A polycystin-type transient receptor potential (Trp) channel that is activated by ATP

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ABSTRACT

ATP and ADP are ancient extra-cellular signalling molecules that in *Dictyostelium* amoebae cause rapid, transient increases in cytosolic calcium due to an influx through the plasma membrane. This response is independent of hetero-trimeric G-proteins, the putative IP3 receptor IplA and all P2X channels. We show, unexpectedly, that this is abolished in mutants of the polycystin-type transient receptor potential channel, TrpP. Responses to the chemoattractants cyclic-AMP and folic acid are unaffected in TrpP mutants. We report that the DIF morphogens, cyclic-di-GMP, GABA, glutamate and adenosine all induce strong cytoplasmic calcium responses, likewise independently of TrpP. Thus, TrpP is dedicated to purinergic signalling. ATP treatment causes cell blebbing within seconds but this does not require TrpP, implicating a separate purinergic receptor. We could detect no effect of ATP on chemotaxis and TrpP mutants grow, chemotax and develop almost normally in standard conditions. No gating ligand is known for the human homologue of TrpP, polycystin-2, which causes polycystic kidney disease. Our results now show that TrpP mediates purinergic signalling in *Dictyostelium* and is directly or indirectly gated by ATP.

KEY WORDS: Trp channel, Polycystin-2, Purinergic signalling, ATP, DIF, *Dictyostelium*

INTRODUCTION

ATP and other purines are ancient signalling molecules used widely in animals as neuro-transmitters and also by protozoa and plants for diverse purposes (Burnstock and Verkhratsky, 2009). The signalling pathway typically consists of release of ATP, its detection by cell surface receptors with consequent signal transduction, and destruction of the signal by ecto-ATPases. Two types of ATP receptor are known in mammalian cells: P2X receptors are gated ion channels, which generally allow calcium into the cell and P2Y receptors are G-protein coupled receptors (GPCRs) (Burnstock, 2007).

Calcium signalling also has ancient origins and it is likely that ancestral single-celled eukaryotes were able to produce Ca\(^{2+}\) gradients across their plasma membrane using calcium pumps and transporters, and activate calcium entry into the cytoplasm through regulated channels in the plasma membrane and the membranes of internal vesicular stores of Ca\(^{2+}\) ions. Changes in free Ca\(^{2+}\) ion concentration could then alter the activity of sensitive proteins and processes in the cytoplasm. Present day microbes use calcium signalling in a wide variety of ways and have recognizable homologues in their genomes to many components of calcium signalling found in mammalian cells (Martinac et al., 2008; Collins and Meyer, 2011; Plattner and Verkhratsky, 2015). These ancient signalling processes can be combined so that ATP causes a cytoplasmic calcium increase.

The social amoeba *Dictyostelium discoideum* grows on bacteria or in liquid media as separate cells (Kessin, 2001). These cells respond to starvation by aggregating together by chemotaxis, to form a multicellular mass and ultimately a stalked fruiting body carrying a mass of spores at its top. In the growth phase the cells are chemotactic to folic acid, which guides them to bacteria, and after starvation they become chemotactic to cyclic AMP (cAMP), which is released periodically from aggregation centres, to which it attracts the amoebae. Both folic acid and cAMP are detected through G-protein coupled receptors (Klein et al., 1988; Pan et al., 2016) and set off a variety of intra-cellular signalling responses, including an influx of Ca\(^{2+}\). The coordinated movement and differentiation of amoebae into stalk cells and spores during development is controlled by small molecule signalling, including by cAMP, the polyketides DIF and MPBD (Morris et al., 1987; Saito et al., 2006), cyclic-di-GMP (Chen and Schaap, 2012) and GABA/glutamate (Taniura et al., 2006; Anjard and Loomis, 2006).

*Dictyostelium* cells also respond strongly to extracellular ATP and ADP, which both cause an immediate and transient increase in cytosolic Ca\(^{2+}\) due to an influx through the plasma membrane (Ludlow et al., 2008, 2009). *Dictyostelium* cells can also release ATP into the medium in micro-molar concentrations (Sivaramakrishnan and Fountain, 2015) and have an ecto-ATPase activity, which degrades ATP (Parish and Weibel, 1980), suggesting that they have a complete set of purinergic signalling components. However, the receptor responsible for the calcium influx in response to ATP is currently unknown.

