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Ca^{2+} signalling is not required for chemotaxis in *Dictyostelium*

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Dictyostelium **cells can move rapidly towards a source of cyclic-AMP (cAMP). This chemoattractant is detected by G-protein-linked receptors, which trigger a signalling cascade including a rapid influx of Ca^{2+}. We have disrupted an inositol 1,4,5-trisphosphate ($InsP_3$) receptor-like gene, *iplA*, to produce null cells in which Ca^{2+} entry in response to chemoattractants is abolished, as is the normal increase in free cytosolic Ca^{2+} ($[Ca^{2+}]_c$) that follows chemotactic stimulation. However, the resting $[Ca^{2+}]_c$ is similar to wild type. This mutant provides a test for the role of Ca^{2+} influx in both chemotaxis and the signalling cascade that controls it. The production of cyclic-GMP and cAMP, and the activation of the MAP kinase, DdERK2, triggered from the cAMP receptor, are little perturbed in the mutant; mobilization of actin into the cytoskeleton also follows similar kinetics to wild type. Mutant cells chemotax efficiently towards cAMP or folic acid and their sensitivity to cAMP is similar to wild type. Finally, they move at similar speeds to wild-type cells, with or without chemoattractant. We conclude that Ca^{2+} signalling is not necessary for chemotaxis to cAMP.**

Keywords: calcium signalling/chemotaxis/cyclic-AMP/*Dictyostelium*/$InsP_3$ receptor

Introduction

Chemotaxis of such diverse amoeboid cells as leukocytes and *Dictyostelium* amoebae appears to be achieved in a similar manner using similar components (Devreotes and Zigmond, 1988; Parent and Devreotes, 1999). *Dictyostelium* amoebae become responsive to cyclic-AMP (cAMP) during development and can move up concentration gradients of as little as a 2% change along their length. Cells become polarized in these gradients in a matter of seconds, as detected by the translocation of the PH-domain proteins CRAC and PKB to the membrane at the front of the cell (Parent *et al*., 1998; Meili *et al*., 1999). When a gradient is imposed upon a cell, it rapidly throws out a pseudopod containing F-actin towards the source, followed by a flow of cytoplasm in the same direction (Gerisch *et al*., 1975; Swanson and Taylor, 1982). Thus, movement involves an extensive re-organization of the actin–myosin cytoskeleton (Mitchison and Cramer, 1996), and, most likely, a re-orientation of membrane exocytosis to the front of the cell (Bretscher, 1996).

One way of investigating chemotaxis is to trace the signal transduction pathways down from the chemotactic receptors to the motile apparatus. Moving and chemotacting cells frequently display transient, graded increases in cytosolic Ca^{2+} that correlate with their direction of movement (Taylor *et al*., 1980; Sawyer *et al*., 1985; Brundage *et al*., 1991; Maxfield, 1993). Since Ca^{2+} entry across the plasma membrane and elevated cytosolic Ca^{2+} levels are caused by chemotactic agents such as cAMP in *Dictyostelium* (Wick *et al*., 1978; Bumann *et al*., 1983; Abe *et al*., 1988; Milne and Coukell, 1991; Saran *et al*., 1994; Schlatterer *et al*., 1994; Yumura *et al*., 1996; Nebl and Fisher, 1997) and F-Met-Leu-Phe in leukocytes, it seems possible that Ca^{2+} signalling may have a key role in chemotaxis. This idea is supported by the Ca^{2+} sensitivity of actin-binding proteins such as α-actinin (Witke *et al*., 1993) and severin (Yamamoto *et al*., 1982), which may have a role in re-organizing the actin cytoskeleton during movement.

However, the signalling pathways stemming from chemotactic receptors are complex and so offer other alternatives. In *Dictyostelium*, cAMP acts through the cAR1 receptor and an essential G-protein to stimulate the production of cyclic-GMP (cGMP) (Mato *et al*., 1977), inositol 1,4,5-trisphosphate ($InsP_3$) (Europe-Finner and Newell, 1987; Van Haastert, 1989) and cAMP, as well as the activation of the MAP kinase DdERK2 and the appearance of membrane-binding sites for PH-domain proteins such as CRAC and PKB.

Physiological manipulation of Ca^{2+} signalling has given unclear results. Nerve growth cones can be guided by local elevation of cytosolic Ca^{2+} levels (Hong *et al*., 2000; Zheng, 2000), whereas Ca^{2+} chelators do not affect neutrophil chemotaxis (Marasco *et al*., 1980; Maxfield, 1993). In some experiments with *Dictyostelium*, chelation of extracellular Ca^{2+} inhibits movement, though not orientation, of chemotacting cells (Malchow *et al*., 1982), but in other cases it is without effect (Van Duijn and Van Haastert, 1992). The difference may be due to varying degrees of depletion of intracellular Ca^{2+} Europe-Finner *et al*., 1984). However, clamping intracellular Ca^{2+} levels by introducing chelators to the cytoplasm strongly inhibits movement, nearly to the point of paralysis (Van Duijn and Van Haastert, 1992; Schlatterer and Malchow, 1993; Unterweger and Schlatterer, 1995). A difficulty with these experiments is that chelators may either insufficiently deplete cytosolic Ca^{2+} to damp down Ca^{2+} signalling, or alternatively they may reduce resting Ca^{2+} levels, with unpredictable consequences. Additional tests are therefore required for the

involvement of Ca^{2+} signalling in movement. One possibility is the genetic manipulation of the Ca^{2+} signalling system.

