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Reversible Inhibition of Movement in the Amoebae *Dictyostelium discoideum* and its Effect on Chemoattractant Recognition*

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The cell fixatives formaldehyde and $KMnO_4$ at low concentrations reversibly inhibit the movement of *D. discoideum* amoebae without directly interfering with cell viability. This inhibition of cell movement is accompanied by the decreased attachment of cells to substratum. When the tenacity and attachment of immobilized cells are artificially increased by compressing cells between two glass surfaces, the amoebae begin to move even in the presence of the fixatives. Amoebae starved for 24 hours, subjected to fixatives and a mineral salt solution in which they remained motionless, maintained chemotactic responses to folic acid and only after a few hours of active locomotion became reactive to cAMP, in contrast to amoebae that reacted to cAMP after starvation in the absence of fixatives.

Key words: Cell movement, *Dictyostelium discoideum*, chemotaxis, folic acid, cAMP, movement inhibition, cell membrane, fixatives

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Abbreviations: *D. discoideum* – *Dictyostelium discoideum*, FA – folic acid

The importance of cell movement during embryogenesis, regeneration, immune response, spread of cancer cells and formation of metastases is commonly appreciated and studied in many laboratories. Nevertheless, there remain some unanswered questions. One is how originally motionless cells commence motile activity, leave their local environment and upon reaching their destination stop moving and remain motionless (ABERCROMBIE & AMBROSE 1962; WILKINSON 1987; VON TSCHARNER BIINO *et al.* 1997). The analysis of cell movement when studied *in vivo* and *in vitro* is often difficult because movement is not continuous. Such behaviour has been described by many studies for various cells both *in vivo* and *in vitro* (WILKINSON 1987; POMORSKI *et al.* 2004; REICHMAN-FRIED *et al.* 2004).

Cell mobilization has been studied especially in relation to the mechanisms of contact inhibition, scatter factors, cytokines, proteolytic enzymes, chemotaxis and galvanotaxis, all of which have gained the attention of many investigators; however, less attention has been paid to factors which immobilize cells (ABERCROMBIE & AMBROSE 1962; JACOBELLI *et al.* 2004; TAMAGNONE & COMOGLIO 2004). The formation and maturation of focal contacts (WÓJCIAK-STOTHARD *et al.* 1997), gap junctions and desmosomes occur later, when cells are already immobilized. In addition reversible prevention of movement, without interfering with cell viability, would have many advantages for experimentalists.

There have been various experimental attempts to reversibly inhibit cell movement, including decreasing temperature, applying viscous media (polyvinyl alcohol, methylcellulose solutions), and cell detachment or cell seeding on non-adhesive substrata (SAYERS *et al.* 1979; JOUNG *et al.* 1987; HASSLEN *et al.* 1996). In this report we describe how modification of the cell membrane is used to reversibly inhibit cell movement. This was achieved by modification of the cell membrane of *D. discoideum* amoebae using fixatives at low concentra-

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tions, without direct impairment of intracellular processes. The investigations were carried out on *D. discoideum* amoebae, although similar qualitative observations have been made on other motile cells.

The second question we addressed concerns the capacity of *D. discoideum* amoebae to change their chemotactic response during starvation. Well fed amoebae respond to folic acid (FA) and pterines, whereas after starvation in mineral salt solutions for 6 or more hours they begin to produce and react to cAMP (KESBEKE *et al.* 1990; MILNE & COUKELL 1991; REYMOND *et al.* 1995; NEBL & FISHER 1997; ARKOWITZ 1999; KOROHODA *et al.* 2002). As we report in this communication, starved and immotile amoebae maintain the capacity to recognize and respond to FA for at least 24 h and require the next several hours of starvation before they become sensitive to cAMP.

Material and Methods

Cell Culture

Dictyostelium discoideum strain AX-2 (ATCC24397) was cultured as described previously (KOROHODA *et al.* 2002; SROKA *et al.* 2002) in medium containing: 14.3g/l meat peptic peptone (Oxoid Ltd, England), 7.15g/l yeast extract (BioMerieux, France), 18g/l maltose (Sigma St. Louis, MO), 1.28g/l Na_2HPO_4 x 12 H_2O, 0.48g/l KH_2PO_4, adjusted to pH 7.4. The cells were grown in suspension at 20°C, harvested at the exponential growth stage, washed three times with Chalkley's solution (14 mM NaCl, 0.27 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$), resuspended in Chalkley's medium and plated in Petri dishes. For chemotaxis studies the cells were starved in Chalkley's medium for 6h at 20°C, resuspended in fresh medium and plated into a special chemotactic chamber described previously (KOROHODA *et al.* 1997; KOROHODA *et al.* 2002), in order to observe the effects of folic acid (FA) and cAMP.

