressing P2Xa–GCaMP2 at 0, 20 and 50 s after ATP application show increased fluorescence at the plasma membrane. Scale bar, 10 \( \mu \)m. (e) ATP (1 mM) increases GCaMP2 fluorescence in cells expressing wild-type P2Xa receptors (filled green squares), but not in cells expressing P2Xa(K67A/K289A) receptors (open green squares) or P2Xa–GFP receptors (black squares). Error bars represent s.e.m. and results are means from \( n = 6 \) cells. (f) Dictyostelium cells co-expressing P2Xa–RFP and either P2Xa–GCaMP2 (filled green squares), P2Xa(K67A/K289A)–GCaMP2 (open green squares) or P2Xa–GFP (filled black squares) were subjected to osmotic shock. The ratio of GFP to RFP fluorescence was measured at three stages throughout the cycle (maturing, docked and ring-to-patch) for each strain (except cells expressing P2Xa(K67A/K289A)–GCaMP2 receptors were not studied at ring-to-patch stage because these cells do not undergo that stage). The GFP/RFP ratio in cells expressing P2Xa–GCaMP2 increased significantly at the later stages of the CV cycle (paired \( t \)-test between P2Xa–GCaMP2/P2Xa–RFP and P2Xa–GFP/P2Xa–RFP gives \( P < 0.001 \) at both docked and ring-to-patch stages). In cells expressing P2Xa(K67A/K289A)–GCaMP2 or P2Xa–GFP, the GFP/RFP ratio remained constant throughout the cycle (error bars represent s.e.m. and results are means from \( n = 10 \) cells). (g) Fluorescence images of Dictyostelium cells co-expressing P2Xa–GCaMP2 and P2Xa–RFP, P2Xa–GFP and P2Xa–RFP or P2Xa(K67A/K289A)–GCaMP2 and P2Xa(K67A/K289A)–RFP during osmoregulation. Arrows indicate ‘ring to patch’ transition of P2Xa and fusion events. Scale bar, 5 \( \mu \)m.
CnrF exhibited GAP activity towards Rab11a, but not Rab8, another CV-localized Rab (Fig. 7a,b). Second, the efficiency of GAP activity was greatly increased by the presence of calcium (Fig. 7a–c). Importantly, this calcium-dependent increase was dependent on the presence of the EF-hand domain (Fig. 7a,b). It is noteworthy, however, that deletion of the EF hands did not completely eliminate GAP activity, as activity could be detected when a high concentration of truncated CnrF was added to the in vitro assay (Fig. 7a,b). Therefore, calcium binding through the EF hand seems to play a crucial role in regulating the efficiency of CnrF Rab GAP activity. This idea was further supported by in vivo studies, because rescue of the cnrF mutant osmoregulation defects required much greater levels of exogenous expression of the EF-hand truncated version of cnrF, when compared with full-length cnrF (Fig. 8a). Similar findings were observed when the EF-hand point mutant was expressed in cnrF mutant cells, although rescue was never seen with the Rab GAP domain point mutant, further highlighting the critical importance of Rab GAP activity for cnrF function (Fig. 8b). Finally, although CnrF was not specifically localized to the CV (Fig. 8c), both full-length and truncated CnrF interacted with P2XA and Rab11a in pulldown assays (Fig. 8d) in the presence and absence of calcium (Fig. 8e). These findings suggest that CnrF exists in a protein–protein complex with Rab11a and P2XA, and that it is the Rab GAP activity, rather than the binding of CnrF to Rab11a, that is regulated by calcium (Fig. 8f).