In higher eukaryotic cells, activation of many cell surface receptors causes the production of $InsP_3$, resulting in Ca^{2+} release from intracellular stores, via the $InsP_3$ receptor. Depletion of stores often triggers a larger influx of Ca^{2+} through the plasma membrane, which is carried by store-operated channels and is referred to as capacitative uptake. $InsP_3$ receptors therefore lie at the heart of Ca^{2+} signalling (Clapham, 1995; Berridge *et al*., 1998). The channel formed by the mammalian receptor is tetrameric and homologues have been identified in *Xenopus*, *Drosophila* and *Caenorhabditis elegans* (Clapham, 1995; Berridge *et al*., 1998; Patel *et al*., 1999). Disruption of the mouse type 1 $InsP_3$ receptor gene usually results in embryonic lethality, with survivors having acute neurological dysfunctions. Mutation of the *Drosophila* $InsP_3$ receptor is fatal beyond the larval stage and inhibits cell growth and differentiation. Mutations of the *C.elegans* $InsP_3$ receptor can delay embryonic development, cause sterility and affect the defecation cycle timing (Dal Santo *et al*., 1999).

We have identified a *Dictyostelium* InsP$_3$ receptor-like gene (*iplA*) and made null mutants. These null cells lack receptor-activated Ca^{2+} entry, and the consequences of this mutation for cell movement and chemotaxis have been investigated.

Results

The iplA gene encodes a protein similar to $InsP_3$ receptors

The cDNA SSA203 was isolated as part of the *Dictyostelium* cDNA sequencing project (Morio *et al*., 1998) and has homology to all $InsP_3$ receptors and to a lesser extent to ryanodine receptors (RyR), the other class of intracellular Ca^{2+} release channels. The 809 bp cDNA was used as probe to isolate a 7.5 kb genomic DNA fragment and the remainder of the *iplA* gene and regulatory elements were isolated by chromosome walking (Figures 1 and 2). The gene, including three small introns, spans 9830 bp with a predicted product of 3177 amino acids and a mass of 361 kDa. No related genes were identified by searching the *Dictyostelium* genomic and cDNA databases with this sequence, nor by low stringency Southern blots using *iplA* as probe (not shown).

$InsP_3$ receptors have an N-terminal ligand-binding domain, a central regulatory domain and a C-terminal channel domain, thought to have six transmembrane segments (M1–M6) (Galvan *et al*., 1999; Patel *et al*., 1999). The predicted product of the *iplA* gene is of similar size and topology with a large hydrophilic N-terminal domain and a small C-terminal domain containing multiple transmembrane spanning segments (Figure 1B) and has scattered homology to $InsP_3$ receptors throughout its length (12% identity to human type 1 and 14% identity to the *C.elegans* receptor; Figure 1A). The M6 region of all $InsP_3$ receptors is highly conserved even with RyR receptors, and the corresponding region of IplA (residues 2998–3071) has 42% sequence identity to the human type 1 receptor and 47% identity to the *C.elegans* receptor (Figure 1A).

The $InsP_3$-binding domain of known $InsP_3$ receptors consists of a number of basic amino acids, scattered over a 350 residue segment near the N-terminus. The *iplA* gene product has 11 and 15% sequence identity across the same region compared with the human type 1 and *C.elegans* receptors, respectively. Only two of the 10 basic residues implicated in ligand binding are conserved in the IplA protein but this area of IplA is rich in basic amino acids, which could serve for IP_3 binding (Yoshikawa *et al*., 1996). On the basis of the overall size, topology and sequence similarities, we propose that IplA is related to intracellular Ca^{2+} channels and in particular to the $InsP_3$ receptor family.

The role of IplA in receptor-stimulated Ca^{2+} signalling

Expression of *iplA* mRNA is low during growth and rises to a peak at ~9 h of development, when chemotactic aggregation to cAMP is near its height; levels then decline through the rest of development (Figure 2). In order to investigate the role of *iplA* in Ca^{2+} signalling, null strains were made by gene disruption in the wild-type Ax2 strain. The null phenotype was confirmed by Southern blot analysis and the absence of the *iplA* transcript (Figure 2). The null mutant grew as wild type, either in shaken axenic cultures or in association with *Klebsiella* bacteria on agar plates, and development was essentially normal, except that smaller fruiting bodies were formed (not shown).

Activation of the plasma membrane cAMP or folic acid receptors initiates Ca^{2+} signalling in wild-type cells. To investigate the response to cAMP, starving cells were pulsed with cAMP for several hours, to bring them to a responsive state ('aggregation competent'), and uptake of $^{45}Ca^{2+}$ measured in response to a saturating cAMP stimulus. Figure 3 shows a typical response from the parental strain: uptake starts after a 5–10 s delay and continues for 20–25 s before the response terminates. In sharp contrast, the *iplA* null strain shows a complete lack of stimulated Ca^{2+} uptake; indeed stimulated uptake was often lower than basal in the mutant. The lack of response in the mutant was not due to a lack of cell surface receptors, since cAMP binding experiments showed comparable receptor numbers to Ax2 (in three experiments, mutant cells bound 2.3, 6.6 and 5.3 pmol cAMP/mg protein compared with 3.3, 7.2 and 5.8 for the wild type) and other receptor-mediated responses are unimpaired (see later). Similar results were obtained with six independent knock-out strains.

Folic acid, a chemoattractant for vegetative cells, is believed to act through an unidentified, G-protein-coupled receptor and it stimulates Ca^{2+} uptake by vegetative Ax2 cells, with a 15–20 s delay. This response was also totally lacking in the *iplA* null strain (Figure 3).