Recording of cell motility

Cell migration was observed with an inverted Hund Wilovert S microscope, recorded with a Hitachi CCD camera, digitized and processed with programs written by P. Jochym and R. Tokarski. Images were recorded every 60 s and each cell was contoured at t_0 and after 60 min.

Cells were considered migrating if their projections after 60 minutes did not share any area with their projections at t_0.

The trajectories of migrating cells were determined by images taken every 30s for 20 min. Tracks of individual cells were generated by the cell's centroid position in each frame, with whole trajectories which were composed of 40 consecutive centroid displacements. The program Mathematica was used for further data processing. Cell trajectories are presented as circular diagrams with the starting point of each trajectory located in the centre of the diagram.

Inhibition of cellular motility

Adherent cells in Chalkley's solution were treated with various concentrations of formaldehyde or potassium permanganate. Cell movement in diluted fixative solutions was recorded for 90 min. with images acquired every 60 seconds. Each recorded cell was contoured after 30 min. and again after a further 30 min. of incubation. The percentage of migrating cells was determined.

Viability test

Cells of *D. discoideum* were incubated in the presence of various concentrations of formaldehyde or potassium permanganate for 24h, and suspensions of cells were mixed with fluorescein diacetate and ethidium bromide solution (DANKBERG & PERSIDSKY 1976; SZYDŁOWSKA *et al*. 1978). The results represent the mean values of three measurements obtained from three independent experiments in which 200 cells were observed in each.

Reversibility of cellular motility inhibition

Cells of *D. discoideum* were incubated in the presence of formaldehyde solutions for 24h, then shaken on a rotary shaker, resuspended in fresh Chalkley's solution, plated in a Petri dish and recorded for 180 min as described above. The percentage of migrating cells was determined 180 min after removal of formaldehyde.

"Chimneying effect" – the ability of *D. discoideum* amoebae to regain migration capability in fixative solutions under a coverslip

Adherent amoebae of *D. discoideum* cells were treated with 0.8 mM formaldehyde or 0.05 mM potassium permanganate in Chalkley's medium. After 30 min. of incubation, when locomotion of the cells had completely ceased, a coverslip was put into the Petri dish to gently cover the cells and the medium was removed beneath the level of the upper surface of the coverslip (KELLER & ZIMMERMANN 1985; KELLER *et al.* 1989, 2002; MALAWISTA & DE BOISFLEURY CHEVANCE 1997; MALAWISTA *et al.* 2000, 2002). After 180 min cell locomotion was recorded for 20 min at time intervals of 30s.

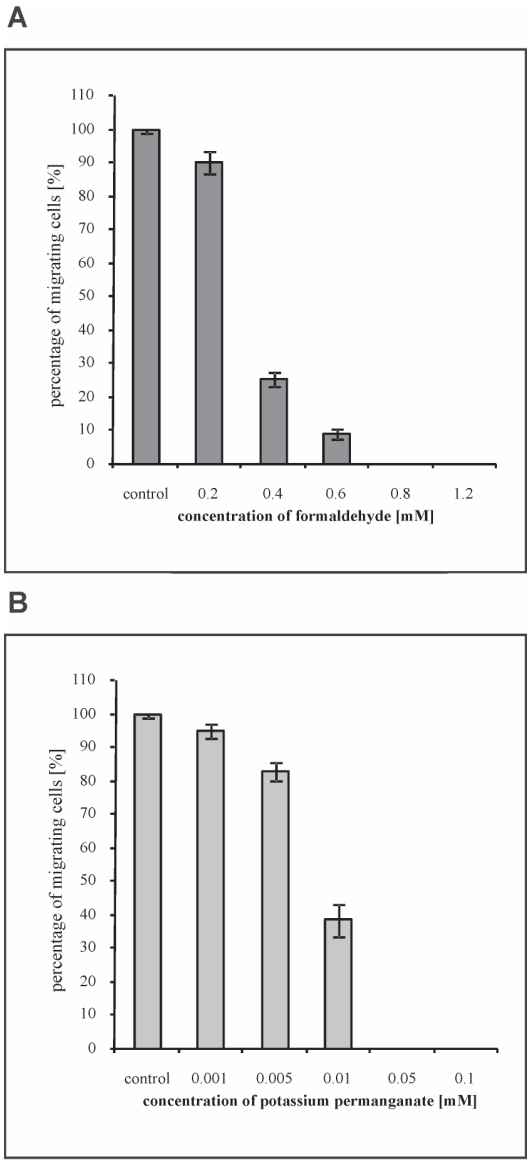

Fig. 1. Percentage of moving amoebae incubated for 0.5 hour in various concentrations of formaldehyde (A) and potassium permanganate (B) in Chalkley's medium.