Figure 6 *cnrF* is required for normal osmoregulation and regulation of Rab11a activity. (a) *cnrF* mutant cells exhibit impaired osmoregulation. Representative bright-field images of cells in KK2, and after 10 and 60 min after changing the media from KK2 to water to induce osmotic shock. Scale bar, 5 μm. (b) Time course of cell rounding and recovery. *cnrF*, *cnrF*(R270A) and *cnrF*(E623Q/D659Q) mutant cells exhibit similar osmoregulation defects to *P2XA* cells. Error bars represent s.e.m. from n = 3 independent experiments, each with 100 cells. (c) Visualization of CV morphology in wild-type, *P2XA*, *cnrF*, *cnrF*(R270A) or *cnrF*(E623Q/D659Q) cells expressing *dajuminGFP* after osmotic shock. All *cnrF* mutants contain many irregularly sized vacuoles at the cell surface that fail to fuse. Scale bar, 5 μm. (d,e) Immunoprecipitation of Rab11a-GTP from wild-type, *P2XA*, *cnrF*, *cnrF*(R270A), *cnrF*(E623Q/D659Q) and CnrF-GFP-overexpressing cells expressing Rab11a-RFP (I, input, B, bound, NB, not bound). The level of Rab11a-RFP expression (input) is comparable between all strains (>1.3-fold difference between highest and lowest). (e) Quantification of GTP- and GDP-bound Rab11a revealed that all mutant strains exhibit a significant (paired t-test *P* < 0.001) 2-2.5-fold increase in the levels of Rab11a-GTP compared with wild-type cells. Furthermore CnrF-GFP-overexpressing cells exhibit a reciprocal 2-fold decrease in GTP-bound Rab11a compared with wild-type (paired t-test *P* < 0.001) cells. Error bars represent s.e.m. from n = 3 independent experiments. Statistical source data for b and e can be found in Supplementary Table 2. Uncropped images of blots are shown in Supplementary Fig. 8.
Figure 7 cnrF encodes a calcium-sensitive Rab11a GAP. (a) GTP hydrolysis of Rab11a or Rab8 by bacterially expressed CnrF. Rabs were incubated with 10 nM or 100 nM GST–CnrF1–670 (full length), GST–CnrF1–410 (truncation that removes EF hands) or GST–disgorgin382–717 in the presence or absence of 20 mM calcium. When putative GAPs were incubated at 10 nM, GST–CnrF1–670 shows significant Rab11a-GTP hydrolysis, but only in the presence of 20 mM calcium (paired t-test \( P < 0.001 \)). When putative GAPs were incubated at 100 nM, both GST–CnrF1–670 and GST–CnrF1–410 show significant Rab11a-GTP hydrolysis. The addition of 20 mM calcium has no significant effect (paired t-test \( P = 0.87 \)). Only GST–disgorgin382–717 shows significant Rab8 hydrolysis (paired t-test \( P < 0.001 \)), which is unaffected by the addition of 20 mM calcium (paired t-test \( P = 0.83 \)). The dashed line represents the mean \( \text{Abs}_{360} \) when 0 nM GAP is added (s.e.m. = 0.00423). Error bars represent s.e.m. from \( n = 3 \) independent experiments, each with 5 technical replicates. (b) Time courses of Rab11a-GTP or Rab8-GTP hydrolysis with 0, 10, 50 or 100 nM GAPs. Error bars represent s.e.m. from \( n = 3 \) independent experiments, each with 5 technical replicates. (c) Calcium-dependent Rab11a-GTP hydrolysis is observed at a wide range of GST–CnrF1–670 concentrations (0.5–5 nM). Results are means of 2 independent experiments, each with 3 technical replicates. (d) In the presence of rate-limiting amounts of Rab11a-GTP (2.5 \( \mu \)M GST–CnrF1–670) exhibits significant levels of calcium-independent Rab11a-GTP hydrolysis. Results are means of 2 independent experiments, each with 3 technical replicates. (e) When Rab11a is present at a non-limiting concentration (25 \( \mu \)M), the presence of 20 mM calcium increases the rate of Rab11a hydrolysis by both 50 nM and 100 nM GST–CnrF1–670. These results suggest that calcium can increase the efficiency of Rab11a hydrolysis by GST–CnrF1–670 at all GAP concentrations tested. Results are means of 2 independent experiments, each with 3 technical replicates. Statistical source data for a–e can be found in Supplementary Table 2.
GTP cycle requires the correct