These results were confirmed by measuring the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in single amoebae. Amoebae were developed until competent to respond to extracellular cAMP then loaded with fura 2-dextran. The resting $[Ca^{2+}]_c$ in Ax2 cells was 76 $\pm$ 29 nM (n = 54) and stimulation with 1 μM cAMP caused a transient 2- to 3-fold increase in $[Ca^{2+}]_c$, consistent with previous results (Yumura *et al*., 1996; Nebl and Fisher, 1997; Sonnemann *et al*., 1997). In agreement with previous results, the change in $[Ca^{2+}]_c$ depended on extracellular Ca^{2+} and was

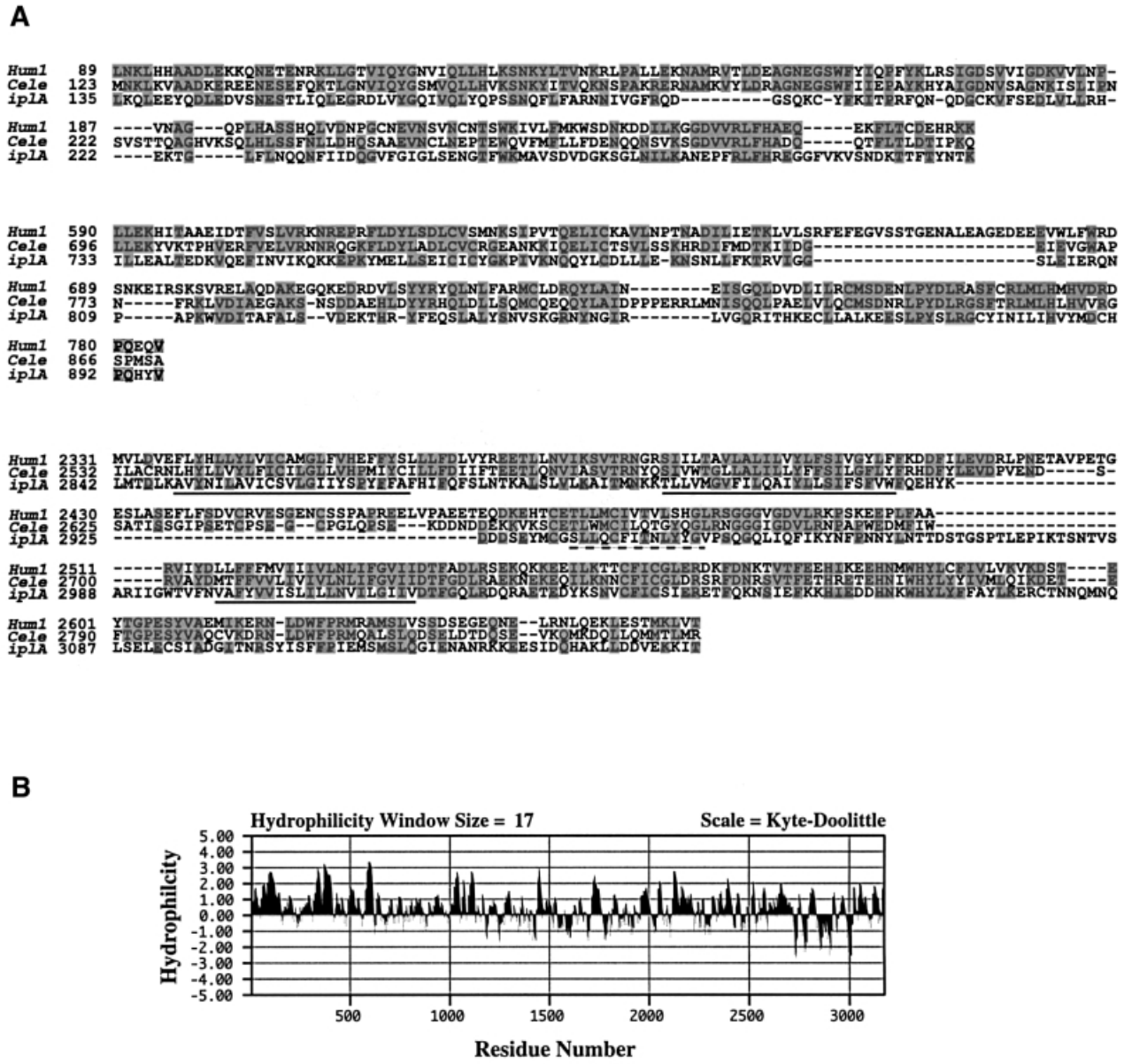

Fig. 1. (**A**) Alignment of the high scoring segments obtained from a Blast search of the non-redundant database at NCBI, using the predicted amino acid sequence of the *Dictyostelium iplA* gene. The corresponding regions of the human type 1 (*Hum1*; DDBJ/EMBL/GenBank accession No. D26070; Yamada *et al*., 1994) and *C.elegans* [*Cele*; accession No. AF168688; *itr-1* gene of Dal Santo *et al*. (1999)] IP_3 receptors were aligned with the *iplA* sequence (accession No. AJ277590) using ClustalW. Identical residues are shaded grey; predicted transmembrane spanning sequences, corresponding to M4–M6 of the human type 1 protein, are underlined and the dashed line indicates the proposed pore-forming region of this channel. (**B**) Hydrophilicity plot of the predicted amino acid sequence of the *iplA* gene (Kyte and Doolittle, 1982).

abolished in Ca^{2+}-free buffer (Nebl and Fisher, 1997). IplA null cells had a resting $[Ca^{2+}]_c$ of 79 ± 15 nM (n = 59), similar to the wild type (77 ± 29; n = 54), but this did not change after stimulation with extracellular cAMP, regardless of whether Ca^{2+} was present or not in the bathing buffer (not shown). Together these data show that IplA is necessary for these examples of chemoattractant-stimulated Ca^{2+} entry across the plasma membrane and that all detectable Ca^{2+} signalling is abolished in the mutant.

