Influx of extracellular Ca^{2+} is necessary for electrotaxis in *Dictyostelium*

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Summary

Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) is a pivotal signalling element in cell migration and is thought to be required for chemotaxis of *Dictyostelium*. Ca^{2+} signalling may also be important for electrotaxis. However this suggestion has been controversial. We show that electric fields direct *Dictyostelium* cells to migrate cathodally and increase $[Ca^{2+}]_i$ in *Dictyostelium* cells, as determined by Fluo-3 AM imaging and $^{45}Ca^{2+}$ uptake. Omission of extracellular Ca^{2+}($[Ca^{2+}]_e$) and incubation with EGTA abolished the electric-field-stimulated $[Ca^{2+}]_i$ rise and directional cell migration. This suggests a requirement for $[Ca^{2+}]_e$ in the electrotactic response. Deletion of *iplA*, a gene responsible for chemoattractant-induced $[Ca^{2+}]_i$ increase, had only a minor effect on the electric-field-induced $[Ca^{2+}]_i$ rise. Moreover, *iplA*-null *Dictyostelium* cells showed the same electrotactic response as wild-type cells. Therefore, *iplA*-independent Ca^{2+} influx is necessary for electrotactic cell migration. These results suggest that the $[Ca^{2+}]_i$ regulatory mechanisms induced by electric fields are different from those induced by cAMP and folic acid in *Dictyostelium* cells. Different roles of the *iplA* gene in chemoattractant-induced and electrically induced Ca^{2+} signalling, and different effects of $[Ca^{2+}]_i$ elevation on chemotaxis and electrotaxis indicate that the chemoattractant and electric cues activate distinctive initial signalling elements.

Supplementary material available online at
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Key words: Calcium, Cell migration, *Dictyostelium*, *iplA*, Electrotaxis

Introduction

Migrating cells frequently display transient, graded increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) that correlate with their direction of movement (Taylor et al., 1980; Sawyer et al., 1985; Brundage et al., 1991; Marks and Maxfield, 1990; Mandeville et al., 1995; Lee et al., 1999). Chemoattractants induce rapid Ca^{2+} entry across the plasma membrane and elevate $[Ca^{2+}]_i$ in many types of cells, including those of *Dictyostelium discoideum* (Wick et al., 1978; Yumura et al., 1996; Nebl and Fisher, 1997). Ca^{2+} signalling is generally believed to play a very important role in directional cell migration in chemical gradients. In support for this, focal Ca^{2+} release induces biased motility of growth cones (Zheng, 2000; Hong et al., 2000). In addition, actin-binding proteins, such as α-actinin and severin are sensitive to $[Ca^{2+}]_i$ (Witke et al., 1993; Yamamoto et al., 1982). Surprisingly, genetic analysis shows that Ca^{2+} signalling is not required for chemotaxis of *Dictyostelium* cells. Disruption of an inositol 1,3,5-trisphosphate [Ins(1,3,5)P_3]-receptor-like gene, *iplA*, in *Dictyostelium* cells abolishes $[Ca^{2+}]_i$ rise in response to chemoattractants (cAMP or folic acid). However, chemotaxis of *iplA*-deficient cells towards sources of cAMP and folic acid is unaffected (Traynor et al., 2000; Schaloske et al., 2005).

Many types of cells respond to direct-current electric fields (EFs) by directional migration, a phenomenon called galvanotaxis or electrotaxis. These include fish and human keratinocytes, human corneal epithelial cells and *Dictyostelium* cells (Cooper and Schliwa, 1986; Nishimura et al., 1996; Zhao et al., 1996; Zhao et al., 1997; Zhao et al., 2002; Pullar and Isseroff, 2005; Pullar et al., 2006). Most cell types are attracted cathodally and repelled anodally. However, some types of cells migrate towards the anode, such as lens epithelial cells and vascular endothelial cells (Wang et al., 2003; Zhao et al., 2004; Bai et al., 2004). A role for EFs in directing cell migration during development and in wound healing has been suggested (Nuccitelli, 2003; McCaig and Zhao, 1997; McCaig et al., 2005). Accumulating experimental evidence suggests a much more important role than previously thought for electric signals in directing cell migration in wound healing (Zhao et al., 2006). EFs are detected at the cornea and skin wounds and manipulation of these naturally occurring wound electric currents prevents healing of the wounds (Illingworth and Barker, 1980; Chiang et al., 1992; Sta Iglesia and Vanable, Jr, 1998; Reid et al., 2005). Disruption of electrical gradients during development induces skeletal and neural abnormalities (Hotary and Robinson, 1992; Hotary and Robinson, 1994; Shi and Borgens, 1995). Whereas asymmetric surface-membrane-protein distribution and biased intracellular signalling are proposed to be involved in electrotaxis (Brown and Loew, 1994; Zhao et al., 2003), the mechanisms that mediate electrotaxis and whether the same or similar signalling elements govern electrotaxis and chemotaxis are largely unknown.

Ca^{2+} signalling has been proposed to play an essential role

in the electrotactic response (Mycielska and Djamgoz, 2004). However this suggestion has been controversial. For example, Ca^{2+} dependence of electrotaxis has been observed in neural crest cells, embryo mouse fibroblasts, and fish and human keratocytes (Cooper and Schliwa, 1985; Onuma and Hui, 1985; Onuma and Hui, 1988; Nuccitelli and Smart, 1989; Nuccitelli and Smart, 1991; Fang et al., 1998; Trollinger et al., 2002). By contrast, two strains of mouse fibroblasts exhibit electrotaxis independent of Ca^{2+} signalling (Brown and Loew, 1994).

