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Independent control of locomotion and orientation during *Dictyostelium discoideum* chemotaxis

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Summary

Chemotaxis is cell movement in the direction of a chemical and is composed of two components: movement and directionality. The directionality of eukaryotic chemotaxis is probably derived from orientation: the detection of the spacial gradient of chemoattractant over the cell length. Chemotaxis was investigated in eukaryotic *Dictyostelium discoideum* cells that were permeabilized by high-voltage discharges. These permeable cells respond chemotactically to extracellular cAMP. However, locomotion is impaired if the Ca^{2+} concentration is clamped at submicromolar concentrations; interestingly, these non-motile cells still form pseudopodia and elongate in the direction of the cAMP gradient. These results imply that locomotion and orientation during *Dictyostelium* chemotaxis are independently regulated.

Key words: chemotaxis, cytosolic calcium, locomotion, electroporation, cyclic AMP, cyclic GMP, *Dictyostelium discoideum*.

Introduction

Chemotaxis is cell motion that is directed by external chemical gradients. Chemotactic responses are present in both prokaryotic and eukaryotic organisms and play an important role in a broad spectrum of functions, such as growth, development and pattern formation, defence system against infections, wound healing, tumour metastasis and food searching. Chemotaxis plays an essential role in the life-cycle of the cellular slime mold *Dictyostelium discoideum*. Under starving conditions multicellular aggregates are formed by chemotaxis of free-living amoebae towards cAMP that is secreted by the cells (Konijn et al., 1967; Janssens and Van Haastert, 1987; Gerisch, 1987; Devreotes, 1989).

A chemotactic cell may obtain both temporal and spatial information on the extracellular gradient of chemoattractant. Motile cells may detect the gradient by combining temporal information with the direction of movement, such that movement persists if the concentration of chemoattractant increases with time. In these cells motion and directionality cannot be separated. This situation is well documented for bacterial chemotaxis (MacNab and Koshland, 1972; Koshland, 1988; Bourret et al., 1989). Chemotaxis in eukaryotes is probably more complex, since cells seem to orient in the gradient, probably by detecting the concentration of chemoattractant over their cell length. In these cells motion and orientation are not necessarily connected, and may be the result of distinct biochemical reactions that are controlled by different mechanisms (Devreotes and Zigmond, 1988). Many biochemical and biophysical responses to chemotactic stimulation of amoeboid cells have been reported, but only a few of them appear to be essential for chemotaxis (Devreotes and Zigmond, 1988). The polymerization of actin is essential for chemotaxis (Wallace et al., 1984), but the second messengers involved in the regulation of actin polymerization are largely unknown. Different types of myosin play a role in pseudopod formation and migration (Fukui et al., 1989). Apparently contradictory reports have been published on the role of intracellular calcium in chemotaxis, showing both a lack of involvement of intracellular calcium and a strong sensitivity of chemotaxis for intracellular calcium concentration changes (e.g. see Marasco et al., 1980; Elferink and Deierkauf, 1985a,b; Meshulam et al., 1986; Europe-Finner et al., 1984; Zigmond et al., 1988; Marks and Maxfield, 1990; Marks et al., 1991). The use of different substrata (Marks et al., 1991), and different roles for local release of calcium and calcium entering the cell from the extracellular medium (Kim and Westhead, 1989) may account for these contradictions.

Recently we obtained controlled conditions for carefully permeabilizing *Dictyostelium* cells by high-voltage discharges. Transport studies and membrane

potential measurements have shown that these cells are permeable to small molecules up to about 450 Da for about 10 min (Van Haastert et al., 1989; Van Duijn et al., 1990). These cells were used to study the effect of intracellular calcium buffering on different aspects of *Dictyostelium discoideum* chemotaxis. The results show that intracellular calcium is important for locomotion but not for orientation and pseudopod formation.

Materials and methods

Materials

[^{3}H]cGMP was from Amersham Int., England. GTPγS, GTP, cGMP and cAMP were from Boehringer (Mannheim, FRG).

Cell culture conditions

Dictyostelium discoideum NC-4(H) amoebae were grown is association with *Escherichia coli* 281 on a solid medium containing 3.3g peptone, 3.3g glucose, 4.5g KH_2PO_4, 1.4g $Na_2HPO_4.2H_2O$ and 15 g agar per liter. Vegetative cells were harvested with cold 10 mM sodium/potassium phosphate buffer, pH 6.5 (PB), and washed free of bacteria by three washes and centrifugations at 150 *g* for 2 min. These cells were used for random movement measurements. To obtain starved cells, the vegetative cells were plated on non-nutrient agar (1.5% agar in PB) at a density of about 1.5×10^6 cells/cm^2 for 4 h at 22°C or 15 h at 6°C, followed by shaking in suspension at 2×10^7 cells/ml in PB for an additional hour. All experiments were performed at room temperature (about 20°C).