The most obvious candidate for this ATP receptor is one or more of the five P2X receptors encoded in the genome, four of which have been shown to be ATP-gated calcium channels in heterologous expression experiments (Fountain et al., 2007; Ludlow et al., 2009; Baines et al., 2013). However, these receptors are largely expressed on the intracellular membranes of the contractile vacuole and have a role in its discharge (Fountain et al., 2007; Ludlow et al., 2009; Sivaramakrishnan and Fountain, 2012; Parkinson et al., 2014). Crucially, a mutant with all five P2X receptors knocked out still retains its calcium response to ATP (Ludlow et al., 2009). The *Dictyostelium* genome only carries a limited set of candidate Ca\(^{2+}\) signalling proteins (Eichinger et al., 2005; Wilczynska et al., 2005), which include two transient receptor potential (Trp) channels (Clapham, 2003; Hardie, 2007). The nearest human homologues of these *Dictyostelium* proteins are mucolipin and polycystin-2, which...
are named after the corresponding genetic diseases (Lima et al., 2012, 2014). There is also a two-pore channel and an IP3-like receptor, IplA (Traynor et al., 2000) and two potential stretch-operated channels: MscS is homologous to the bacterial small conductance mechanosensitive channel (Martinac et al., 2008) and a homologue of the eukaryotic Piezo mechanosensitive channel (Coste et al., 2010).

The role of extra-cellular ATP signalling in the Dictyostelium life-cycle is not yet clear. ATP has been reported to affect various processes, including cellular aggregation, possibly by enhancing cyclic AMP signalling (Mato and Konijn, 1975; Perekalin, 1977), and recovery from hypo-osmotic stress (Sivaramakrishnan and Fountain, 2015).

We sought to identify the channel mediating the purinergic response of Dictyostelium cells by knocking out candidate calcium channels and assessing the response of the mutant cells to ATP using a reporter for cytoplasmic calcium. In this way we show that the polycystin-type Trp channel, TrpP is essential for the response, and either is the ATP receptor, or closely coupled to it. We also show for the first time that a number of endogenous effector molecules including DIF, GABA and cyclic-di-GMP trigger calcium signals, and that these responses are independent of TrpP.

RESULTS
Visualising calcium signalling using the cameleon FRET reporter
In order to characterise the Ca\(^{2+}\) signalling triggered by ATP, we first set up a convenient assay to measure changes in cytosolic calcium concentration. The cameleon series of FRET-based, genetically encoded calcium reporters do not require loading into cells, nor do they require an added cofactor, unlike aequorin (Nagai et al., 2004; Horikawa et al., 2010). We initially characterized the well-studied response of starved, developing (aggregation-competent) cells to the chemoattractant cAMP (Abe et al., 1988; Milne and Coulkell, 1991; Nebel and Fisher, 1997; Nebel et al., 2002) (Fig. 1D). We found that the higher affinity cameleon YC2.60 (K_d 95 nM) was not saturated by a maximal dose of cAMP or ATP and could robustly detect sub-maximal doses, unlike the lower affinity YC3.60 (K_d 215 nM) and thus was ideal for our purposes (Fig. S1).

Using this reporter, we found that stimulation with a uniform concentration of cAMP causes a transient increase in cytosolic Ca\(^{2+}\) levels after a delay of 6.0±1.0 s (mean±s.e.m.; n=7), with a peak at 22.1±4.3 s (n=7) and return to baseline by 58.1±3.2 s (n=7) (Fig. 1D). Half-maximal response is somewhat variable at 87±71 nM (n=4) cAMP (Fig. S2).

cAMP signalling is mediated through a family of G-protein coupled receptors, principally cAR1 (Klein et al., 1988). These receptors appear during development and are only expressed at low levels in growing cells, which accordingly show little or no response to cAMP. However vegetative cells do respond to folic acid (Nebel et al., 2002), which is also a chemoattractant and is detected by a G-protein coupled receptor (Pan et al., 2016): as expected, folate induces a delayed Ca\(^{2+}\) response, similar to that produced by cAMP (Fig. 1C).