The role of IplA-dependent calcium uptake in chemotaxis

We investigated the role of Ca^{2+} uptake in the chemotactic response at several levels: immediate signal transduction events, actin cytoskeletal responses and chemotaxis itself.

Role of Ca^{2+} influx in signal transduction from the cAMP receptor. Extracellular cAMP activates guanylyl cyclase, with a short delay and later adenylyl cyclase, leading to the accumulation of cGMP and cAMP. Both responses adapt, so that a continuous stimulus only produces a transient response. Guanylyl cyclase activity adapts after ~10 s and since activity in cell lysates is strongly inhibited by high nanomolar Ca^{2+} levels (Janssens and De Jong, 1988), it seemed possible that the Ca^{2+} influx might be responsible for adaptation. However, with the possible exception of the earliest time point, cGMP accumulates after stimulation in both mutant and wild-type cells with very similar kinetics and extents (Figure 4). This result also eliminates an alternative suggestion, that Ca^{2+} influx triggers the production of cGMP (Small *et al*., 1986). ACA, the major

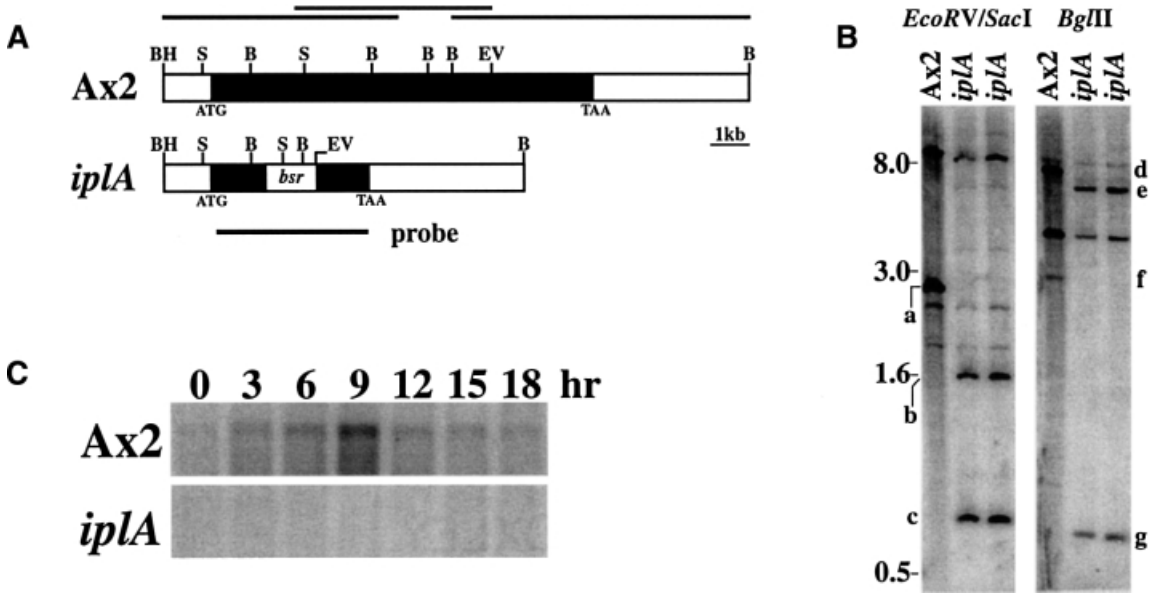

Fig. 2. Disruption of the *iplA* locus by homologous recombination. (**A**) Organization of the *iplA* locus. The 9.8 kb *iplA* gene in Ax2 (black shading) is reduced to 1.4 kb of 5′ and 1.4 kb of 3′ coding sequences flanking the 1.35 kb blasticidin S transferase cassette (*bsr*) in the *iplA* strain. The black lines above the Ax2 locus show the position of the genomic clones isolated from mini-libraries and used for sequencing. Restriction sites are *Bam*HI (BH), *Bgl*II (B), *Eco*RV (EV) and *Sac*I (S). (**B**) Southern blot analysis. Genomic DNA from Ax2 and two null clones was digested with *Eco*RV and *Sac*I to check disruption of the 5′ end of the *iplA* locus or with *Bgl*II to check the 3′ end. At the 5′ end, the 2.7 kb *Sac*I band (a) encompassing the start codon in Ax2 is reduced to a 1.7 kb fragment (b) in the *iplA* clones and an additional 0.64 kb *Sac*I–*Eco*RV fragment (c) is liberated from the *bsr* casssette. The 7.6 kb *Bgl*II fragment (d) from the 3′ end of the *iplA* gene and flanking sequence in Ax2 is replaced with a 6.4 kb fragment (e) in the *iplA* clones and a 0.72 kb band (g) encompassing part of the *bsr* cassette and 5′ sequence. The 3.0 kb *Bgl*II fragment (f) from the middle to near the 5′ end of the *iplA* gene in Ax2 is deleted in the *iplA* clones. Blots were probed with the *iplA* disruption cassette as denoted by a thick black line in (A). Bands common to Ax2 and *iplA* clones are due either to fragments outside the targeted region, or to fragments hybridizing to the actin promoter/terminator sequences within the *bsr* cassette. Approximately 5 μg of DNA were loaded per lane. (**C**) Developmental expression of the *iplA* transcript in Ax2. Amoebae were aggregating by 6 h, formed mounds by 9 h and fruited by 18 h. The transcript is absent in the *iplA* strain. Ten micrograms of total RNA from each time point were loaded.

adenylyl cyclase, is relatively indifferent to the presence of Ca^{2+} in biochemical assays and, as expected, cAMP accumulation by mutant and wild type is very similar.