Using the genetically tractable organism *Dictyostelium discoideum*, we aimed to elucidate the role of Ca^{2+} signalling in electrotaxis. Unlike many types of cells, developed *Dictyostelium* cells migrate well in low-Ca^{2+} and Ca^{2+}-free buffers (Korohoda et al., 2002). This allows to, more reliably, investigate the role of Ca^{2+} signalling in electrotaxis. Combining Ca^{2+} imaging, radioactive Ca^{2+} measurements, extracellular Ca^{2+} ($[Ca^{2+}]_e$) depletion and genetic manipulation, we investigated the role of Ca^{2+} signalling in electrotaxis of *Dictyostelium* cells. We show that in *Dictyostelium* cells: First, EFs induce long-lasting $[Ca^{2+}]_i$ elevation compared with no-EF controls, which is different from rapid onset and short-lived $[Ca^{2+}]_i$ increase induced by chemoattractants. Second, there are two components of $[Ca^{2+}]_i$ rise in electrotaxis. The first component is dependent on the *iplA* gene and is not essential for EF-directed cell migration. The second component of the Ca^{2+} influx is *iplA* independent and is required for electrotaxis in *Dictyostelium*. These results contrast with those for chemotaxis in which the $[Ca^{2+}]_i$ rise primarily depends upon the *iplA* gene, whereas the EF-induced $[Ca^{2+}]_i$ rise is largely independent of *iplA*. A chemoattractant-induced $[Ca^{2+}]_i$ rise is not essential for chemotactic migration, whereas an EF-induced $[Ca^{2+}]_i$ rise due to Ca^{2+} influx is required for electrotactic migration. Thus, these findings suggest an important difference between the mechanisms underlying electrotaxis and chemotaxis.

Results

Dictyostelium cells migrate cathodally in an EF

A direct-current EF induced *Dictyostelium* cells to migrate directionally towards the cathode (Zhao et al., 2002) (Fig. 1). By contrast, the same batch of cells showed random migration when not exposed to EFs (Fig. 1). Direction of migration, which is a measure of how the cells move in a particular direction, was assessed as cosine θ (see Materials and Methods). In the absence of an EF the cosine θ value was close to zero (0.07±0.11) indicating random cell migration. Direction of migration increased significantly to 0.91±0.02 when the cells were exposed to an EF (15 V/cm), indicating that almost all cells migrated directly towards the cathode (Fig. 2). Reversal of the field polarity caused rapid reversal of the direction of migration (Fig. 2A; see also supplementary material Movie 1). Trajectory speed was not significantly altered in the presence of an EF, but displacement speed increased and this increase was maintained on field reversal (Fig. 2B).

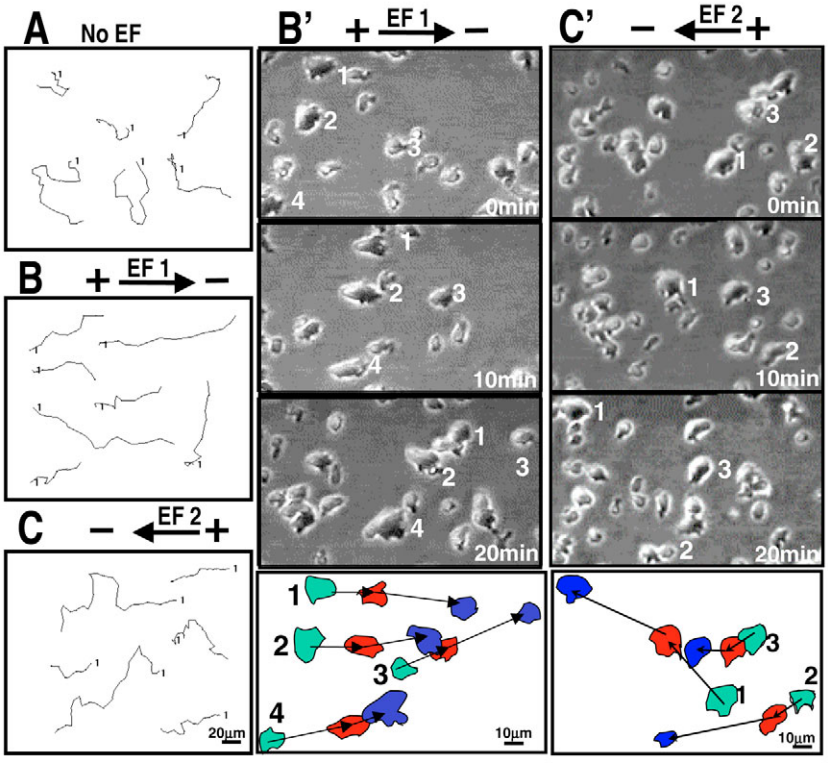

Fig. 1. *Dictyostelium* cells migrate directionally in an electric field. (A,B,C) Trajectories plotting the movement of the cell centres of AX2 cells in (A) the absence of an electric field, (B) the presence of an electric field and (C) following reversal of field polarity. (B',C') Consecutive micrographs show migration of cells in an electric field of 15 V/cm. (supplementary material Movie 1). Numbers in A-C' indicate individual cells.

EFs induce Ca^{2+} signalling in *Dictyostelium* cells

Imaging developed *Dictyostelium* cells loaded with Fluo-3 AM under control condition (no EF) produced a very steady and consistent decline in the ratio of $F:F_0$ (average from individual cells), where F is the fluorescence intensity at a defined time and F_0 the fluorescence intensity at time 0 (Fig. 3A). Varying the duration of excitation illumination verified that the steady decline of the $F:F_0$ ratio was mainly due to photobleaching. Imaging the cells at a fixed exposure time every 15, 5 or 2 seconds showed a well-correlated increase in the decline of the $F:F_0$ ratio with time, whereas increasing the duration of intervals between successive excitations to every 30 seconds resulted in much reduced decline in $F:F_0$ with time (data not shown, for details see Materials and Methods). The technique used to image $[Ca^{2+}]_i$ with Fluo-3 AM in *Dictyostelium* cells was further verified by stimulation with cAMP, which induced a significant elevation in the $F:F_0$ ratio. Consistent with previous observations (Yumura et al., 1996; Lusche et al., 2005) the elevation in the $F:F_0$ ratio lasted between 15 and 30 seconds (Fig. 3A; P=0.001, control vs cAMP-treated cells 15 seconds after the addition of cAMP, n=17).