**TrpP mediates the calcium response to ATP**
We found that ATP and ADP robustly evoke transient increases in cytosolic Ca\(^{2+}\) from both vegetative and aggregation-competent cells (Fig. 1A,B; for a response to ADP, see Fig. 3), confirming previous work (Ludlow et al., 2008, 2009). There is a minimal delay of around 1 s (1.3±0.2 s n=7) before onset of the response to ATP, which is similar to the mixing time, and a mean rise time of 11.0±1.4 s (n=7) from baseline to peak response. The kinetics of this purinergic response clearly differ from those to folic acid and cAMP, having a much shorter lag before onset. The half-maximal response was at 1.1±0.4 µM (n=5) for ATP and 1.6±1.5 µM (n=3) for ADP in aggregation-competent cells (Fig. S2). These values were obtained only from those cells producing a response, which was more than 90% at saturating doses, but fell off at lower ATP concentrations (Fig. S2).

Genetically, the response to ATP does not depend on any of the P2X receptors encoded in the Dictyostelium genome (Ludlow et al., 2009). To try and establish the signalling route used by ATP we therefore examined the effect of mutating other signalling proteins. First, we tested dependence on hetero-trimeric G-proteins using a null mutant in the only G subunit encoded in the genome (Wu et al., 1995) and found that the response to ATP was unaffected (Fig. 2C). In contrast the responses to folate and cAMP were abolished (Fig. 2D-F). Similarly, the response in a mutant of IplA, the homologue of the endoplasmic reticulum channel activated by IP3, to ATP was also intact (Fig. 2A,B) (Traynor et al., 2000). Thus, the purinergic response does not appear to require either hetero-trimeric G-proteins or IplA (Ludlow et al., 2008), in both cases differentiating it from the GPCR-mediated responses to cAMP and folate.
We therefore investigated other candidate calcium signalling proteins and made null mutants in three potential channels: the mechano-sensitive channel homologue, MscS (dictyBase DDB_G0277253; http://dictybase.org) and two Trp channels, one a mucolipin homologue, McIIN (dictyBase DDB_G0291275) (Lima et al., 2012), the other a polycystin-2 homologue, which we call TrpP (gene, trpP; the protein is also known as PKD2; dictyBase DDB_G02772999) (Lima et al., 2014). In case of redundancy, we made a triple mutant lacking all three proteins and in our initial experiments used the uptake of 45Ca2+ to measure the response. To our surprise, we found that the fast responses to ATP and ADP are essentially abolished in this triple mutant (Fig. S3A). Testing the single mutants individually showed that the rapid ATP and ADP responses are abolished in the TrpP null mutant (Fig. 3A,C; Fig. S3B), but unaffected in the other mutants (Fig. S3C,D).

The abolition of responses to ATP and ADP in trpP– mutant cells was confirmed using the cameleon FRET reporter in both vegetative and aggregation-competent cells (Fig. 3A,C; Fig. S4). Responsiveness could be restored by expressing TrpP under the control of its own promoter, demonstrating that the phenotype is due to the loss of TrpP and not a secondary mutation introduced elsewhere in the genome (Fig. 3B,D). In addition, a C-terminal fusion of TrpP to GFP is largely localised to the plasma membrane in living cells, consistent with TrpP acting as a plasma membrane channel (Fig. S5).

Thus we conclude that TrpP mediates the fast calcium responses to ATP and ADP and is likely gated, directly or indirectly, by ATP and ADP.

Purinergic and chemoattractant Ca2+ signalling use genetically distinct pathways

We have shown above that purinergic signalling does not require either Gβ or IplA, whereas these proteins are required for chemoattractant signalling by folic acid and cAMP – although there are contradictory reports on the importance of Gβ for cAMP signalling (Milne and Devreotes, 1993; Nebl et al., 2002; Ludlow et al., 2008). Both folate and cAMP calcium signalling depend on an influx of extracellular calcium through the plasma membrane, but the channel responsible has not been identified (Nebl and Fisher, 1997). In principle this channel could be TrpP, to which these ligands might couple indirectly through their respective GPCRs. However, we found that the responses to both cAMP and folate remain intact in TrpP null cells, showing that TrpP is not their influx channel (Fig. 3G; Fig. S4E). Thus the pathways of chemoattractant and purinergic signalling are genetically distinct.