DdERK2 is a MAP kinase that is necessary for proper cell polarization during chemotaxis (Segall *et al*., 1995). It becomes phosphorylated and activated shortly after cAMP receptor stimulation. Activation can conveniently be detected by a mobility shift of DdERK2 in western blots and Figure 4 shows that the enzyme is activated with similar kinetics in Ax2 and *iplA* null cells.

Role of Ca^{2+} signalling in cytoskeletal responses. A sudden elevation in cAMP levels causes cells to slow down briefly and round up (‘cringe’), before resuming normal movement within ~1 min (Futrelle *et al*., 1982). This cringe response correlates with a rapid increase in cellular F-actin content, followed by a drop and then a second rise as motility resumes (McRobbie and Newell, 1983; Hall *et al*., 1988). *iplA* null cells show a cringe response to cAMP indistinguishable from wild type (not shown) and the behaviour of their actin cytoskeleton after cAMP stimulation is also very similar (Figure 5).

Role of Ca^{2+} signalling in movement and chemotaxis. Chemotaxis was tested in small populations of cells contained in droplets of buffer on a hydrophobic agar surface. These cells can orientate and move towards a nearby droplet of chemoattractant (Konijn, 1970). In this test, *iplA* null cells chemotact to both cAMP and folic acid (not shown) and are essentially as sensitive to cAMP as their parent (Figure 6). Movement and chemotaxis were analysed in more detail by filming cells in perfusion chambers (Table I). Both mutant and parental cells moved at similar speeds and with similar persistence in the absence of chemoattractant. In both cases vegetative cells moved more slowly than aggregation-competent cells, as previously noted. Both strains chemotaxed to cAMP with a similar speed, persistence and chemotactic index.

Discussion

Chemotaxis of *Dictyostelium* cells to cAMP can be initiated by stimulation of cAR1, a G-protein-coupled receptor, and must be controlled by signal transduction events from this receptor. An influx of Ca^{2+} starts 5–10 s after receptor stimulation, producing an elevation of cytosolic Ca^{2+} levels and there have been many suggestions that this is important for chemotaxis.

The sequence homologies of the *iplA* gene product clearly place it in the $InsP_3$/RyR receptor family of ligand-gated Ca^{2+} channels and the defective Ca^{2+} signalling of the null mutant supports this assignment. Such genes are absent from the yeast *Saccharomyces cerevisiae* and, to our knowledge, *iplA* is the first example from outside the animal kingdom, although homologous sequences are also present in the genomes of parasitic protozoa. *Dictyostelium* cells produce $InsP_3$, both through a conventional phospholipase C and by an unconventional route from higher inositol phosphates (Van Dijken *et al*., 1995)

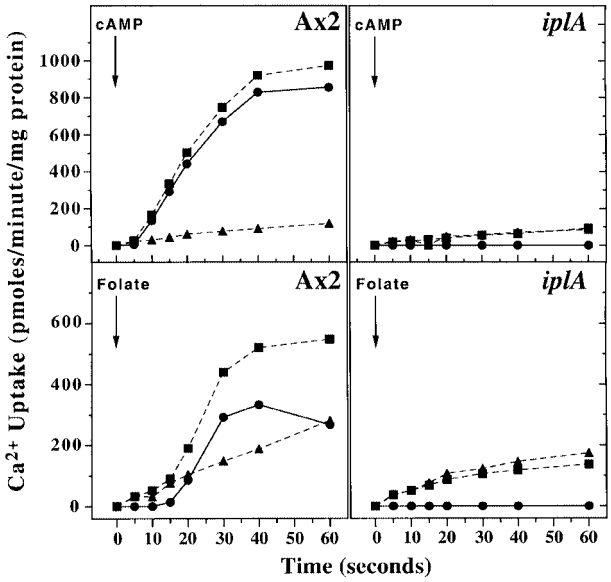

Fig. 3. Chemoattractant-stimulated Ca^{2+} uptake is abolished in *iplA* cells. At zero time, 1×10^7 aggregation-competent amoebae (upper two panels) were added to buffer containing 100 μM $CaCl_2$, 5 μCi/ml of $^{45}CaCl_2$ with (squares) or without (triangles) 100 μM cAMP. At the times indicated, the reaction was stopped and cell-associated $^{45}Ca^{2+}$ determined. In the lower two panels, 1×10^7 vegetative amoebae were stimulated in the same conditions with (squares) or without (triangles) 100 μM folic acid. The stimulated uptake (circles) in all panels is the difference between the uptake in the presence and absence of chemoattractant.

and there is evidence that $InsP_3$ can cause release of Ca^{2+} from internal stores (Europe-Finner and Newell, 1986; Flaadt *et al*., 1993); however, biochemical evidence that IplA is an $InsP_3$ receptor is still lacking.

Removal of the *iplA* gene product by homologous recombination results in a clean biochemical defect: the abolition of Ca^{2+} entry stimulated by cAMP or folic acid. We can envisage two major possibilities for how IplA controls Ca^{2+} uptake. Either IplA is the plasma membrane channel through which Ca^{2+} enters the cell or, more likely, it is the membrane channel of an intracellular Ca^{2+} store, which, when drained, indirectly activates Ca^{2+} entry through the plasma membrane, in a manner analogous to capacitative uptake in vertebrate cells (Putney, 1986; Barritt, 1999). The linkage from the cAR1 receptor to IplA is also of interest, since stimulated Ca^{2+} entry is partially independent of G-proteins (Milne *et al*., 1995; Hall *et al*., 1999). These issues are being investigated further.