For imaging the effects of an EF on $[Ca^{2+}]_i$ an exposure time of 200 miliseconds and intervals of 15 seconds were chosen. This minimised photobleaching but at the same time

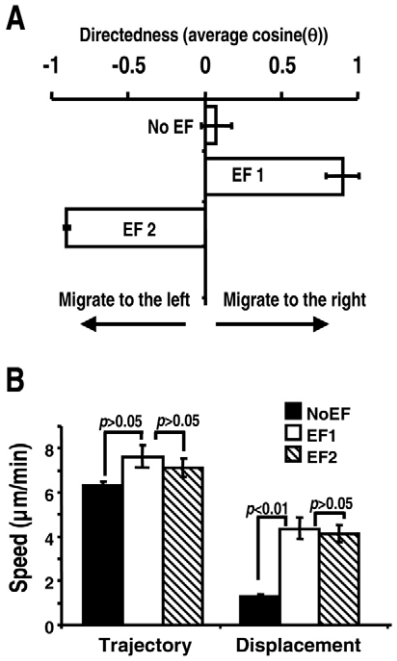

Fig. 2. Quantification of the directional migration of *Dictyostelium* cells in an electric field. (A) Direction of cell migration without and with an EF (EF1, cathode on the right) and reversal of polarity (EF2, cathode to the left). (B) Trajectory and displacement speeds; average of 33-43 cells from three independent experiments.

allowed the capture of short-duration $[Ca^{2+}]_i$ spikes which were observed ~200 seconds after the EF was switched on. These sporadic spikes of $[Ca^{2+}]_i$ rise were observed in about 40-50% of cells exposed to electric fields (Fig. 3B; Fig. 4E,I; supplementary material Movie 2). Control cells that were not subjected to an EF did not show these high-magnitude spikes (Fig. 3C). Analysis of cell movement showed that the spikes were often correlated with changes in the direction of migration. Nine cells in which 13 spikes were identified were closely examined. In 11 cases the Ca^{2+} rise correlated with the change of direction of the cell (>45 degrees). In two cases the peak occurred without any change in migration direction, and six turnings were not accompanied by $[Ca^{2+}]_i$ spikes.

Ca^{2+} spikes were not synchronised between cells, however, when compared with cells not exposed to an EF; averaging of the $F:F_0$ ratio still revealed an overall increase in $[Ca^{2+}]_i$ in those cells exposed to an EF. (Fig. 3A). This EF-induced $[Ca^{2+}]_i$ elevation is significant as early as 15 seconds after the EF is switched on (P=0.01) and an $F:F_0$ ratio of 0.94±0.24 was maintained for at least 255 seconds. This elevation is significant compared with control cells not exposed to an EF ($F:F_0$=0.46±0.12; P<0.001). This type of $[Ca^{2+}]_i$ elevation, induced by an EF, persisted for the entire duration in which the cells were examined (Fig. 4L). Compared with control cells, this long-lasting $[Ca^{2+}]_i$ rise contrasts with the short $[Ca^{2+}]_i$ rise that occurs in response to stimulation with cAMP and folic acid (Fig. 3A,D) (Yumura et al., 1996).

$^{45}Ca^{2+}$ uptake confirms that EFs induce Ca^{2+} signalling

To confirm the effect of EF stimulation on Ca^{2+} signalling, uptake of $^{45}Ca^{2+}$ by *Dictyostelium* cells was measured.

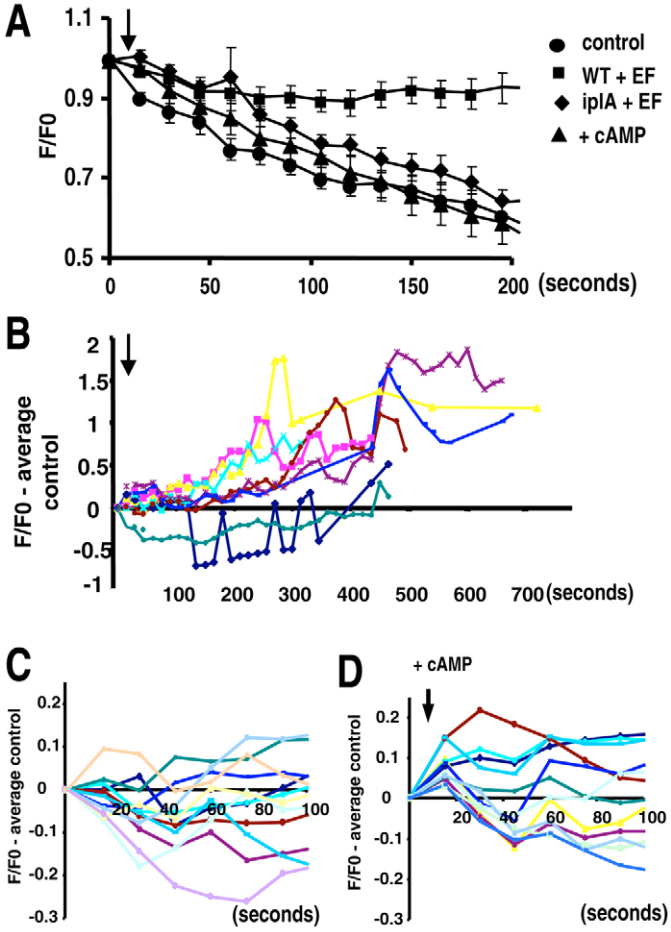

Fig. 3. A direct-current EF induces $[Ca^{2+}]_i$ elevation compared with the control. (A) $F:F_0$ ratio of control cells and *iplA* mutants exposed to an EF or cAMP. (B-D) Relative fluorescence intensity ($F:F_0$ is the average $F:F_0$ ratio of control cells) obtained for (B) individual wild-type cells in, (C) an EF under control conditions and (D) following stimulation with cAMP. Arrows indicate start of EF exposure (in B) or addition of cAMP (in D).

Exposure of cells to an EF for 10 minutes increased the level of intracellular $[^{45}Ca^{2+}]_i$ significantly. Wild-type (AX2) *Dictyostelium* cells showed an 284±36% increase in $[^{45}Ca^{2+}]_i$ after 10 minutes in an EF (Fig. 5). These two methods, Ca^{2+} imaging and $^{45}Ca^{2+}$-uptake measurement, demonstrated that EF stimulation increases $[Ca^{2+}]_i$ in *Dictyostelium* cells and an elevation of $[Ca^{2+}]_i$ lasts longer compared with that following cAMP stimulation.