We also noticed that TrpP null cells occasionally show a small, delayed response to ATP, which can also be elicited by buffer alone (Fig. S6; Table S1). A similar delayed response to ATP is seen in wild-type cells treated with Zn2+ to inhibit the primary response (Ludlow et al., 2008). This response might be due to a stretch-operated channel, which is activated by the physical stresses of mixing in ligand or buffer. Since the timing of the delayed responses overlaps with those depending on IplA, we created a double trpP–/iplA– mutant, and found that the response is completely abolished (Fig. S6). Indeed, this double mutant lacks a calcium response to all ligands tested (illustrated for ATP and cAMP in Fig. S6) but remains sensitive to the calmodulin inhibitor, calmidazolium, which is known to elevate cytosolic Ca2+ levels by Ca2+ release from stores (Schlatterer and Schalouske, 1996).

Ca2+ signalling is induced by DIF, cyclic-di-GMP and GABA independently of TrpP

The differentiation and behaviour of Dictyostelium cells during multicellular development is controlled by a number of small signalling molecules, in addition to cAMP, but whether these molecules cause rapid changes in cytosolic Ca2+ levels is not known. To determine the scope of calcium signalling mediated by TrpP, we tested whether these endogenous signals could trigger Ca2+ signals, and if so, whether the response depends on TrpP, or not.

The DIFs are a family of chlorinated polyketides that induce stalk cell differentiation during development (Morris et al., 1987; Masento et al., 1988), particularly those of the fruiting body basal disc (Saito et al., 2008). DIF has rapid effects on protein...
phosphorylation and transcription (Sugden et al., 2015; Williams et al., 1987) but its receptor is unknown.

We found that DIF-1, the major species, causes a robust increase in cytosolic Ca\(^2+\) in aggregation-competent cells after a delay of about 5.5±1.2 (\(n=7\)) seconds, reaching a peak at around 15.5±2.1 (\(n=7\)) seconds (Fig. 1F). Half-maximal response is at around 20 nM (Fig. S1; EC\(_{50}\) 23.3±3.7 nM; \(n=3\)), which is well within the range inducing cell differentiation and below the estimated physiological concentration (Kay, 1998). Vegetative cells give similar but weaker responses, consistent with the effects of DIF-1 early in development (Fig. 1E) (Wurster and Kay, 1990; Fukuzawa et al., 2001).

DIF-2 also elicits a response from both vegetative and aggregation-competent cells (Fig. 1G,H). It has 40% of the activity of DIF-1 in a cell differentiation assay but is equipotent in causing a Ca\(^2+\) response in aggregation-competent cells. A second polyketide, MPBD, is important both during early development and for proper spore maturation (Saito et al., 2006; Narita et al., 2014). It failed to evoke a calcium response from vegetative and aggregation-competent cells at 1 µM (not shown).

We found that cyclic-di-GMP, which is required for stalk cell maturation (Chen and Schaap, 2012; Song et al., 2015), caused a delayed Ca\(^2+\) response from aggregation-competent cells at 125 µM (Fig. 4D). Dictyostelium has a GABA signalling system, which is active during development (Taniura et al., 2006; Anjard and Loomis, 2006; Wu and Janetopoulou, 2013) and we found that GABA and L-glutamate both evoke a cytoplasmic calcium signal from aggregation-competent cells, although this was erratic (Fig. 4B,C). Finally, adenosine, which affects prestalk and prespore patterning in development (Schaap and Wang, 1986) also causes a calcium response (Fig. 4A). The calcium responses to GABA and L-glutamate are independent of TrpP, remaining intact in the null mutant, but do depend on IplA (Fig. S7).

ATP causes cell blebbing

We sought to establish the role of purinergic signalling in Dictyostelium biology by examining, first cellular responses to ATP, and then the TrpP mutant phenotype.

Early reports suggested that extracellular ATP stimulates aggregation centre formation in small drop assays of starving cells (Mato and Konijn, 1975; Pererekalin, 1977), but we could not reproduce this effect using up to 1 mM ATP (not shown). We investigated the effects of ATP on cell motility in detail. ATP was not a chemoattractant for aggregation-competent cells at a range of concentrations, and a uniform concentration of ATP did not enhance or inhibit chemotaxis to cAMP (Table S2). Neither was there a significant effect of a uniform concentration of ATP on the speed of

Fig. 3. ATP-stimulated calcium signalling requires the Trp channel, TrpP. (A,C,E,G) [Ca\(^{2+}\)]\(_c\) transients stimulated by ATP (A) and ADP (C) are abolished in TrpP null cells, whereas [Ca\(^{2+}\)]\(_c\) transients stimulated by cAMP (G) and DIF-1 (E) are essentially normal. (B,D,F,H) Purinergic responses are restored by over-expression of TrpP in the TrpP- strain (B,D), whereas responses to DIF-1 (F) and cAMP (H) are little affected by over-expression of TrpP. Aggregation-competent TrpP null cells expressing the [Ca\(^{2+}\)]\(_c\) reporter cameleon YC2.60 were used, with as indicated, a plasmid for expression of TrpP (pDT50). Changes in the fluorescence ratio with time are presented with each panel showing the mean ratios ± s.e.m. (grey bars) of 6-15 cells. The data is representative of at least three independent experiments. The arrow indicates when the compound was added.