Whatever the mechanism, removal of IplA function is an effective way of ablating Ca^{2+} uptake, stimulated through the cAMP and folic acid receptors. Since the basal cytosolic Ca^{2+} concentration is not affected in the mutant, the *iplA* null strain provides a discerning test for the role of receptor-stimulated Ca^{2+} uptake in signal transduction and chemotaxis.

Our results show that, apart from the defect in Ca^{2+} influx, all other aspects of the chemotaxis signalling cascade investigated are unimpaired in the *iplA* mutant (cGMP and cAMP production and DdERK2 phosphorylation). Likewise, the actin–cytoskeletal response to an

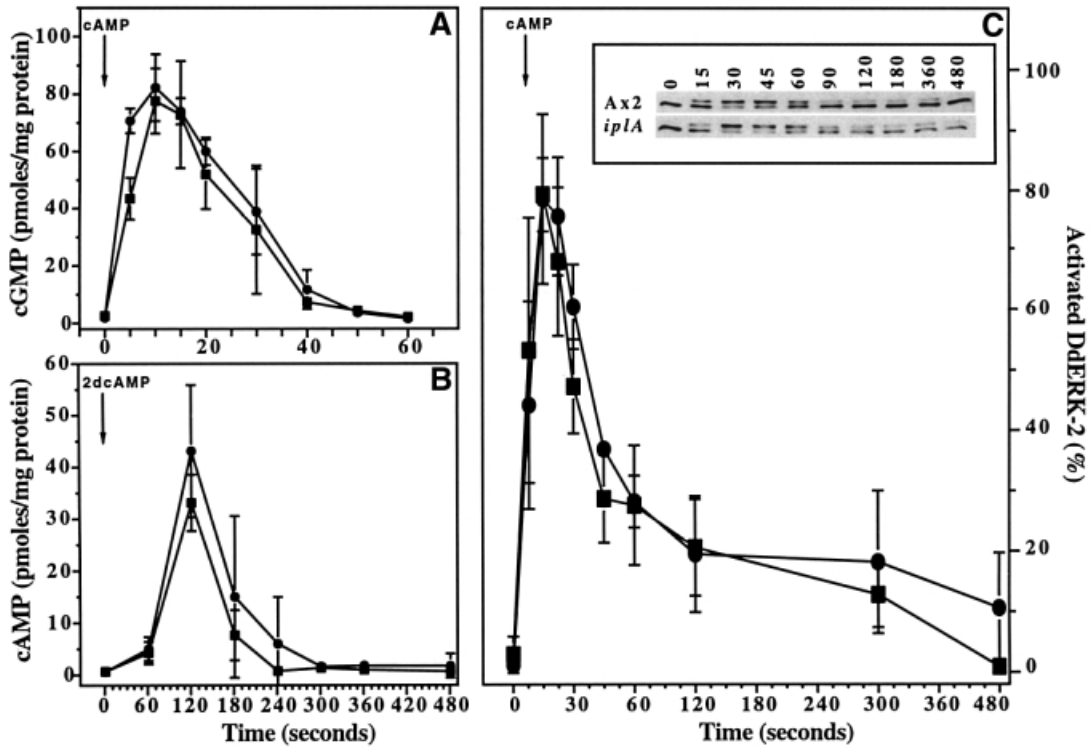

Fig. 4. Signal transduction events in response to cAMP. In all panels, aerated suspensions of aggregation-competent Ax2 (squares) and *iplA* (circles) cells are compared. (**A**) cGMP response; 1 μM cAMP stimulus. (**B**) cAMP relay response; cells at 1×10^8/ml, 10–20 μM 2dcAMP stimulus. (**C**) Activation of DdERK2; cells at 2×10^7/ml, 1 μM cAMP stimulus. Inset shows western blot using a polyclonal antiserum against DdERK2. The unactivated form (lower band) and the phosphorylated, activated form of DdERK2 (upper band) were quantified by densitometry. Results are the mean ± SD of at least three separate experiments.

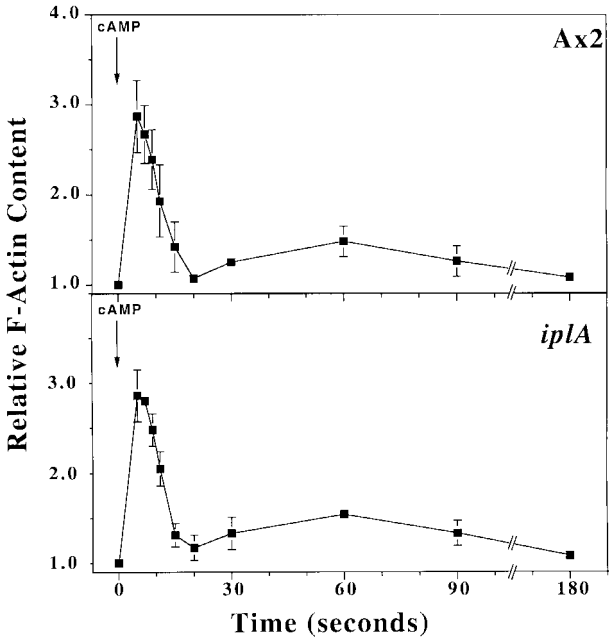

Fig. 5. Actin polymerization in response to cAMP. Aerated suspensions of 1.2×10^7/ml aggregation-competent cells in PBC were stimulated with 1 μM cAMP and the F-actin associated with the triton-insoluble cytoskeleton determined. Results are the mean ± SD of three separate experiments.