The *iplA*-null mutation does not prevent EF-induced increase in $[Ca^{2+}]_i$

iplA-null cells do not show an agonist-induced $[Ca^{2+}]_i$ $^{+}$ increase (Traynor et al., 2000). We then asked whether such a mutation has an effect on EF-induced $[Ca^{2+}]_i$ increase. Under control conditions in the absence of an EF, the amount $^{45}Ca^{2+}$ present in *iplA* null cells was not different from that in wild-type cells. Similarly to wild-type cells, EF stimulation (15 V/cm) of *iplA*-null cells resulted in a rapid elevation of $[Ca^{2+}]_i$ (15 seconds after switching the EF on, P=0.048, Fig. 3A) compared with control cells. However, the magnitude of this

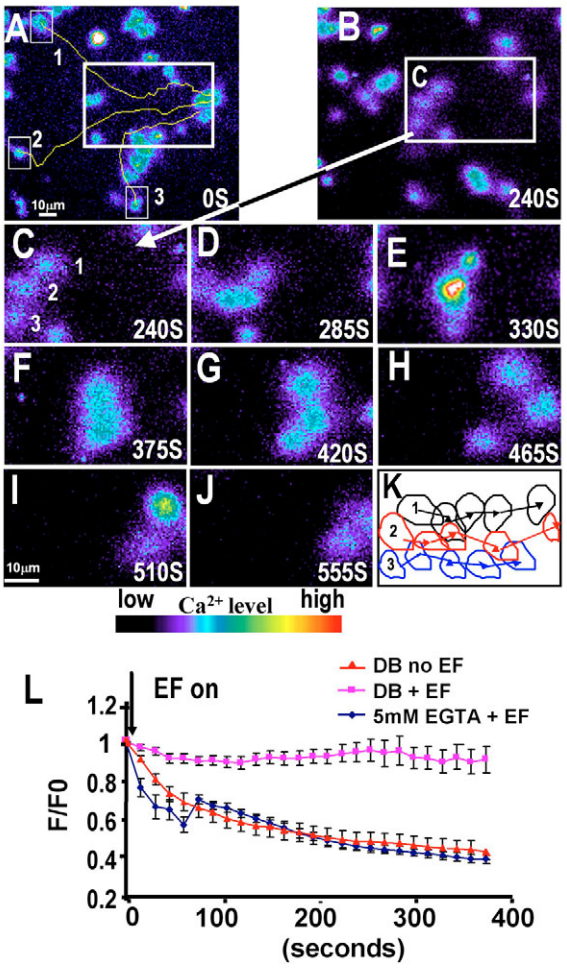

Fig. 4. A direct-current EF induces $[Ca^{2+}]_i$ elevation from the control and later-appearing $[Ca^{2+}]_i$ spikes. (A-J) Fluorescent images of Fluo-3-AM-loaded cells. (A,B) Entire field at time 0 and 240 seconds. The EF was switched on immediately after the frame in A was taken and the cells moved to the right (towards the cathode). (C-J) Selected field showing details of migrating cells and the $[Ca^{2+}]_i$ spikes (E,I). (K) Tracks of the three cells shown in A-J. (L) EF exposure significantly increases $[Ca^{2+}]_i$ (P=8^{-9}), which is prevented in the presence of 5 mM EGTA (n=29-30 cells; supplementary material Movie 2). Numbers in A and C indicate individual cells.

rise was lower than that observed in wild-type cells (120 seconds after EF exposure, the F:F_0 ratio for mutants was 0.79±0.03 compared with 0.089±0.03 for wild type, P<0.05). Furthermore, compared with wild-type cells, the duration of the EF-induced Ca^{2+} response was comparatively short. (160 seconds compared with 500 seconds in wild-type cells). During Ca^{2+} imaging, the cells migrated actively towards the cathode; however, some cells did not migrate well, presumably owing to strong fluorescence illumination. We further confirmed these observations by determining $^{45}Ca^{2+}$ uptake. A 10-minute exposure to an EF resulted in a significant increase in $^{45}Ca^{2+}$ influx in *iplA*-null cells (186±24%) (Fig. 5). Consistent with our Fluo-3 AM data, the magnitude of Ca^{2+} rise in *iplA*-null cells was lower compared with wild-type *Dictyostelium* cells (P<0.05).

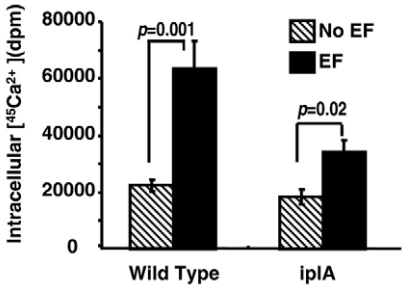

Fig. 5. A direct-current EF induces $^{45}Ca^{2+}$ influx. EF stimulation increases the amount of radioactive $^{45}Ca^{2+}$ (dpm) detected within both wild-type and *iplA*-null mutant cells (average of four independent experiments).

Electrotaxis does not require the *iplA* gene

In the absence of an EF, *iplA*-null cells migrated randomly with a directedness value of 0.08±0.14. Consistent with their wild type counterparts, *iplA* null cells show significant $[Ca^{2+}]_i$ change compared to control conditions in the absence of an EF and migrate to wards the cathodal in response to EF stimuli. In an EF, *iplA* null cells migrated towards the cathode with a increase in the direction of migration of 0.90±0.04, a value virtually the same as that of wild-type cells (Fig. 7A). Additionally, reversal of the polarity of the EF reversed the direction of cell migration (Fig. 6; supplementary material Movie 3). Thus the *iplA* gene and its products are not required in the directional sensing mechanisms in response to an EF. The *iplA* gene does, however, appears to play a minor role in EF-induced increase in displacement speed. Under conditions without an EF, *iplA*-null cells migrate randomly with a trajectory- and displacement-speed similar to wild type cells (Fig. 7B,C). Following exposure to an EF the average displacement speed of the *iplA* mutants increased significantly; however, this increase was significantly less than that of wild-type cells (Fig. 7C).