Cell migration in response to chemoattractants

Migration of cells in developing concentration gradients of folic acid (FA) and cAMP was observed in a chemotactic pocket-like chamber, as previously described (KOROHODA *et al.* 1997; KOROHODA *et al.* 2002). The effects of folic acid (0.5 mM) or cAMP (1μM)), both in Chalkley's medium, were examined (i) in cells taken directly from the growth medium (Fig. 3A, B), (ii) in cells starved in mineral salt solutions for 24 h (Fig. 3 C, D), (iii) in cells starved in mineral salt solutions in the presence of diluted fixatives added directly after washing out fixatives (Fig. 3 E, F) and (iv) 6 h later when these cells were permitted to move actively without fixatives (Fig. 3 G, H).

Results

Cell fixatives are commonly used to kill cells and to stabilize their structures for light or electron microscopy. Formaldehyde and potassium permanganate are often used for fixation of cells in light microscopy and electron microscopy, respectively. Formaldehyde is known to stabilize cell membranes without affecting their immunological and electrochemical properties, and potassium permanganate is often used for fixation of membranous structures (SEAMAN & COOK 1965; GINGELL & TODD 1975; HANNIG *et al.* 1990). Formaldehyde and glutaraldehyde stabilize both natural biological membranes and black lipid membranes or liposomes, and modify ion fluxes across membranes. At low concentrations these fixatives do not kill the cells but modify their membranes (PRZESTALSKI *et al.* 1978; HASSLEN *et al.* 1996; JOVTCHEV *et al.* 2000).

In the first experiments we estimated nonlethal concentrations of these two fixatives, permitting

Table 1

Effect of formaldehyde diluted in Chalkley's medium on viability and locomotion of *D. discoideum* amoebae

	Concentration of formaldehyde					
	0 mM	0.2 mM	0.4 mM	0.6 mM	0.8 mM	1.2 mM
Viability of cells incubated for 24 hours in the presence of formaldehyde as estimated with the cell viability test	97.73±0.93	97.65±0.38	93.44±3.77	88.56±6.65	87.96±8.19	75.49±8.52
Percentage of cells migrating after 30 min incubation in the presence of formaldehyde	99.33±0.67	89.88±3.28	25.04±2.19	8.97±1.54	0	0
Percentage of cells migrating 3h after removal of formaldehyde, in the presence of which the cells were preincubated for 24h	94.07±2.68	92.14±2.98	86.02±4.19	82.52±10.02	63.00±5.00	41.81±11.11

Note: Values are given as the mean ± SEM.

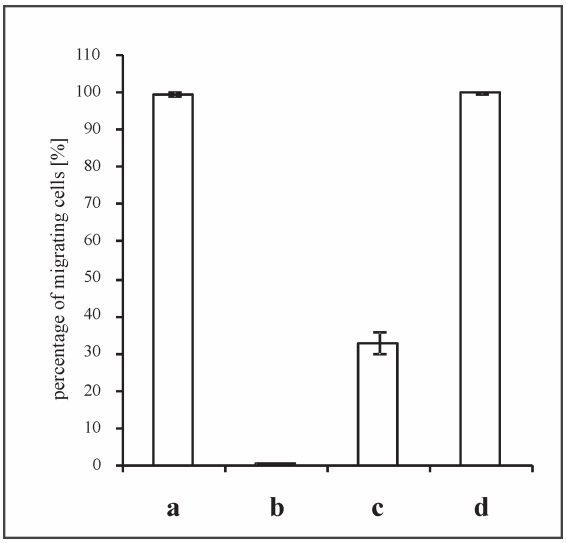

Fig. 2. The percentage amoebae moving: a – under control conditions (in Chalkley's medium), b – in the presence of 0.8 mM formaldehyde in bulk solution, c – in the presence of 0.8mM formaldehyde, 2 hours under the coverslip, d – in the presence of 0.8mM formaldehyde, 3 hours under the coverslip.

the treated *D. discoideum* cells to recover after prolonged treatment.