a

![Graph](image)

**DISCUSSION**

Precise regulation of the *Dictyostelium* CV cycle requires the correct spatial and temporal regulation of a Rab small GTPase cascade, as well as the activity of the CV-localized P2XA receptor ion channel. We show that activation of P2XA results in localized increases in calcium concentration, which activates a calcium-sensitive Rab GAP and in turn downregulates its target Rab11a, which is required for vesicle fusion with the plasma membrane (Fig. 8f). Interestingly, earlier studies

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**Figure 8** *cnrF* is a cytosolic calcium-dependent Rab11a GAP that interacts with P2XA and Rab11a. (a) Rescue of the *cnrF* mutant null phenotype by full-length (1–670) *cnrF–RFP* and a truncated (1–410) form of *cnrF–RFP* that lacks the EF hands. Cell lines were selected on the basis of RFP expression levels. *cnrF*/*cnrF* low (2064 A.U.), *cnrF*/*cnrF* high (10106 A.U.) and *cnrF*/*cnrF* wild-type (9987 A.U.) cells all show an indistinguishable hypo-osmotic shock response to wild-type (paired t-test *P* > 0.01) cells. However, *cnrF*/*cnrF* low (2111 A.U.) cells fail to rescue the mutant phenotype (paired t-test *P* > 0.05). Error bars represent s.e.m. and results are means of *n* = 3 independent experiments, each with 100 cells. Statistical source data for a and b can be found in Supplementary Table 2. (c) *cnrF–GFP* expression in wild-type cells. Scale bar, 5 μm. (d) Rab11a and P2XA (but not Rab8a) bind GST–CnrF (E623Q/D659Q) or GST–CnrF1–410, but not GST–Rab GAP421 control (a related *Dictyostelium* EF-hand-domain-containing Rab GAP (DDB_G0275421)). (e) Rab11a binding to CnrF is not calcium dependent. Varying amounts of protein from cells expressing Rab11a:RFP were incubated with beads bound with GST–CnrF (E623Q/D659Q) or GST–CnrF1–410 in 20 mM Ca²⁺ or 100 mM EGTA. (f) Proposed model of P2XA-regulated vesicle fusion. Maturing vacuoles express P2XA, Rab11aGTP and drainin. Once tethered, P2XA undergoes a ‘ring to patch’ transition so that P2XA is expressed only at the plasma membrane contact site. Concentration of P2XA activity leads to a localized increase in calcium ions as they pass through the active channel. Consequently, P2XA-bound CnrF is activated as Ca²⁺ binds to the EF-hand domain on CnrF, leading to the hydrolysis and inactivation of Rab11a–GTP, and therefore inactivation of drainin at the plasma membrane contact site. Uncropped images of blots are shown in Supplementary Fig. 8.
have revealed that Rab11a localization does not change on vacuole fusion\cite{22,23,25}. However, the Rab11a–GTP specific binding protein, drainin, is removed from the membrane at the fusion site, as predicted by our model\cite{22,23}. Furthermore, expression of constitutively active Rab11a blocks this redistribution of drainin\cite{22}. These findings suggest that the rate of Rab11a effector dissociation is faster than the rate of removal of inactivated Rab11a–GDP from the CV membrane. It is therefore likely that CV fusion requires a change in Rab11a activity at the site of vacuole fusion, but not redistribution of Rab11a itself, only its effector drainin. Thus, this study provides a mechanistic link between P2X ion channel activity and Rab-regulated vesicular trafficking.

Our data provide further evidence that P2X receptors can play a role in regulating intracellular events, in addition to their well-established role in regulating responses to extracellular ATP. However, it is important to note that P2X receptors are also expressed on intracellular membranes in some cell types of higher organisms\cite{39,40}. Furthermore, evidence is emerging to support their functional importance. For example, P2X4 receptors have been shown to localize to exocytic lamellar bodies, intracellular vesicles found within alveolar type II epithelial cells\cite{39}. Expression of dominant-negative P2X4 in these cells has led to the suggestion that endogenous P2X4 is required to stabilize and expand the fusion pore to allow sufficient release of lung surfactant\cite{39}, although the molecular mechanism has not been determined.