$[Ca^{2+}]_e$ is necessary for electrotaxis

To assess the requirement of $[Ca^{2+}]_e$ for electrotaxis, we tested what effect an increase of $[Ca^{2+}]_e$ (to 0.6 mM) and depletion of $[Ca^{2+}]_e$ (by using Ca^{2+}-free buffer or extracellular medium without Ca^{2+} but containing 5 mM EGTA) has on electrotaxis. Neither elevation nor depletion of $[Ca^{2+}]_e$ significantly affected cellular morphology or migratory behaviour in the absence of an EF (Fig. 8A,C, Fig. 9). No difference in basal migration (without an EF) was observed between the two $[Ca^{2+}]_e$-depletion conditions (a, Ca^{2+}-free buffer extracellularly; b, Ca^{2+}-free buffer with 5 mM EGTA) and the data in Figs 8, 9 are a combination of the two. Increasing the concentration of EGTA above 5 mM did, however, result in rounded-up *Dictyostelium* cells and in the inhibition of random cell migration. To assay whether 5 mM EGTA was sufficient to alter an EF-induced rise of $[Ca^{2+}]_i$, we monitored $[Ca^{2+}]_i$ by measuring Fluo-3 AM fluorescence intensity. These experiments confirmed that 5 mM EGTA are indeed effective at preventing the EF-induced $[Ca^{2+}]_i$ rise and suggest that $[Ca^{2+}]_e$ is required for the EF-induced Ca^{2+} response in *Dictyostelium* (Fig. 4L).

Having confirmed that our experimental conditions did not significantly alter basal migration, and that the protocol used

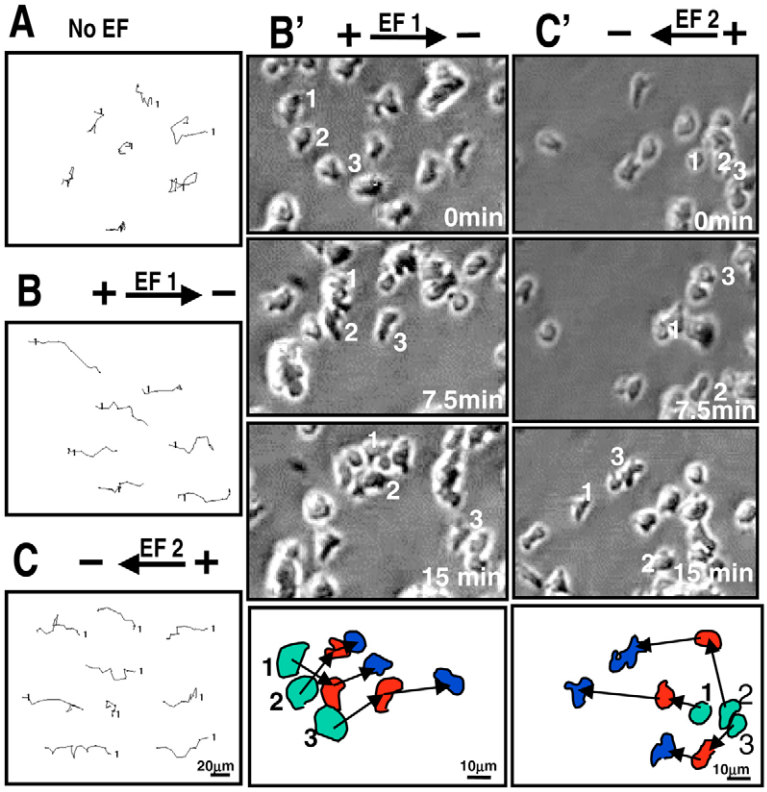

Fig. 6. *iplA* gene is not required for electrotaxis. (A-C) Trajectories plotting the movement of cell centres under control conditions (A), in the presence of an electric field with the cathode to the right (B) and on reversal of field polarity (C). (B',C') Consecutive micrographs show directional migration of cells in an electric field of 15 V/cm at 0, 7.5 and 15 minutes (supplementary material, Movie 3). Numbers in A-C' indicate individual cells.

to deplete $[Ca^{2+}]_e$ did indeed prevent an EF-induced Ca^{2+} response, we investigated whether these conditions affected electrotaxis. Increasing $[Ca^{2+}]_e$ to 0.6 mM did not have detectable effects on electrotaxis, and cells migrated effectively towards the cathode (Fig. 8B). The direction of migration towards the cathode as well as the trajectory and displacement migration speeds remained the same as in cells kept in normal developing buffer buffer with 0.2 mM Ca^{2+} (Fig. 9). By contrast, depletion of $[Ca^{2+}]_e$ completely abolished the directional migration of *Dictyostelium* cells in an EF. In the absence of Ca^{2+} (achieved by Ca^{2+} omission or the addition of 5 mM EGTA to the extracellular medium) both AX2 cells and *iplA*-null cells lost the EF-induced directional response, while, however, maintaining active migration (Fig. 8D, Fig. 9A; see also supplementary material, Movie 4; data of *iplA*-null cells are not shown). These results indicate that the EF-induced $[Ca^{2+}]_i$ rise, which is prevented by removal of Ca^{2+} from the extracellular medium, is required for electrotaxis.

Discussion

The molecular mechanisms underlying EF-directed cell migration are poorly understood. The slime mould *Dictyostelium* show robust EF-guided migration and offer a good model to study electrotaxis (Zhao et al., 2002). Using this genetically tractable organism, we have investigated the role of Ca^{2+} signalling in electrotaxis. We show in this report that EFs induce a significant and sustained $[Ca^{2+}]_i$ elevation compared with the control in *Dictyostelium* cells. This is different from the transient rise of $[Ca^{2+}]_i$ induced by chemoattractants (Yumura et al., 1996). The persistent $[Ca^{2+}]_i$ elevation comprises an *iplA*-independent element and an *iplA*-dependent element. Late-appearing $[Ca^{2+}]_i$ spikes in cells exposed to an EF appear to contribute to the sustained $[Ca^{2+}]_i$ elevation from the control (Fig. 3A,B). The *Dictyostelium iplA* gene, is a key regulator of agonist-induced Ca^{2+} flux (Traynor et al., 2000; Schaloske et al., 2005). By contrast, this study demonstrates that the *iplA* gene is not essential for EF-induced $[Ca^{2+}]_i$ and directional migration. Instead, an *iplA*-independent, EF-induced Ca^{2+} rise that is dependent upon the presence of $[Ca^{2+}]_e$ is required for directional migration of *Dictyostelium* cells in an EF.