Electroporation conditions

Cells were resuspended in electroporation buffer consisting of 1.5 mM $MgCl_2$ and 20 mM Hepes, pH 7.0. Cells were electroporated at a density of 10^8cells/ml in a workshop-built apparatus; two 7 kV/cm pulses with a RC-time of 210 μs, separated by a 5-s interval, were applied (Van Haastert et al., 1989; Van Duijn et al., 1990). After permeabilization, cells were immediately incubated in the extracellular solution used for the chemotaxis and random movement measurements and allowed to adhere to a glass coverslip. Measurement of chemotaxis and random movement was started 75 s after applying of the voltage pulses and was ended within 8 min, unless specified otherwise. The glass coverslip was mounted to an open-bottom teflon culture dish and cells were observed using ×100 objective magnification with phase-contrast oil-immersion optics (Ince et al., 1985), or ×40 phase-contrast objective magnification.

Chemotaxis and locomotion measurements

Chemotaxis of starved cells towards a microcapillary (Ø 2-3 μm) filled with bath solution containing 10^{-5} M of the chemoattractant cAMP was studied. A $t=0$ a short pressure pulse was applied to the microcapillary to stimulate the cells. At different time intervals photographs of the cells were taken. The distances between the tip of the microcapillary and the cells were measured from the photographs. Subtraction of these distances from the distance at $t=0$ resulted in the values for the distances moved towards or away from the microcapillary at the different time points. These distances were plotted as a function of time (Van Duijn et al., 1990). Orientation of the cells in the chemoattractant gradient was measured from the photographs. Orientation is defined as the angle formed by the polarity line of the cell with the line from the tip of the capillary to the centre of the cell. The mean angle is 45° in random orientated cells and 0° in perfectly orientated cells.

Guanylate cyclase and cGMP assays

Starved cells were washed in buffer containing 40 mM Hepes, pH 7.0, and resuspended in buffer containing 40 mM Hepes, 3 mM $MgCl_2$, 50 μM GTPγS, 11.8 mM EGTA and different concentrations of $CaCl_2$, to obtain final concentrations of free calcium in the range 0 to 10^{-6} M. The cells were lysed by passage through a Nuclepore filter (pore size 3 μm). At 30 s after lysis, the guanylate cyclase reaction was started by mixing equal volumes of lysate with a mixture containing 10 mM dithiothreitol and 0.6 mM GTP. The reaction was terminated with perchloric acid at 0, 30 and 60 s, and cGMP was measured in the neutralized extracts by radioimmunoassay (Van Haastert and Van der Heijden, 1983; Janssens et al., 1989).

For the determination of the basal cGMP content, electropermeabilized cells were incubated at different concentrations of extracellular free calcium for 40 s. Subsequently the reaction was terminated with perchloric acid and the cGMP content of neutralized samples was determined by radioimmunoassay (Van Haastert and Van der Heijden, 1983).

Results

To determine the role of intracellular calcium in the chemotactic response of the cellular slime mold *Dictyostelium discoideum*, chemotaxis of electropermeabilized cells was measured under different extracellular calcium conditions. In these experiments two aspects of chemotaxis were considered: locomotion and orientation (pseudopod formation). cGMP measurements were used to determine the effect of extracellular calcium buffering on the intracellular free calcium concentration in electropermeabilized cells.

Manipulation of intracellular calcium concentration in electropermeabilized cells

The cytosolic Ca^{2+} concentration of permeable cells is most likely determined by the extracellular Ca^{2+} concentration, but the quantitative relationship is difficult to address. *Dictyostelium* cells show a very poor uptake of fluorescent calcium probes and a rapid extrusion of these indicators from the cytoplasm. Therefore, cytoplasmic free calcium measurement with fluorescent probes has been shown to be extremely difficult in these cells. We have used an indirect method to monitor intracellular Ca^{2+} concentrations that is based on the inhibition of guanylate cyclase activity by Ca^{2+} (Janssens et al., 1989). In vitro, membrane-bound guanylate cyclase activity is strongly regulated by nanomolar Ca^{2+} concentrations with half-maximal inhibition at 40 nM Ca^{2+} (Fig 1). We studied the guanylate cyclase activity in electropermeabilized cells incubated in different extracellular calcium concentrations by measuring the cGMP content of these cells. Electropermeabilized cells were incubated briefly (40 s) with different extracellular Ca^{2+} concentrations. Basal cGMP levels decreased maximally 50% with a sensitivity towards the Ca^{2+} concentration like that of the activity of guanylate cyclase in vitro (Fig. 1). The basal cGMP concentration of control cells is not affected by extracellular Ca^{2+} (Van Haastert, 1985). These exper-