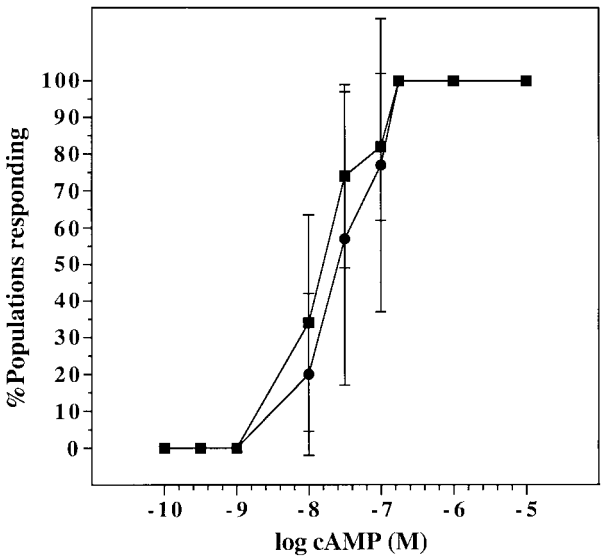

Fig. 6. Chemotactic sensitivity to cAMP. Small droplets containing 50–200 aggregation-competent Ax2 (squares) or *iplA* amoebae (circles) were placed on hydrophobic agar within 1 mm of a similarly sized droplet containing cAMP at the concentrations indicated. Chemotaxis was scored by microscopy 20–60 min later. Seven to 10 populations were scored for each concentration of cAMP and the results are given as the mean ± SD of three separate experiments.

Table I. Motility measurements for wild-type and *iplA* cells

		Speed (μm/min)	(*n*)	Persistence	Chemotactic index
Vegetative	Ax2	7.8 ± 3.2	(64)	0.45 ± 0.24	n.a.
	iplA	7.4 ± 3.2	(71)	0.44 ± 0.18	n.a.
Agg.-competent[a]	Ax2	10.9 ± 4.0	(29)	0.50 ± 0.12	n.a.
	iplA	9.9 ± 2.4	(24)	0.44 ± 0.09	n.a.
cAMP gradient[b]	Ax2	12.2 ± 3.4	(54)	0.73 ± 0.14	0.74 ± 0.13
	iplA	13.6 ± 4.0	(78)	0.64 ± 0.19	0.76 ± 0.13

[a]Aggregation-competent cells.
[b]Aggregation-competent cells in a gradient of 0–100 nM cAMP.
n.a., not applicable.
Data shown are mean ± SD.

elevation in cAMP concentration follows normal kinetics. Most tellingly, chemotaxis to cAMP is normal in the mutant with respect to sensitivity, accuracy of orientation and persistence of movement. It remains possible that some very local, brief or minor release of Ca^{2+} may still occur in stimulated *iplA* null cells, which was not detected by our imaging techniques and is necessary for chemotaxis. With this caveat, our results show that Ca^{2+} signalling is not essential for *Dictyostelium* chemotaxis. A similar conclusion has been reached by non-genetic means for leukocyte chemotaxis (Zigmond, 1977; Marasco *et al*., 1980).

A role for cAMP as a second messenger in chemotaxis can probably be eliminated since cells in which the major adenylyl cyclase, ACA, has been ablated, are able to chemotact (Pitt *et al*., 1992); a more likely role for intracellular cAMP is in regulating gene expression, through activation of protein kinase A. On the other hand, there is increasing evidence that signalling through cGMP is important, since several chemotactic defects correlate with alterations in its synthesis or breakdown (Ross and Newell, 1981; Kuwayama *et al*., 1993) and recent work also suggests a key role for the production of membrane-binding sites for PH-domain proteins (Parent *et al*., 1998; Meili *et al*., 1999). The current work now seems to eliminate an essential role for Ca^{2+} signalling in chemotaxis and therefore considerably narrows the search for the chemotactic steering mechanism.

Materials and methods

Materials

The sodium salt of 2′-deoxyadenosine 3′:5′-cyclic monophosphate (2dcAMP) and TRITC-phalloidin were from Sigma. Fura 2-dextran (10 kDa) was from Molecular Probes.

Cell culture and development

Amoebae were grown in shaken suspension (Watts and Ashworth, 1970) or in association with *Klebsiella aerogenes* on SM agar plates (Kay, 1987) at 22°C. Cells were developed in shaken (180 r.p.m.) suspension at 2×10^7/ml in PBC (2.1 mM Na_2HPO_4, 14.9 mM KH_2PO_4, 100 or

250 μM $CaCl_2$ pH 6.2) for 1 h then pulsed every 6 min with 100 nM cAMP for 3–7 h.

Gene disruption

Ax2 cells at 1–2 × 10^6/ml were harvested from axenic medium, washed twice in KK_2 (3.9 mM K_2HPO_4/16.5 mM KH_2PO_4 pH 6.1), once in E buffer (KK_2 plus 50 mM sucrose), resuspended at 1.25 × 10^7/ml in E buffer and 0.8 ml electroporated (1.6 kV at 3 μF) in an ice-cold cuvette (4 mm gap) with 12.5 μg of *Apa*I–*Sac*II restricted pDT5. After 10 min on ice, $CaCl_2$ and $MgCl_2$ were added to 1 mM. After 15 min at room temperature, the cells were dispensed into four 9 cm tissue culture dishes and growth medium added. Five cuvettes were processed in each disruption experiment. Selection at 10 μg/ml blasticidin S was started 15–18 h later in the presence of heat-killed *Klebsiella*. After 7–10 days, resistant cells were plated in association with *Klebsiella* on SM agar plates to obtain clones that were screened by PCR and Southern blot analysis to verify disruption of the *iplA* gene. The initial *iplA* null strain, HM1028, had a deletion/insertion 3′ end of the gene and was used in early work, but was later superceded by HM1038 and HM1049, both of which had all but the initial 1.4 kb of the 5′ and 1.4 kb of the 3′ end of the gene deleted (Figure 2). The phenotypes of all these strains were indistinguishable.