EFs induce a rise in $[Ca^{2+}]_i$

Using two different methods, Ca^{2+} imaging and $^{45}Ca^{2+}$-uptake measurement, we have demonstrated that *Dictyostelium* cells elevated $[Ca^{2+}]_i$ in response to EFs (Figs 3-5). The *iplA* gene, which is orthologous to those encoding eukaryotic Ins(1,3,5)P_3 receptors, has recently been identified in *Dictyostelium* cells (Traynor et al., 2000). This *iplA* gene is thought to represent a membrane channel of an intracellular Ca^{2+} store and mediates agonist-induced Ca^{2+} fluxes (Schaloske et al., 2005). Deletion of *iplA* in *Dictyostelium* cells completely abolishes the $[Ca^{2+}]_i$ rise induced by cAMP or folic acid (Traynor et al., 2000). Also, in *iplA*-null cells the EF-induced $[Ca^{2+}]_i$ rise is reduced (our observations), indicating that a significant component of the $[Ca^{2+}]_i$ elevation is mediated by *iplA* products, i.e. Ins(1,3,5)P_3 receptors controlling Ca^{2+} release from intracellular Ca^{2+} stores (Fig. 5). However, EFs still induced a significant increase in $[Ca^{2+}]_i$, even in the absence of the *iplA* gene (Fig. 5). However, agonist-induced $[Ca^{2+}]_i$ increase is abolished in the *iplA*-null cells (Traynor et al., 2000) This suggests that the electrotactic response induces $[Ca^{2+}]_i$ regulatory mechanisms that are different from those underlying the $[Ca^{2+}]_i$ rise induced by chemoattractants.

The *iplA*-independent mechanisms of $[Ca^{2+}]_i$ elevation is yet to be determined. In wild-type cells, we noticed later-appearing $[Ca^{2+}]_i$ spikes (~200 seconds after an EF was switched on) (Fig. 3B). These spikes contribute, at least partially, to the maintained elevation of $[Ca^{2+}]_i$. Another possibility for the sustained elevation in $[Ca^{2+}]_i$ might involve passive influx. It is estimated that in a cell of 10-μm diameter, exposed to an EF of 100 mV/mm, the cathodal side of cell membrane would depolarise by ~5 mV, whereas the membrane facing the anode hyperpolarises by the same amount (Patel and Poo, 1982; Poo, 1981). Presuming the dependence of depolarisation and/or hyperpolarisation on electric field strength is linear, an electric field of 1000 mV/mm would cause the cathodal side of cell membrane to depolarise by ~50 mV, whereas the anodal side of membrane hyperpolarise by ~50 mV. This is significant, because *Dictyostelium* cells have a membrane potential of ~19-39 mV (Van Duijn et al., 1988). The resulting hyperpolarisation could increase the electromotive force

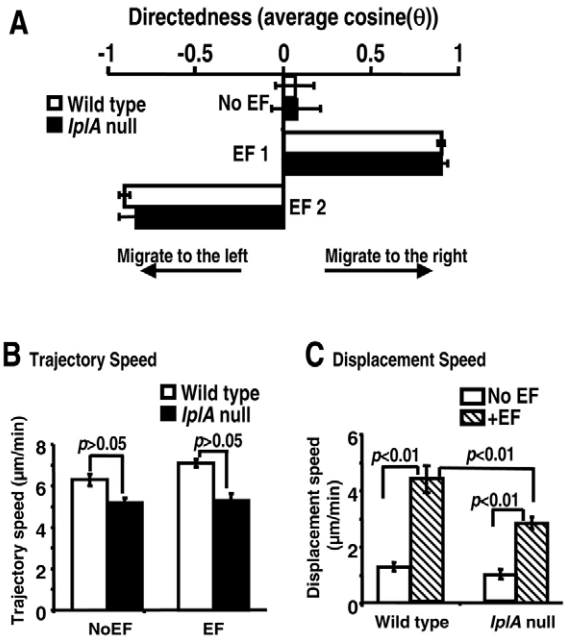

Fig. 7. The *iplA* gene is not required for electrotaxis. (A) *iplA*-null cells and AX2 cells migrate towards the cathode with similar direction of cell migration. (B) The deletion of *iplA* does not significantly affect the trajectory speeds. (C) Displacement speeds increased significantly in both types of cells following field stimulation, but the increase of that of *iplA*-null cells was significantly less than that of AX2 cells (P=0.004; data are the average of 21-33 cells from three independent experiments).

driving Ca^{2+} passively into the cell (Cooper and Keller, 1984). However, Ca^{2+} fluxes are also mediated by voltage-gated channels and mechanosensitive channels. Voltage-gated Ca^{2+} channels (VGCCs) might not play a significant role here because it has been suggested that, when conducted by VGCCs, the rise in $[Ca^{2+}]_i$ would be biased towards the cathodal side (Mycielska and Djamgoz, 2004), i.e. towards the front side of the migrating cell in case of *Dictyostelium*. Nevertheless, it was shown that migrating fibroblasts in the later stages of wound healing exhibit an increasing gradient of free Ca^{2+} from the front to the rear (Hahn et al., 1992; Gough and Taylor, 1993). Thus it is more probable that the site of higher Ca^{2+} rise occurs at the rear side of the migrating cells. Local Ca^{2+} rises might function to trigger focal adhesion disassembly (Giannone et al., 2004), thus enabling the rear-edge detachment during migration process. The role of mechanosensitive channels in electrotactic response of *Dictyostelium* cells is yet to be tested. Further work is required using this cell model of electrotaxis to examine the mechanism by which $[Ca^{2+}]_e$ modulates the intracellular molecular machinery to drive the field-directed response.