As the function of P2X receptors in CV fusion requires intact ATP-binding residues, this raises the question of how channel activity is regulated. So far, only ATP has been shown to act as an efficient ligand for P2XA (ref. 34), although where the ATP comes from and how its levels are controlled are unknown. It does, however, seem likely that the ATP must come from the vacuole itself, rather than the extracellular space, as hypo-osmotic shock and activation of P2XA occurs when the cells are placed in water. Furthermore, the putative ATP-binding domain of P2XA has been shown to reside within the lumen of the CV (refs 35,41). One possibility, therefore, is that ATP, although present throughout the CV cycle, acts as a permissive ligand that is able to activate the receptors only when the ionic conditions or pH encountered are optimal. Some support for this idea comes from our recent finding that the ionic and proton environment encountered by the ATP-binding domain (that is, that of the lumen of the vacuole) has a large effect on the level of P2XA activation\cite{34}. Moreover, the CV contains a high concentration of proton pumps (V-type ATPases)\cite{42}, and best estimates suggest that the vesicles may be acidified to pH 6.2 (ref. 43). Under these conditions the P2XA channel is rapidly desensitized, and may remain so under conditions of continued acidification\cite{34}. However, when the vacuole fills with water, the proton concentration would fall, possibly allowing ATP to better activate P2XA. Alternatively, regulation of ATP translocation could conceivably allow ATP levels to reach the threshold concentration for P2XA activation only when vacuoles are ready to fuse\cite{35}. However, this raises more questions as to the exact concentration of ATP in the CV, and the identity of the ATP transporter.

Many studies have highlighted the importance of calcium in the regulation of vesicle fusion events. They have revealed that calcium is released from the vacuole lumen and that required changes are localized and fast. Indeed, measurements using the P2XA–GCaMP2 sensor described in this study reveal that the calcium concentration around the CV increases almost twofold before fusion. We estimate that this results in a localized calcium concentration of 100–200 nM, assuming basal cytoplasmic levels are 50–90 nM (ref. 44). However, in many systems, studies of the role of calcium changes have been hampered by the lack of available genetic tools. Most notably, identification of specific calcium channels responsible for release has proved elusive\cite{7,19,19}. Our studies thus provide a significant advance as they reveal that activity of a calcium-permeable ion channel within vesicles can affect vesicle fusion. Most importantly, we have also demonstrated a molecular mechanism by which calcium efflux through ion channels can regulate Rab-dependent vesicle fusion events. Several lines of evidence suggest that this is likely to represent a conserved mode of action. For example, the key principles and regulatory components of different intracellular vesicular trafficking mechanisms are broadly conserved at the molecular level. Most importantly, sequence comparisons reveal that the genomes of higher organisms contain several homologues of the calcium-dependent Rab GAP, CnrF (Supplementary Fig. 8), which represent good candidates for ion channel or calcium-dependent regulation.

Understanding how the sequential activation of Rab GTPases is achieved during vesicle trafficking is a central question in cell biology. Several different solutions to this problem have been proposed. For example, in the Rab GEF cascade, activation of one Rab results in the recruitment of effectors, including the GEF for the downstream Rab\cite{2,45}. Counter cascades of Rab GAP recruitment serve to amplify the cascade\cite{46,46} or prevent premature activation of the next Rab in the cascade\cite{47}. However, this in turn raises the question of how regulatory Rab GEFs or Rab GAPS are activated at the right place and time. Our studies thus provide one simple solution to this problem as they illustrate a mechanism by which a constitutively expressed Rab GAP can be temporally and spatially activated to correctly regulate the activity of one or more different Rabs to allow vesicle fusion.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

K.P. performed most of the experiments. A.E.B. performed the electrophysiology, P2XA point mutant and GCaMP2 studies; T.K. created the CnrF Rab GAP knockout strain; N.G. performed the bioinformatic analysis of EF-hand-containing Rab GAPS; L.B. created the P2XA point mutants for expression in HEK cells. C.R.L.T. and R.A.N. conceived of the study and wrote the manuscript. All authors discussed the results, and contributed to writing or commenting on the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Plasmid construction. For deletion of the P2XA and putative Rab GAP genes, a flexed blasticidin cassette was cloned within genomic fragments flanking the coding sequence of each gene. Linearized construct was transformed into AX4 cells by electroporation followed by blasticidin selection and confirmation of gene deletion by PCR. The blasticidin cassette was then removed by Cre recombinase.