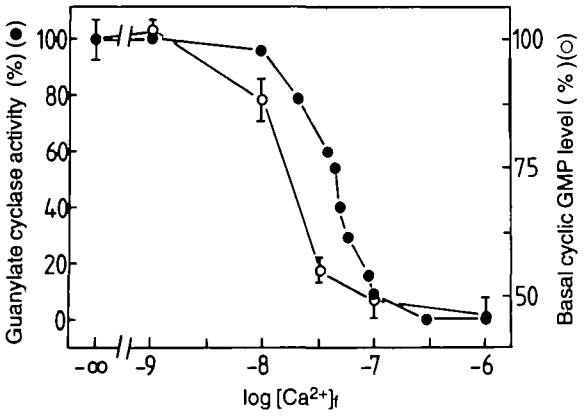

Fig. 1. Guanylate cyclase activity in membranes (●), and basal cGMP levels in permeable *D. discoideum* cells (○) as a function of different Ca^{2+} concentrations.

iments reveal that the intracellular Ca^{2+} concentration can be effectively clamped in electropermeabilized *Dictyostelium* cells using strongly buffered extracellular Ca^{2+} concentrations. Maximal inhibition is not complete, possibly because the 40-s period with Ca^{2+} was not sufficient to degrade all basal cGMP or because not all *Dictyostelium* guanylate cyclase activity is Ca^{2+}-dependent.

Calcium-dependent chemotaxis in electropermeabilized cells

Chemotaxis of electropermeabilized cells bathed in solutions with different free calcium concentrations was studied. Two important aspects of chemotaxis, locomotion and orientation, were analyzed. Fig. 2 shows chemotaxis of electropermeabilized cells towards a capillary filled with cAMP. In the presence of extracellular Ca^{2+}, permeable cells clearly move towards the capillary (Fig. 2A). However, in the absence of extracellular Ca^{2+} (Fig. 2B), permeable cells seem to elongate in the direction of the capillary, but there is no movement towards the capillary. Fig. 2C reveals that these electropermeabilized cells in a Ca^{2+}-free medium actively form pseudopods on the edge of the cell in the direction of the capillary.

These experiments show two unexpected results: cells with holes in the plasma membrane can still perform chemotaxis, and intracellular Ca^{2+} may be required for locomotion, but not for orientation. These experiments were extended using different concentrations of extracellular Ca^{2+}, and the results were quantitated by measuring the distance that the cells moved towards the capillary (chemotactic locomotion) and the polarity of the cells relative to the tip of the capillary (orientation). In non-permeabilized cells, the speed of chemotactic locomotion is independent of the extracellular Ca^{2+} concentration (Fig. 3A). In contrast, electropermeabilized cells do not move in a Ca^{2+}-free solution; locomotion is restored by about 35% at a free extracellular Ca^{2+} concentration of 1 μM and nearly completely restored at 1 mM Ca^{2+} (Fig. 3A). We conclude that locomotion during chemotaxis of electropermeabilized cells can be controlled by buffering of the intracellular calcium concentration.

Orientation of cells was measured in control cells and in electropermeabilized cells in the presence and absence of extracellular Ca^{2+} (Fig. 3B). Under all experimental conditions tested, cells showed normal pseudopod formation upon cAMP stimulation (cf. Fig. 2C), and normal orientation in the cAMP gradient (Fig. 2B). The mean polarity axis of the cells relative to the position of the capillary is about 45° before chemotactic stimulation; this value is predicted for random movement. Upon cAMP stimulation the mean angle reduces to about 10°, indicating efficient orientation of the cells towards the capillary. This orientation is essentially identical in control and permeabilized cells and is independent of the extracellular Ca^{2+} concentration (Fig. 3B). We conclude that orientation of the cells in a chemoattractant gradient is independent of the basal intracellular calcium concentration. These experiments imply that non-motile cells can sense and orient in a cAMP gradient. Hence, locomotion and orientation/pseudopod formation during amoebal chemotaxis are different processes, which can be independently regulated.