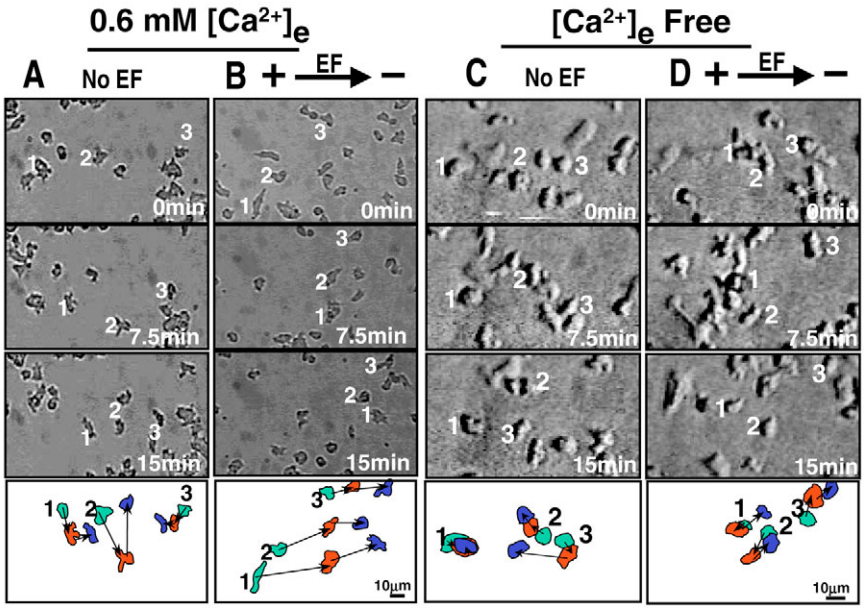

Fig. 8. Depletion of $[Ca^{2+}]_e$ abolishes electrotactic response. (A,C) Varying $[Ca^{2+}]_e$ concentrations did not affect cell migration in control conditions. (B) Increasing $[Ca^{2+}]_e$ to 0.6 mM did not affect the electrotactic response. (D) depletion of $[Ca^{2+}]_e$ (by using Ca^{2+}-free buffer or addition of 5 mM EGTA) completely abolished the electrotactic response, while the cells still migrate actively. (supplementary material, Movie 4). Numbers in A-D indicate individual cells.

$[Ca^{2+}]_i$, the *iplA* gene and electrotactic migration

Evidence from our experiments supports the role of Ca^{2+} signalling in electrotaxis. First, EFs induce sustained $[Ca^{2+}]_i$ elevation above the control (Figs 3-5). Second, depletion of $[Ca^{2+}]_e$ completely abolished $[Ca^{2+}]_i$ rise and electrotactic response (Figs 8, 9).

As discussed above, EF-induced $[Ca^{2+}]_i$ elevation is mediated by two mechanisms, one *iplA* dependent the other *iplA* independent. Deletion of *iplA* in *Dictyostelium* cells does not have an effect on their directional migration in an EF, which suggests that a full $[Ca^{2+}]_i$ rise is not required for directional sensing (Figs 6, 7). However, the *iplA*-independent EF-induced $[Ca^{2+}]_i$ elevation is essential because removal of Ca^{2+} from the extracellular medium completely prevents the electrotactic response. This may explain why most reports suggest a role for Ca^{2+} signalling in electrotaxis, (Cooper and Schliwa, 1985; Onuma and Hui, 1985; Nuccitelli and Smart, 1989; Nuccitelli and Smart, 1991; Fang et al., 1998; Trollinger et al., 2002; Mycielska and Djamgoz, 2004), whereas one report reports no $[Ca^{2+}]_i$ change (Brown and Loew, 1994). This discrepancy may be accounted for by the requirement for different levels of $[Ca^{2+}]_i$ rise that are required for the electrotactic response in different cell types. Indeed, Brown and Loew (Brown and Loew, 1994) did not detect any change in Fura-2-dextran fluorescence intensity on exposure of the fibroblast cell lines NIH3T3 or SV101 to an EF. It might be that these cells do not, as the authors conclude, elevate $[Ca^{2+}]_i$ in response to field exposure. However, it might also be true that the Ca^{2+} rise is too small to be detected by the Ca^{2+}-imaging methods available

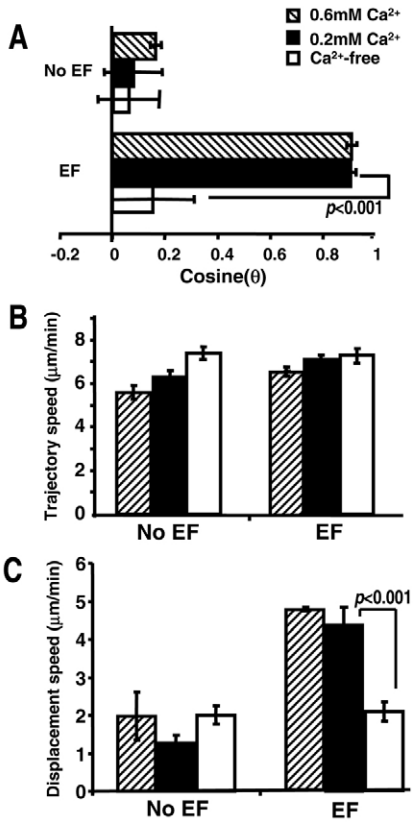

Fig. 9. Requirement of $[Ca^{2+}]_e$ for electrotactic migration. (A-C) Effects of varying $[Ca^{2+}]_e$ concentrations on (A) direction of cell migration, (B) trajectory speed and (C) displacement speed. Data are the average of 30-72 cells from at least three independent experiments.

at that time. If this hypothesis is correct, one might expect that a small amount of Ca^{2+} remains in the extracellular medium despite the presence of 1 mM EGTA. In the case of the NIH 3T3 and SV101 cells, this small amount of residual Ca^{2+} may be sufficient to promote electrotactic behaviour and would explain the discrepancy of these findings.

In summary, direct-current EFs induce sustained $[Ca^{2+}]_i$ elevation above control level in *Dictyostelium* cells. This is different from the rapid $[Ca^{2+}]_i$ transient caused by chemoattractants. EF-induced $[Ca^{2+}]_i$ elevation includes two components: one is dependent upon the *iplA* gene; the other is independent of the gene. Depletion of $[Ca^{2+}]_e$ abolishes electrotaxis of *Dictyostelium* cells. The elevation of $[Ca^{2+}]_i$ is essential for EF-directed cell migration. These results suggest a crucial role for an *iplA*-independent rise of $[Ca^{2+}]_i$ in electrotaxis. As well as identifying important control elements of electrotaxis, this study also indicates a possible signalling divergence between chemotaxis and electrotaxis.