Identification of P2XA-interacting proteins. For identification of interacting proteins, GluTrap (Chromek Technology) was employed. A total of 3 × 10^4 cells expressing either GFP or P2XA–GFP were lysed in 250 µl buffer (50 mM Tris HCl, 0.5% Triton, 2 mM MgCl₂ and 150 mM NaCl, supplemented with protease inhibitor (Complete; Roche)). The lysate was centrifuged for 15 min at 16,000g and 75 µl of supernatant was incubated with 15 µl GFP-Trap agarose beads under slight agitation for 1 h at 4°C. The beads were washed according to the manufacturer’s protocol, and bound proteins were eluted by boiling in SDS sample buffer. Proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue-R. For protein identification, bound proteins were run into the top of an SDS–PAGE gel, excised and then analysed by mass spectrometry using the Waters Q-TOF Micro with Waters CapLC chromatography system after overnight trypsinisation.

Rab11a–GTP measurement assay. Rab11a activity was measured in all strains using a conformation-specific antibody that specifically recognizes Rab11a–GTP (NewEast Biosciences catalogue number 26919). For in vitro precipitation of Rab11a–GTP, 1 µg bacterially expressed GST–Rab was incubated with 20 µM EDTA and either 100 µM GTP-γ-S or 1 mM GDP for 30 min at 30°C. GTP/GDP loading was stopped by placing the tubes on ice and adding 60 mM MgCl₂. Reaction volume was adjusted to 1 ml with lysis buffer (50 mM Tris-HCl at pH 8, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA and 1% Triton X-100) before incubating with 1 µl of anti-Rab11a–GTP antibody and 20 µl of protein A/G agarose (50% slurry) for 1 h at 4°C with gentle agitation. Beads were washed three times in lysis buffer before elution of bound protein by boiling in 20 µl of 2X SDS sample buffer. Proteins were separated by SDS–PAGE and immunoblotted for GST using an anti-GST antibody (Abcam catalogue number ab6615). For in vivo precipitation of Rab11a–GTP, 1 × 10^5 cells expressing Rab11a–GFP were lysed in 1 ml lysis buffer (50 mM Tris-HCl, at pH 8, 150 mM NaCl, 10 mM MgCl₂, 1 mM EDTA and 1% Triton X-100). Lysate was then incubated with 1 µl of anti Rab11a–GTP antibody and 20 µl of protein A/G agarose (50% slurry) for 1 h at 4°C with gentle agitation. Beads were washed three times in lysis buffer before elution of bound protein by boiling in 20 µl of 2X SDS sample buffer. Proteins from input, bound and not bound fractions were separated by SDS–PAGE and immunoblotted for GFP using an anti-GFP antibody (Santa Cruz Biotechnology catalogue number sc-20477) was used as a loading control for each sample. The ratio of bound Rab11a–GFP (Rab11a–GTP) and unbound Rab11a–RFP (Rab11a–GDP) was calculated for each clone after normalizing against the actin loading control. Densitometry analyses of western blots were performed in ImageJ. All blot analyses were in the linear range.