Discussion

In this study we investigated the role of intracellular calcium in the regulation of two essential aspects of amoeboid chemotaxis: locomotion and orientation. Electropermeabilized *D. discoideum* cells were used to permit the manipulation of the intracellular calcium concentration by bathing the cells in solutions with different calcium concentrations. Intracellular free calcium measurements with fluorescent calcium probes are extremely difficult in the cellular slime mold *D. discoideum*. This is due to poor uptake and rapid extrusion of these indicators from the cytoplasm. Therefore, we used a different approach to investigate the effect of different extracellular calcium concentrations on the intracellular calcium concentration in electropermeabilized cells. Since guanylate cyclase activity in *D. discoideum* cells is strongly calcium-dependent (Janssens et al., 1989; Fig. 1), basal cGMP levels in electropermeabilized cells at different extracellular calcium concentrations may be used as indicators for intracellular calcium concentration. The identical sensitivity to calcium of the basal cGMP level in electroporated cells and the guanylate cyclase activity in vitro suggests that in electropermeabilized cells the intracellular free calcium concentration is determined by the extracellular calcium concentration.

Locomotion of electropermeabilized cells is strongly dependent on the extracellular calcium concentration, whereas extracellular calcium has no effect on locomotion of non-permeabilized cells (Figs 2A,B, 3). This effect is not due to rapid calcium-dependent healing of the plasma membrane, because electropermeabilized cells bathed in 1 mM Ca^{2+} have zero membrane potential (Van Duijn et al., 1990). It is likely that