The response to DIF is totally dependent on IplA in both vegetative and aggregation-competent cells but is maintained in G\(_{\beta}\)-null cells, suggesting that it does not depend on a heterotrimeric G-protein (Fig. 2G-I). The response also remains essentially unchanged in TrpP null cells and so does not depend on TrpP (Fig. 3E,F).

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chemotaxing cells or on the random movement of either vegetative or starving cells (Table S3).

In the course of the FRET measurements of calcium, we noticed that ATP addition causes cells to bleb vigorously (Fig. 5A). Blebs occur when the plasma membrane becomes detached from the underlying F-actin cortex, and is driven out by fluid pressure. Dictyostelium cells can move using blebs instead of pseudopods (Yoshida and Soldati, 2006; Zatulovskiy et al., 2014; Tyson et al., 2014) and blebbing is also induced by cAMP.

Morphologically, blebs induced by ATP resemble those induced by cAMP, with their characteristic rapid expansion, smooth curvature and residual scar of F-actin, representing the former cortex. A new cortex is then rapidly rebuilt on the exposed membrane of the bleb. However, ATP induces blebbing much faster than the published timing for cAMP (Langridge and Kay, 2006), with a delay of only 3-7 s compared to around 25 s for cAMP (Fig. 5B). ATP-induced blebbing requires myosin-II and is abolished in null mutants of either the myosin-II heavy chain or the essential light chain (Fig. S8), as is cAMP-induced blebbing (Zatulovskiy et al., 2014).

The signalling and other events triggered by ATP and cAMP differ significantly: ATP does not cause the transient actin polymerization characteristic of the period before blebbing starts in cells stimulated with cAMP (Langridge and Kay, 2006) (Fig. 5C). Nor does ATP stimulate PIP3 production, as measured in live cells by recruitment of the PH-CRAC reporter to the plasma membrane (Parent et al., 1998), or activate the MAP kinase ErkB (Kosaka and Pears, 1997) or the AKT homologue PKB (Meili et al., 1999), as

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**Fig. 5. ATP addition induces cellular blebbing.** (A) Ax2 cell stimulated with 30 µM ATP at t0 and expressing the F-actin reporter GFP-ABP120 observed by fluorescence confocal and DIC microscopy. The cell starts to bleb after 14 s (white arrows) and stops by 29 s. When first formed, the blebs are almost devoid of F-actin but leave an F-actin scar behind (red arrowheads). Scale bar: 10 µm. (B) Comparison of the timings of ATP- and cAMP-induced bleb formation (cAMP data reproduced with permission from Langridge and Kay, 2006). The mean±s.e.m. for onset of each of the events are indicated for ATP (green) and cAMP (red). The small squares indicate the times of earliest onset and cessation of each event for the cells (n=31) in this data set. (C) Actin polymerization in cells in response to 100 µM ATP, 1 µM cAMP or buffer only. The data shown is representative of three experiments, but the slight F-actin depolymerization seen in this example did not repeat in response to ATP. Aggregation-competent cells were used throughout.
The purinergic calcium response is significantly different to the responses evoked by the chemotactants folic acid and cAMP, which are mediated by GPCRs. It seems that at least two basic modes of calcium signalling can be distinguished in *Dictyostelium*: ‘GPCR-dependent’ signalling (cAMP and folic acid) whose onset is delayed for 5–10 s after the stimulus, and which depend on IplA and at least partially on Gβ; and ‘purinergic’ signalling (ATP and ADP) which has a rapid onset of less than 1 s, is independent of Gβ and IplA, but depends on TrpP.