GST pulldown and GAP assays. GST-fused proteins were expressed in BL21 (DE3) bacteria and purified on glutathione–Sepharose beads. GAP assays were performed as previously described. Briefly, 10 nM or 100 nM GAPs were incubated in the presence of 2.5 µM purified Rab pre-loaded with GST. The amount of released inorganic phosphate was measured using the EnzChek Phosphate Assay Kit (Invitrogen) in 96-well plates according to the manufacturer’s instructions. Changes in absorbance were measured at 360 nm using a BioTek Synergy HT plate reader. Five technical and three independent biological replicates were performed. For GST pulldowns from Dictyostelium cell lysates, AX4 cells expressing Rab11a–RFP, Rab8–RFP or P2XA–RFP were incubated at 4°C for 1 h with 10 µg of GST–GAP bound to glutathione–agarose beads. After washing the beads three times, bound proteins were eluted by boiling in SDS sample buffer. Eluted proteins were run on SDS–PAGE gel and probed with anti-RFP antibody (Chromotek catalogue number 3D5).
Supplementary Figure 1 A P2XA deletion mutant exhibits defects in osmoregulation. A. Schematic to show the generation of a P2XA deletion construct for use in homologous recombination. 1.6kb downstream and 1.4kb upstream of the P2XA coding sequence were amplified and cloned either side of the floxed blasticidin cassette in pLPBLP. B. Bright field images of cells in KK2, and after 10 min and 60 min after changing the media from KK2 to water to induce osmotic shock. Wild-type cells round up within 10 minutes, but fully recover to their normal shape within 60 minutes in water. P2XA- cells also round up within 10 minutes, but fail to recover their shape even after 60 minutes in water. P2XA- cells expressing P2XA:GFP behave like wild-type cells. Wild-type cells treated with 10uM copper fail to recover their shape after 60 minutes in water. Scale bar = 20μm C. Time course of cell rounding and recovery. Wild-type cells (black squares, solid line) round up for 20-30 min and then regain their normal shape by 40 minutes. P2XA- cells (red square, solid line) become rounder than wild type cells and stay round throughout the entire time course. P2XA- cells expressing P2XA:GFP (red square, dashed line) are similar to wild-type cells, but get slightly rounder and take longer to regain their shape. Wild-type cells treated with 10 μM copper (black square, dashed line) stay round throughout the entire time course. Error bars represent s.e.m. and results are means of n=3 independent experiments, each with 120 cells. Statistical source data for Supplementary Fig 1C can be found in Supplementary Table 2.
Supplementary Figure 2 P2XA mutants correctly localise to the contractile vacuole. Fluorescent images of RFP tagged mutants expressed in wild-type *Dictyostelium*. Fluorescence shows that P2XA(R63A), P2XA(R285K) and P2XA(K67A/K289A) all correctly localise to the contractile vacuole in *Dictyostelium*. Scale bar = 5μm.
Supplementary Figure 3 P2XA is specifically expressed on CV’s at all stages of the cycle and undergoes a ‘ring to patch’ transition. A. Localization of Rab7:GFP and P2XA:RFP in wild-type cells. No co-localization is observed. Scale bar = 5 μm. B. Localization of Dajumin:GFP and P2XA:RFP in wild-type cells at the maturation (top panel) and ‘ring to patch’ (bottom panel) stages of the CV cycle. P2XA:RFP co-localizes with Dajumin:GFP during maturation, but localization of P2XA:RFP is restricted to the plasma membrane contact site of the CV during the ring to patch transition stage. Scale bar = 5 μm. C. Localization of drainin:GFP and P2XA:RFP in wild-type cells at the maturation (top panel) and ‘ring to patch’ (bottom panel) stages of the CV cycle. P2XA:RFP co-localizes with drainin:GFP at all stages of the cycle. Scale bar = 5 μm. D. Localization of rab8a:GFP and P2XA:RFP in wild-type cells. P2XA:RFP co-localizes with rab8a:GFP during the ‘ring to patch’ transition stage of the CV cycle. Scale bar = 5 μm.
Supplementary Figure 4 GCaMP2 acts as a calcium sensor in HEK cells. A. Confocal images of P2XA-GCaMP2 expressed in HEK cells before (control), during (+ATP) and after (wash) stimulation with ATP (1mM-10mM). Increases in fluorescence can be seen when stimulated with 100mM, 300mM and 1mM ATP. B. GCaMP2 acts as a sensor for P2XA activity. Fluorescent confocal images of HEK cells expressing P2XA-GCaMP2, P2XA-GFP and P2XA(K67A/K289A)-GCaMP2 over time. Following stimulation with 1mM ATP, P2XA-GCaMP2 cells show a transient increase in fluorescence at 20 seconds, whilst there is no change in fluorescence for P2XA-GFP or P2XA(K67A/K289A)-GCaMP2. Scale bar = 10 μm.
Supplementary Figure 5 GCaMP2 acts as a calcium sensor Dictyostelium cells. A. Time course of cell rounding and recovery of P2XA- cells expressing P2XA-GCaMP2. Over-expression of P2X-GCaMP2 in the P2XA- background (green) rescues the osmoregulation phenotype so that cells transiently round up after 10 mins in water but are able to recover their shape within 60 mins. Error bars represent s.e.m. and results are means of n=4 independent experiments. Statistical source data for Supplementary Fig 5A can be found in Supplementary Table 2. B. Fluorescence LUT images of Dictyostelium cells expressing Actin15-GCaMP2 or Actin15-GFP. Increases in fluorescence are observed in cells expressing Actin15-GCaMP2 after ATP stimulation (30mM) in the presence of extracellular Ca^{2+} only. No changes in fluorescence are observed in cells expressing Actin15-GFP in the presence or absence of Ca^{2+}. Scale bar = 15 μm. C. Plot of changes in fluorescence of cells expressing Actin15-GCaMP2 or Actin15-GFP over time following 30mM ATP stimulation. Error bars represent s.e.m. and results are means of n=5 independent experiments, each with 5 cells. Increases in fluorescence are observed in cells expressing Actin15-GCaMP2 only in the presence of extracellular Ca^{2+}. © 2014 Macmillan Publishers Limited. All rights reserved.
Supplementary Figure 6 Validation of Rab11a-GTP specific antibody. A. The anti rab11-GTP antibody is specific for rab11-GTP in vitro. Rab11a-GST or Rab8-GST were loaded with either GTPγS or GDP, incubated with anti rab11-GTP antibody and then immunoprecipitated using protein A/G agarose beads. Bound protein was probed with an anti-GST antibody. The anti rab11-GTP antibody could only immunoprecipitate bacterially expressed rab11a when loaded with GTP. B. The anti rab11-GTP antibody is specific for rab11a-GTP in vivo. Immunoprecipitation of rab11a-GTP from wild-type cells expressing Rab11a-RFP, Rab7-RFP or Rab8-RFP. Lysate from each strain was incubated with anti rab11-GTP antibody and immunoprecipitated using protein A/G agarose beads. Bound and unbound proteins were probed with an anti RFP antibody. (I = input, B = bound, NB = not bound). The anti rab11-GTP antibody could only immunoprecipitate RFP from cells expressing rab11a-RFP. C. The anti rab11-GTP antibody works in the linear range for protocols used throughout the manuscript. Top panel: Lysate from AX4 cells expressing rab11a-RFP was used neat, at a 1:2 dilution or a 1:4 dilution for immunoprecipitation of rab11a-GTP using the anti rab11-GTP antibody. Bottom panel: Densitometry analyses of bound (rab11a-GTP) and unbound (rab11a-GDP) bands reveal that the GTP/GDP ratios remain constant for each sample dilution. Error bars represent s.e.m. and results are means of n=3 independent experiments. Statistical source data for Supplementary Fig 6C can be found in Supplementary Table 2.
### Supplementary Figure 7