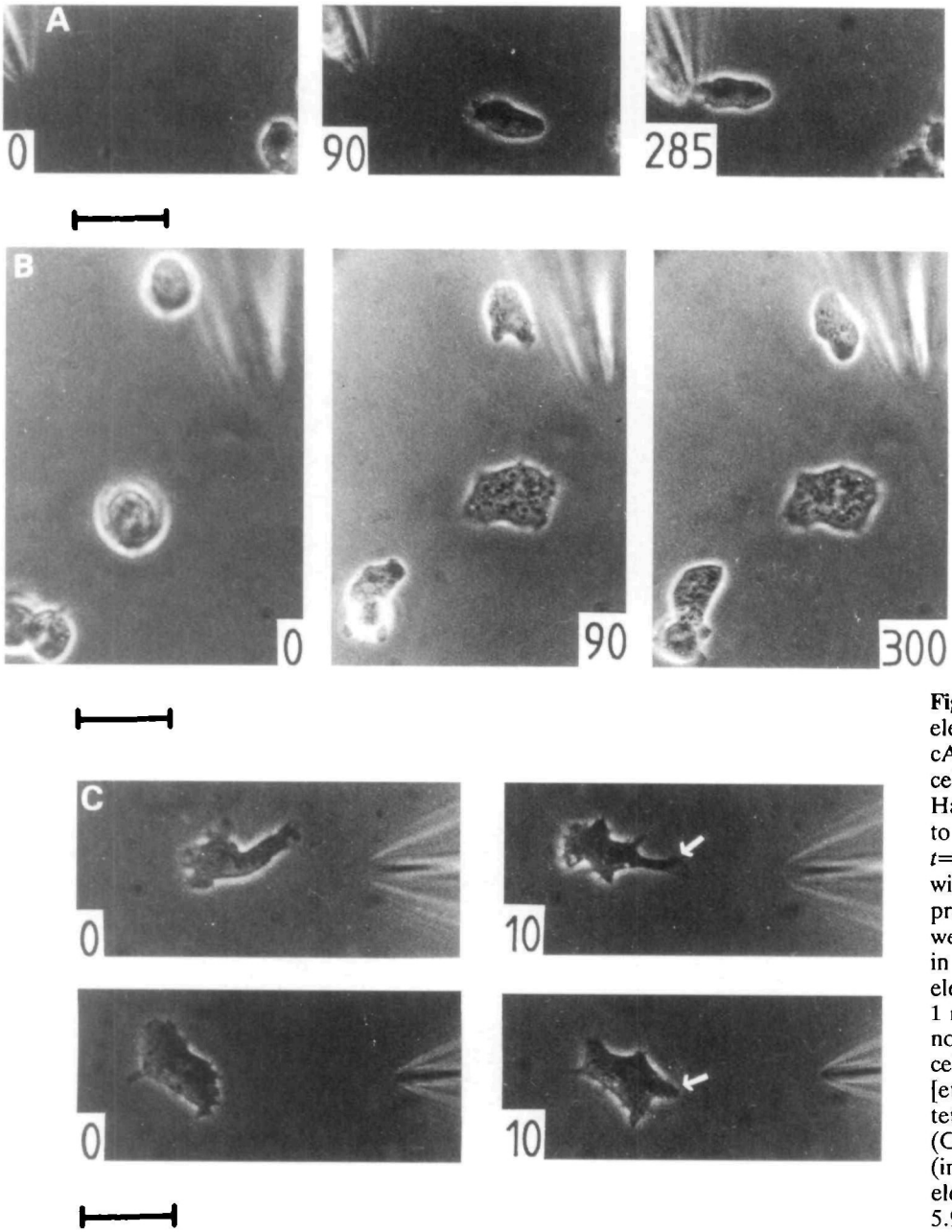

Fig. 2. Chemotaxis of electropermeabilized cells in a cAMP gradient. *Dictyostelium* cells were electroporated (Van Haastert et al., 1989), transferred to a coverslip, and stimulated at $t=0$ with a microcapillary filled with 10^{-5} M cAMP by means of a pressure pulse. The photographs were made at the indicated times in seconds. (A) Chemotaxis of electroporated cells in buffer with 1 mM Ca^{2+}. (B) Orientation but no locomotion of electroporated cells in buffer with 5.9 mM [ethylene-bis(oxyethylenenitrilo)] tetraacetic acid (EGTA). (C) Pseudopod formation (indicated by the arrows) of electroporated cells in buffer with 5.9 mM EGTA. Bars, 20 μm.

EGTA removes intracellular calcium in electropermeabilized cells, and that addition of extracellular calcium is required to restore the intracellular calcium concentration. This suggests that intracellular calcium is essential for locomotion of *Dictyostelium* cells. Cell movement consists of the formation of a new pseudopod, the attachment of the pseudopod to the substratum, the transfer of cell mass into the new pseudopod and retraction of old pseudopods. Intracellular calcium is not required for pseudopod formation, but could be important for each of the other reactions.

Intracellular calcium is also not required for the orientation of newly formed pseudopods in the direction of the cAMP gradient. These results imply that locomotion and pseudopod formation/orientation can be separated, and that *Dictyostelium* cells do not have to move in order to orient themselves in a spatial gradient of chemoattractant.

Both pseudopod extension and locomotion are generally associated with actin and myosin polymerization, but their regulation and function during chemotaxis are uncertain (McRobbie, 1986; Devreotes and Zigmond, 1988; Fukui et al., 1989). Fukui and coworkers reported the presence of myosin I mainly in the pseudopodia of stimulated *Dictyostelium* cells, whereas myosin II is concentrated in the posterior of the cell (Fukui et al., 1989). F-actin, assembled after chemotactic stimulation, is localized in the pseudopods (Condeelis et al., 1988). It is proposed that actomyosin I contributes to the forces that cause extension at the leading edge of the cell, while actomyosin II at the rear plays a role in the movement of the cell mass (Fukui et al., 1989). Genetically engineered *Dictyostelium* cells