Materials and Methods

Cell culture and development

Wild-type (AX2) and *iplA*-null cells were grown in suspension and shaken (120-150 rpm) or were grown on tissue culture Petri dishes in HL5 medium. At 4-6 hours prior to experimentation, cells were starved for 1 hour in developing buffer (10 mM Na_2HPO_4-$7H_2O$, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, pH 6.2) and then pulsed with 100 μM cAMP every 6 minutes in while shaken in suspension.

EF stimulation

Developed cells were seeded in a specially constructed trough as described (Zhao et al., 1996). For EF application, two agar salt bridges were used to connect Ag_2Cl_2 electrodes in beakers of developing buffer to pools of excess buffer at either sides of the chamber. In control experiments, the electric fields were not switched on. Unless stated otherwise, a field strength of 15 V/cm was used. Field strengths were measured directly at the beginning and at the end of the experiment.

Quantification of cell behaviour

Cells were visualised with an inverted microscope using a 20× objective lens. Images were captured onto videotapes, digitalised and analysed with a frame interval of 30 seconds using Metamorph software (Universal Imaging Corp) (Zhao et al., 1996; Zhao et al., 2002). The direction of migration of cells was assessed as cosine θ, where θ is the angle between the EF vector and a straight line connecting start and end positions of a cell. A cell moving directly to the cathode would have a directedness of 1, whereas a cell moving directly to the anode would have a direction of migration of –1. Randomly moving cells have a direction of migration of zero. The average direction of migration of a population of cells gives a quantification of directionality of migration of the cells. Trajectory speed is the total distance travelled by a cell divided by time. Displacement speed is the straight-line distance from starting and ending points of a cell divided by time.

$[Ca^{2+}]_i$ imaging

$[Ca^{2+}]_i$ was imaged with Fluo-3 AM (Invitrogen) on an inverted microscope (Zeiss, Axiovert S100T). *Dictyostelium* cells were loaded for 45-60 minutes with 2.2 μM Fluo-3 AM dissolved in dimethyl sulfoxide (final concentration 0.05%) and Pluronic F-127 (Invitrogen, final concentration 0.005%). After loading, the cells were centrifuged (1400 ***g***, 2 minutes, 23°C) and washed twice with developing buffer (or Ca^{2+}-free developing buffer as appropriate). The cells were placed in an electrotactic chamber for ~10 minutes before application of EFs. The microscope is equipped with a cooled CCD camera (Quantix, Photometrics) and an objective lens of FLUAR 40× (oil immersion lens NA 1.3). The imaging system is operated by MetaMorph software (Universal Imaging Corp). A 100 W mercury lamp and a 450-490 nm filter were used for excitation and emission was recorded using a 495-nm long pass beam splitter and emission filter 500-550 nm. The exposure time was 200 mseconds using the sampling rate of 15 seconds between frames. When not imaging, the shutter was closed.

The technique of monitoring $[Ca^{2+}]_i$ using Fluo-3-AM loaded *Dictyostelium* cells was verified with cAMP, a chemoattractant for *Dictyostelium* which is known to elevate Ca^{2+}. Additionally, the effects of photobleaching were examined by varying the imaging frequency while maintaining the same exposure time. In the pilot study, a steady and consistent decline in fluorescence intensity was observed throughout the course of each experiment in the absence of an EF. Increasing the imaging frequency (one frame every 2 seconds and one frame every 5 seconds) resulted in a well-correlated increase in the rate of decline of the fluorescence intensity. Fluorescence intensity declined much less when the cells were imaged at a reduced frequency (one frame every 15 seconds or 30 seconds) (data not shown). This correlation suggests that the decline occurring during imaging was mainly due to photobleaching. EFs also induced spikes of $[Ca^{2+}]_i$ rises which became obvious ~200 seconds after the EF was switched on (Fig. 3C, Fig. 4E,I). We chose to image cells with frame intervals of 15 seconds. This reduced the effects of photobleaching while permitting the detection of the short duration Ca^{2+} spikes. This Ca^{2+} imaging technique was also confirmed using cAMP stimulation. Developed *Dictyostelium* cells loaded with Fluo-3 AM were stimulated with cAMP (5×10^{-8} M). cAMP induced a rapid and short-lasting rise in $[Ca^{2+}]_i$ (Fig. 3A).

The data are presented as the ratio ($F:F_0$) of fluorescence intensity at a defined time point (F) over the intensity immediately prior to onset of an EF (F_0). The effects of EF exposure were determined by comparing the time course of $F:F_0$ ratios from cells exposed to an EF together, with the time course of $F:F_0$ ratios from the control (not EF-treated) cells. The average control curve (average $F:F_0$ ratio from non-stimulated cells) was subtracted from the curves obtained for single EF-stimulated and cAMP-stimulated cells.

$^{45}Ca^{2+}$-uptake experiments

Dictyostelium cells (2.5×10^6) in 0.5 ml of developing buffer containing 40 μCi of $^{45}Ca^{2+}$ (specific activity of 30 μg Ca^{2+}/Ci, Amersham Biosciences) were subjected to an EF for 10 minutes. The cells were then centrifuged (1000 ***g***, 5 minutes, 4°C) and washed with 1.0 ml of ice-cold developing buffer. This re-suspension-centrifugation step was repeated twice before the radio-labelled *Dictyostelium* pellet was dissolved overnight in scintillation cocktail (Packard) and measured by a liquid scintillation counter measuring the decay. 1 disintegration per minute (dpm) was equivalent to 1.35^{-13} g Ca^{2+} or 3 fmol.

Statistics

Statistical analysis was preformed using Microsoft Origin 6.0 software or Microsoft Excell. All data are expresses as the mean ± s.e.m. Paired and unpaired *t*-tests were used to compare responses within and between experiments, respectively. $P<0.05$ was considered significant. Unless otherwise stated, *n* numbers represent the total

number of *Dictyostelium* cells. Data were collected from at least three individual experiments.

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