An evolutionarily conserved group of Rab GAPs containing a TBC Rab GAP domain and EF hands. A conserved group of Rab GAPs containing a TBC Rab GAP domain and EF hands from different phyla. Proteins from *D. discoidium* (CnrF, DDB_G0295717 and DDB_G0275421), *S. pombe* (NP_596678), *P. tetraurelia* (XP_001441542), *D. rerio* (NP_001120987), *X. tropicalis* (NP_001107313), *M. musculus* (NP_001104774), *H. sapiens* (NP_942568) are shown. A. Protein structure is conserved throughout evolution. B. Alignment of the TBC Rab GAP domain. Homologous sequences were identified using the conserved domains database (Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, Deweese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thaini N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* 2011 Jan;39(Database issue):D225-9. Epub 2010 Nov 24. [PubMed PMID: 21109532]). The alignment was computed using MUSCLE requesting 16 iterations (MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004, 32:1792–1797.). The aligned TBC Rab GAP domain was extracted using CLC Genomics workbench (www.CLCbio.com). C. Alignment of the EF hand domain. Homologous sequences were identified using the conserved domains database (CDD ref). The alignment was computed using MUSCLE requesting 16 iterations (MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004, 32:1792–1797.). The aligned EF hand domain was extracted using CLC Genomics workbench (www.CLCbio.com).
Supplementary Figure 8 Uncropped scans of western blots included in figures.
Supplementary Table Legends