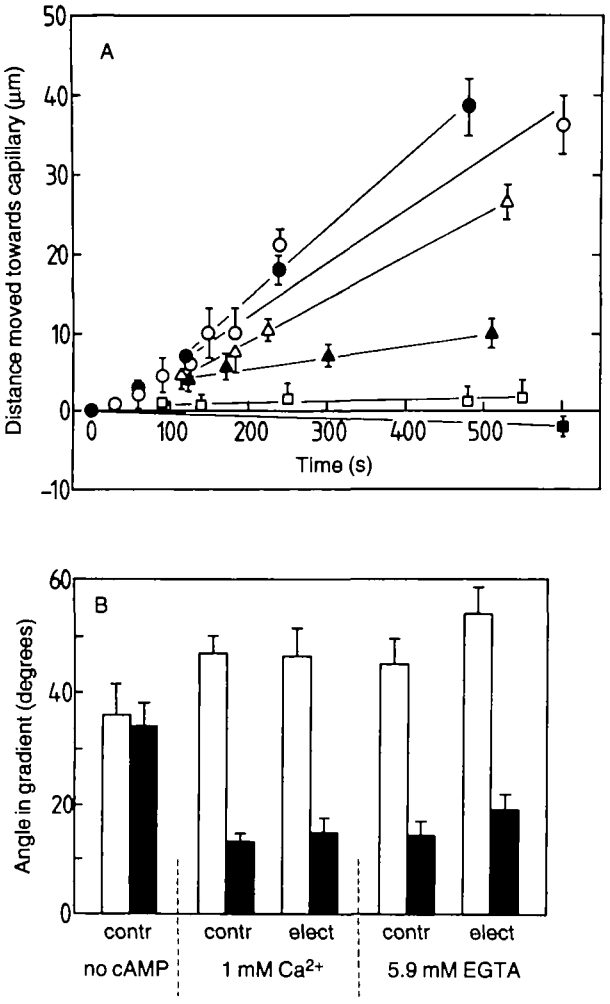

Fig. 3. Quantitation of locomotion and orientation of *Dictyostelium* cells in chemotactic gradients. Control or electropermeabilized cells were incubated at different extracellular free Ca^{2+} concentrations and stimulated with cAMP. (A) The distance moved in the direction of the microcapillary; (●) intact cells in 1 mM free Ca^{2+} (mean of 69 cells); (○) intact cells bathed in 5.9 mM EGTA (mean of 31 cells); (△) permeable cells in 1 mM free Ca^{2+} (mean of 23 cells); (▲) permeable cells in 10^{-6} M free Ca^{2+} (5.9 mM EGTA and 5.0 mM Ca^{2+}; mean of 24 cells); (□) permeable cells in 5.9 mM EGTA (mean of 36 cells); (■) intact cells towards a microcapillary without cAMP (mean of 31 cells). Bars indicate ± s.e. The distance between the tip of the microcapillary and the cells was measured at different time intervals and subtracted from the distance at $t=0$, thus obtaining chemotactic locomotion. (B) Orientation (± s.e.) of control (contr) and electropermeabilized (elect) *Dictyostelium* cells in a cAMP gradient bathed in 1 mM free Ca^{2+} or no free Ca^{2+} (5.9 mM EGTA). The open and filled bars represent, respectively, orientation just before and 450-600 s after application of the cAMP pulse.

that lack myosin II show a decreased velocity of cell movement but a normal rate of pseudopod formation (Wessels et al., 1988), supporting this hypothesis.

The formation of myosin filaments has been suggested to be mediated by cGMP (Liu and Newell, 1988). In electropermeabilized *Dictyostelium* cells, cAMP induces a normal cGMP response (Van Duijn et al., 1990). The association of actin filaments with the Triton-insoluble cytoskeleton in *Dictyostelium* is proposed to be mediated by the receptor-stimulated production of inositol 1,4,5-trisphosphate and the release of Ca^{2+} from internal stores (Europe-Finner and Newell, 1986a, b; Europe-Finner et al., 1989), but recent studies in other organisms show that the formation of actin filaments is calcium-independent (Downey et al., 1990; Greenberg et al., 1991). We observed that electropermeabilized cells bathed in strongly buffered Ca^{2+} solutions still show pseudopod formation. This suggests either (i) that actin polymerization is calcium-independent or (ii) that local release of Ca^{2+} is still able to regulate actin polymerization leading to pseudopod formation. Local and global increases in cytosolic Ca^{2+} have different effects in chromaffin cells (Kim and Westhead, 1989). In electropermeabilized *Dictyostelium* cells that are bathed in strongly buffered Ca^{2+} solutions, the global increase in cytosolic Ca^{2+} is most likely prevented, but local changes in the Ca^{2+} concentration may still occur to some extent. During locomotion both actin assembly and disassembly may be necessary. The local formation of pseudopodia, and thereby orientation of the cell in a chemotactic gradient, may depend only on calcium-independent actin assembly, not on calcium-dependent disassembly (Downey et al., 1990). This hypothesis is supported by the presence of low cytosolic Ca^{2+} concentration (10^{-9} to 10^{-8} M) in pseudopods of locomoting *Amoeba proteus*, while a high cytosolic Ca^{2+} concentration (1.5×10^{-7} M to 2.0×10^{-7} M) is present in the rear and retracting pseudopods (Gollnick et al., 1991). The experiments presented show that the mechanism of chemotaxis consists of different processes. These processes (e.g. locomotion or pseudopod formation) can be activated independently and show different sensitivity to the cytoplasmic calcium concentration. The fact that during complete chemotaxis all these processes, with different calcium sensitivities, have to occur in the same cell at the same time implies that cytoplasmic calcium gradients and local calcium concentration changes must be essential for efficient chemotaxis.

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