We also show for the first time that the stalk cell-inducing morphogen DIF (Morris et al., 1987), whose receptor is unknown, causes a fast, transient calcium response in the physiological concentration range. The characteristics of this response – delayed and IplA-dependent – are more consistent with the G-protein-dependent mode of signalling, but surprisingly we find that this response is largely independent of IplA. Although not studied in detail we also found delayed calcium responses to di-cyclic-GMP (Chen and Schaap, 2012), L-glutamate and GABA (Anjard and Loomis, 2006) but there was no response to the polykette MPBD (Saito et al., 2006).

TrpP is well conserved between dictyostelid species (Sucgang et al., 2011; Heidel et al., 2011; Urushihara et al., 2015) arguing that purinergic signalling must also have a conserved role. We found that ATP is not a chemoattractant for aggregation-competent cells and does not modulate chemotaxis to CAMP, nor could we detect a chemotactic defect in TrpP null mutants. Growth of TrpP null cells in liquid medium was also normal, contrary to a recent report (Waheed et al., 2014) and development only slightly perturbed. It has been reported that TrpP is required for rheotaxis (Lima et al., 2014) but TrpP mutants made in our laboratory strain showed no such defect. Such discrepancies are not unknown in the *Dictyostelium* literature, and are most likely accounted for by genetic background effects, or secondary mutations introduced during gene knock-out (Bloomfield et al., 2008; Schildle et al., 2004; Pollitt et al., 2006; Sivaramakrishnan and Fountain, 2013).

The one clear effect we can detect of adding ATP or ADP to cells is to induce almost immediate blebbing. Blebs form where the plasma membrane detaches from the underlying cortex and is driven outwards by fluid pressure, and blebs are being increasingly recognised as an alternative to pseudopods to drive cell motility, particularly when cells face mechanical resistance (Yoshida and Soldati, 2006; Zatulovskiy et al., 2014; Tyson et al., 2014). Blebbing induced by ATP differs in interesting ways from that induced by the chemotactant cAMP (Langridge and Kay, 2006); in particular, it starts more quickly, there is no global polymerisation of actin, and neither PI3-kinase nor the MAP kinase, ErkB, are activated. Surprisingly, blebbing induced by ATP does not depend on TrpP, implying that another purinergic receptor must be responsible.

TrpP is homologous to the polycystin-2 or TRPP class of vertebrate Trp channels, which are also found in non-metazoan organisms (Venkataram and Montell, 2007) and include the human PKD2 protein (Mochizuki et al., 1996; Wu et al., 1998). PKD2 has been studied intensively as a cause of the severe genetic disorder autosomal dominant polycystic kidney disease, in which fluid-filled cysts grow within the kidney, and eventually disrupt its function (Chapin and Caplan, 2010). PKD2 cooperates with a large extracellular protein, PKD1, and together they form plasma membrane cation channels of high calcium permeability (Hanaoka et al., 2000; Gonzalez-Perrett et al., 2001; Yu et al., 2009). However, we can detect no clear homologue of PKD1 in the *Dictyostelium*
genome (Eichinger et al., 2005), and if one exists, it must be very divergent.

To our knowledge, no gating agonist has been reported for PKD2. In electrophysiological experiments it has been suggested to have an appreciable intrinsic conductance (Gonzalez-Perrett et al., 2001), although this is disputed (Yu et al., 2009), and there is also the possibility of mechanical gating. Our results showing that the primary response to extracellular ATP in Dictyostelium is mediated by a PKD2 homologue, is therefore both surprising and promising, raising the possibility that gating by ATP may be a more widespread feature of these channels.

MATERIALS AND METHODS
Cell cultivation, development, transfection and selection
Ax2 (Kay Laboratory strain; dictyBase DBS0235521), with minimal chromosomal duplications (Bloomfield et al., 2008) was used as parental stock; strains are listed in Table S5, and were renewed from frozen stocks every month. Cell procedures were at 22°C, unless otherwise stated. Cells were grown in HL5 with glucose (Formedium), plus 200 µg/ml dihydrostreptomycin, either in shaken suspension at 180 rpm, or in tissue culture dishes (Hirst et al., 2015). Development was initiated by washing cells free of growth medium in KK2C (16.5 mM KH2PO4, 3.9 mM K2HPO4, 2 mM MgSO4, 0.1 mM CaCl2 pH 6.1) and settling 1×10^8 cells from 4 ml onto 30 ml of 1.8% Oxoid L28 agar/KK2C in a 9 cm diameter petri dish. After 10 min, excess buffer was aspirated off. Submerged development was observed with 2×10^6 cells under 2 ml of KK2C in 3.5 cm tissue culture dishes. Total RNA was extracted (RNeasy kit, Qiagen) from 5×10^7-1×10^8 developing cells and cDNA synthesised from 10.5 µg RNA for each timepoint (SuperScript First-Strand Synthesis System for RT-PCR, Life Technologies) using oligo(dT)12-18 for semi-quantitative PCR or a 1:1 mixture of random hexamers/oligo(dT)12-18 for cDNA cloning. Standard curves were established with cDNA dilutions (1:10 to 1:20,000) for each primer pair. The PCR reaction contained in 50 µl: 50 pmole of each primer, cDNA, 2 mM MgCl2, 200 µM dNTPs and 2.5 units Taq polymerase; PCR was run for 25 cycles.