**Supplementary Table 1** Table of proteins that are pulled down by P2XA using a GFP trap. Table of proteins that were eluted bound to GFP trap beads incubated with lysate from wild type cells expressing P2XA-GFP. The number beside each protein represents the number of peptide matches.

**Supplementary Table 2** Statistical source data

Supplementary Video Legends

**Supplementary Video 1** *dajumin:*GFP expression in wild type cells
Live cell fluorescence imaging of a wild-type cell expressing *dajumin:*GFP during CV cycling (imaged between 0 and 330 seconds after osmotic shock).

**Supplementary Video 2** *dajumin:*GFP expression in P2XA− cells
Live cell fluorescence imaging of a *P2XA−* cell expressing *dajumin:*GFP during CV cycling (imaged between 60 and 210 seconds after osmotic shock).

**Supplementary Video 3** *drainin:*GFP expression in wild type cells
Live cell fluorescence imaging of a wild-type cell expressing *drainin:*GFP during CV cycling (imaged between 60 and 190 seconds after osmotic shock).

**Supplementary Video 4** *drainin:*GFP expression in P2XA− cells
Live cell fluorescence imaging of a *P2XA−* cell expressing *drainin:*GFP during CV cycling (imaged between 60 and 190 seconds after osmotic shock).

**Supplementary Video 5** *rab8:*GFP expression in wild type cells
Live cell fluorescence imaging of a wild-type cell expressing *rab8:*GFP during CV cycling (imaged between 240 and 350 seconds after osmotic shock).

**Supplementary Video 6** *rab8:*GFP expression in P2XA− cells
Live cell fluorescence imaging of a *P2XA−* cell expressing *rab8:*GFP during CV cycling (imaged between 240 and 350 seconds after osmotic shock).

**Supplementary Video 7** P2XA-GCaMP2 in HEK cells
Live cell fluorescence imaging of a HEK cells expressing P2XA-GCaMP2 and stimulated with ATP (1mM) which causes a transient increase in fluorescence.

**Supplementary Video 8** P2XA-GFP in HEK cells
Live cell fluorescence imaging of a HEK cells expressing P2XA-GFP and stimulated with ATP (1mM). No increase in fluorescence is observed.

**Supplementary Video 9** P2XA-GCaMP2/P2XA-RFP co-expressing cell
Live cell fluorescence imaging of a wild-type cells co-expressing P2XA-GCaMP2 and P2XA-RFP and imaged in water during osmotic shock.

**Supplementary Video 10** P2XA-GFP/P2XA-RFP co-expressing cell
Live cell fluorescence imaging of a wild-type cells co-expressing P2XA-GFP and P2XA-RFP and imaged in water during osmotic shock.

**Supplementary Video 11** P2XA(K67A/K289A)-GCaMP2/P2XA(K67A/K289A)-RFP co-expressing cell
Live cell fluorescence imaging of a wild-type cells co-expressing mutant (K67A/K289A) P2XA-GCaMP2 and mutant (K67A/K289A) P2XA-RFP and imaged in water during osmotic shock.