Cloning, sequencing and plasmid construction

The 809 bp insert of clone SSA203, from Dr T.Morio of the *Dictyostelium* cDNA sequencing project (University of Tsukuba, Japan), was used to isolate a 7.5 kb *Bgl*II fragment from a size-fractionated Ax2 genomic mini-library. A 5′ *Eco*RI fragment of 750 bp from this clone was used to probe a size-fractionated *Eco*RI mini-library, yielding a 5 kb genomic fragment. A 2.6 kb *Cla*I fragment of this was used to screen a size-fractionated *Bam*HI mini-library yielding a 6 kb clone encompassing the 5′ end of the gene and its promoter. DNA was sequenced using an ABI377 Prism with Thermo Sequenase dye terminator kits (Amersham). The position of introns was confirmed with the Titan™ reverse transcription–PCR system (Roche) using polyA$^+$ RNA isolated from Ax2 at the mound/tipped mound stage of development. All mini-libraries, inserts and PCR products were cloned into pBluescript II KS for further manipulation and sequencing.

The *iplA* disruption cassette used to generate the null strains HM1038 and HM1049 was constructed by PCR. The fragments corresponding to the 5′ end of the *iplA* gene (nucleotides 1304–2636 of sequence submitted to DDBJ/EMBL/GenBank) and 3′ end (nucleotides 9713–11201) were ligated into pRHI119, on either side of the blasticidin S resistance cassette, as *Apa*I–*Hin*dIII and *Not*I–*Sac*II fragments giving plasmid pDT5.

cAMP, cGMP, DdERK2 and F-actin assays

Amoebae were developed for 5–6 h in shaken suspension, washed once in PBC and resuspended at 10^8/ml in PBC. Suspensions were aerated using a fish tank pump for 10 min before beginning the experiment. Cells were stimulated with 10–20 μM 2-deoxy-cAMP for cAMP experiments and 1–2 μM cAMP for cGMP and DdERK2 experiments. For cyclic nucleotide assays, 300 μl of cell suspension were transferred to an ice-cold microfuge tube containing 15 μl of 35% perchloric acid and dithiothreitol to a final concentration of 5 mM at the times indicated. The suspension was neutralized with 80 μl of 50% (w/v) $KHCO_3$ and the precipitate pelleted by centrifugation. Cyclic nucleotides were assayed in 10–100 μl of the supernatant by radioisotopic dilution with the kits from Amersham. For the DdERK2 band shift assay, 100 μl aliquots were removed from aerated suspensions of aggregation-competent amoebae at 1 × 10^7/ml in PBC and added to an equal volume of 2× SDS–PAGE loading buffer; 30 μl aliquots were analysed by western blotting with DdERK2 antiserum 79-5 (Wang and Segall, 1998). F-actin associated with the triton-insoluble cytoskeleton was determined spectroscopically using TRITC-labelled phalloidin (Peracino *et al*., 1998).

Ca^{2+} uptake assay

Folic acid responses were assayed in vegetative amoebae grown on bacteria; all other assays used cells pulsed in shaken suspension for 4–6 h. Ca^{2+} uptake was measured as before (Milne and Coukell, 1991). Cell suspensions [10^8/ml in H-buffer (20 mM HEPES, 5 mM KCl pH 7.0)] were aerated prior to assay and uptake initiated by mixing 100 μl of cell suspension with 100 μl of assay mix (H-buffer containing ~0.5°C $^{45}CaCl_2$, 100 μM $CaCl_2$ and 500 μM $CoCl_2$) supplemented with stimulant or vehicle control. At the indicated times $^{45}Ca^{2+}$ uptake was stopped by the addition of 100 μl of ice-cold H-buffer containing 775 mM $CaCl_2$. Cells were pelleted for 5 s in a microcentrifuge (13 000 r.p.m.), washed in 1 ml of ice-cold H-buffer containing 10 mM $CaCl_2$, the pellet solubilized in 100 μl of 1% SDS and radioactivity counted.

Measurement of $[Ca^{2+}]_c$

Amoebae developed for 2–4 h in suspension were loaded with the Ca^{2+} indicator fura 2-dextran (10 kDa) by electroporation (Schaloske *et al*., 1998), except that dye loading was at 10–15 mg/ml. Measurements were made 1–4 h after loading on glass coverslips in 0.5–1.0 ml buffer (5 mM HEPES, 5 mM KCl pH 7.0), usually with 1 mM $CaCl_2$. Cells were observed with a 100× lens (Nikon, NA 1.3) on a Nikon inverted microscope with excitation by a Xenon lamp using 340 and 380 nm bandpass filters in a rotating filter wheel. Exposures were 0.6–0.8 s at each wavelength, with 4–6 s between sets. Images were captured with a CCD camera (Princeton Electronics) and processed using Metafluor software (Universal Imaging Corporation). Calibration was with the calcium calibration kit 1 from Molecular Probes.

Cell movement and chemotaxis

Vegetative or aggregation-competent amoebae were observed by video microscopy in a Dvorak-Stotler chamber (for random movement) or a Dunn chamber (chemotaxis of aggregation-competent amoebae). Time-lapse videos were captured and analysed by NIH Image software (Tuxworth *et al*., 1997). All experiments were performed in PBC buffer.

Accession number

The DDBJ/EMBL/GenBank accession No. for the *iplA* sequence is AJ277590.

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