Cells were transformed by electroporating 17.5 µg of gene disruption cassette, freed of plasmid backbone by restriction digest, or 30 µg of...
supercoiled plasmid DNA into 4×10^9 cells (Pang et al., 1999; Hirst et al., 2015). Over-expression cell lines were selected and maintained in tissue culture dishes with HL5 plus 20-40 µg/ml G418, whereas trpP knockout clones were isolated by plating 60-240 cells/well in 96 well plates with 200 µl HL5 plus 10 µg/ml blasticidin S (InvivoGen). DNA was extracted from confluent wells after 10-14 days (Quick-gDNA MiniPrep, Zymo Research) and screened using primers PC2S26 and PC2S27 (primer sequences are given in Table S6) located outside the disruption cassette. Knockout clones were distinguished by the size of the PCR product and the presence of unique restriction sites introduced into the locus by the disruption cassette (Hirst et al., 2015).

Plasmids

Primer sequences are given in Table S6. To construct the trpP knockout vector (pDT27) the 5′ homology was amplified using oligos PC2KO1 plus PC2KO2 and ligated into the Apal site of pLPBLP (Faix et al., 2004) and the 3′ homology amplified using oligos PC2KO3 plus PC2KO4 and ligated as a NotI/SacII fragment into the corresponding sites of the vector containing the 5′ homology. The trpP disruption cassette was liberated from pDT27 by digestion with KpnI and SacII prior to transfection of the cells. The trpP CDS (without a stop codon) was amplified by RT-PCR jDNA using oligos PCL5 plus PCL3 and ligated into the BamHI/Xhol sites of pDT29 creating pDT33 with an in frame C-terminal fusion of GFP(S65T). The plasmid pDXA-3CA was made by digesting pDXA-3C with KpnI and SacI to remove the start codon from the A15 leader in the MCS (Manstein et al., 1995). GFP(S65T) was amplified using oligos RAGFP9 plus RAGFP10, then ligated into the XhoI site of pDXA-3C giving pDT29. To construct trpP driven by its own promoter, a silent restriction site was introduced into pDT33 using mutagenic primers PC2S47 and PC2S48, changing +54A of trpP from genomic DNA. The PCR product, digested with SalI and HindIII, was ligated into the XhoI and SalI sites of pET28a (Horikawa et al., 1993) removing the intact pDXA-3C CDS with its own promoter, which was ligated into XhoI/SpeI sites of pMD304 (Veltman et al., 2009) giving pDT41. The cameleons YC2.60 and YC3.60 (with Aequorea victoria codons) were removed from their pBIG vectors by partial digestion with BamHI/SacI and ligated into the same sites in pET28a (Horikawa et al., 2010), providing a template to amplify both cameleons using oligos YC367 plus YC368. The PCR products were ligated into the BamHI/SpeI sites of the shuttle vector pMD344 (Veltman et al., 2009), removed as Ngm/MIV fragments and ligated into the corresponding site in pDT41 giving pDT48 (YC3.60) and pDT50 (YC2.60). Finally, pDT48 was used as the template to amplify the trpP CDS plus promoter oligos PC2S59 plus PC2S70, with the product ligated into the XhoI/BglII sites of pMD323 (Veltman et al., 2009) giving pDT68.

Microscopy

Vegetative cells were harvested from tissue culture plates, washed three times in HKC buffer (10 mM HEPES, 10 mM KCl, 250 µM CaCl2, pH 6.8) by centrifugation (300×g for 2 min) and resuspended at 10^9/ml in HKC. Cells were plated at 10^4 cells/cm² in 8-well Lab-Tek™ chambered coverslips with 300 µl HKC/well. Coverslips were incubated in a moist atmosphere for up to 1 h before use. Aggregation-competent cells were prepared by pulsing with cAMP for 3.5-5.5 h after 1 h starvation in shaking suspension (Traynor and Kay, 2007), or by plating 10^5 washed cells per 35 mm tissue culture dish in 2 ml of HKC, incubating at 22°C for 1 h, then 15°C overnight (15-17 h), before returning to 22°C for at least 1 h, until they become elongated. Cells were harvested in fresh MKC by pipetting up and down and transferred to a chambered coverslip, where they normally formed long streams and aggregated after 2-4 h at 22°C. Confocal images were obtained using a Zeiss LSM 710 or 780 microscope with Zen 2010 software. Dunn chamber and micropipette chemotaxis assays and under-agarose motility assays were as described (Fets et al., 2014; Zatulovsky et al., 2014). Images were analysed with ImageJ, Fiji (Schindelin et al., 2012) and Excel (Microsoft) software.

Calcium Imaging

Vegetative or aggregation-competent cells were challenged with effectors added as a 100 µl bolus at 5× final concentration to individual wells of a chambered coverslip. Mixing time was less than a second. Time-lapse images were collected on a Zeiss Axivert 200 inverted microscope using a 40× C-Apochromat W Corr M27 lens (NA 1.2) and a Cascade II 512 EMCCD camera (Photometrics) controlled by Metamorph software (Molecular Devices). Cells were illuminated with a Lambda LS light source (Sutter Instruments) containing a 175W xenon bulb through a neutral density filter (Chroma Technology Corp, ND 2.0 A) with 1% transmittance and an excitation filter (ET436/20+ Chroma Technology Corp) that ensured only CFP was illuminated. The CFP and YFP emission light was separated using a beam splitter (Optical Insights, Dual View, filters D480/30, D535/40 and 505/50 dichromatic mirror). Exposure times were 100-250 msec and images captured at 3-7 sec with a binning of 2 unless otherwise stated. Images were analysed using Imaris (Kerr and Schafer, 2006), Volocity (Perkin Elmer) and ImageJ. For each image the CFP (C) and YFP (Y) average fluoresence intensities, obtained by subtracting the background intensities (Ybg and Cbg) from the measured intensities (Ymeas and Cmeas) and ratios (R) were corrected for bleed-through from the CFP channel into the YFP measured at 0.683 using Eqn (1) in the Microsoft Excel software package.

\[
R = \frac{Y(Y_{\text{meas}} - Y_{\text{bg}})}{C(C_{\text{meas}} - C_{\text{bg}})} - 0.683
\]

Response kinetics and peak areas were determined using GraphPad Prism.

Rheotaxis

Sheer stress was created by a flow of KK2C driven by hydrostatic pressure in an Ibidi µ-Slide 1.0 2 flow chamber (tissue culture treated, luer): 6.4 and 8.7 ml/min produced pressures of 3 and 4.5 Pa according to the manufacturer’s lookup table (http://ibidi.com/fileadmin/support/application_notes/AN11_Shear). Cells in the chamber were filmed (1 frame/sec; binning of 2) using a Zeiss Axiovert S100 inverted microscope with motorised stage (Prior) and an ORCA-ER camera (Hamamatsu) controlled by µManager (Edelstein et al., 2014) software. ImageJ was used to analyse the movies.

Acknowledgements

We should like to thank Mario de Bono for use of the calcium-imaging microscope. The pBIG YC2.60 and 3.60 plasmids were obtained from Kazuki Horikawa (Division of Bioimaging, Institute of Biomedical Sciences, Tokushima University Graduate School, Tokushima City, Japan) and Takeharu Nagai (The Institute of Scientific and Industrial Research, Osaka University, Osaka, Japan.). The Gq null strain was courtesy of Peter Devreotes and Jane Borelis (Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, USA).

Competing interests

The authors declare no competing or financial interests.

Author contributions

D.T. and R.R.K. conceived and designed the experiments. D.T. performed most of the experiments. R.R.K. wrote the manuscript with input from D.T.

Funding

Medical Research Council (U105115237 to RRK).

Supplementary information

Supplementary information available online at http://biologists.org/lookup doi:10.1242/bio.020685.supplemental

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