Figure 1A shows the percentage of amoebae moving after remaining for 0.5 hour in solutions containing various concentrations of formaldehyde. The 0.8 mM concentration of formaldehyde fully inhibited cell movement within 30 minutes (Fig. 1A). When cells were incubated for 24 hours in this solution they assumed a spherical shape and remained motionless, but nevertheless, they accumulated neutral red (data not shown) which is often used as an indicator of cell viability (LASAROW *et al.* 1992). $KMn0_4$ solutions in Chalkley's medium showed that 0.05 mM potassium permanganate achieved similar immobilization (Fig. 1B).

In Table 1 the effects of incubation of *D. discoideum* cells in solutions of 0.2 mM to 1.2 mM formaldehyde in Chalkley's solution for 24 hours are shown. The cells survived after 24 hours (or even 48, data not shown) of incubation in the presence of 0.8 mM formaldehyde. After removal of formaldehyde and washing, more than 60 % of the cells resumed locomotion within 3 hours (Table 1).

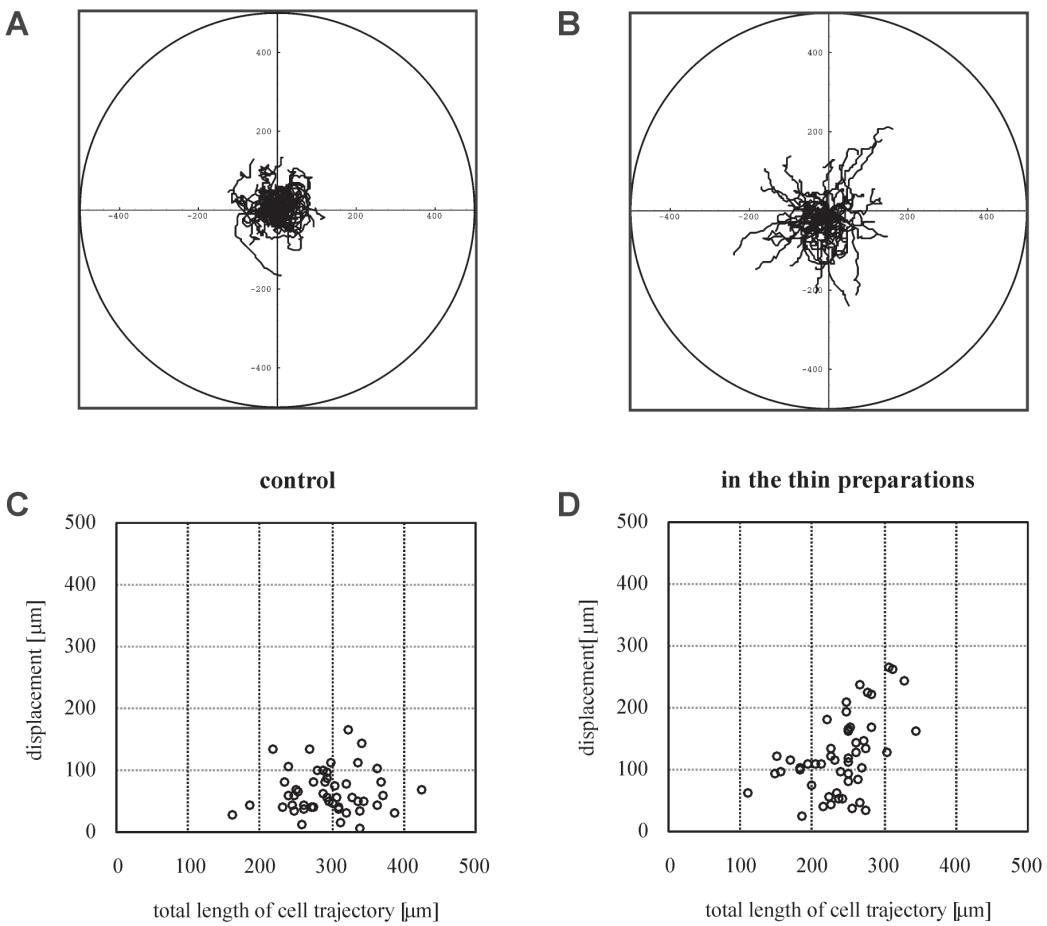

Fig. 3. A, B – Circular diagrams of cell trajectories for *D. discoideum* amoebae (n=50) moving in control isotropic conditions in the absence of fixatives (A) and in the thin preparations in the presence of 0.8 mM formaldehyde. C, D – Scatter correlation diagrams of total length of cell trajectories and displacements of *D. discoideum* cells migrating in control conditions (C) and in the thin preparations in the presence of 0.8mM formaldehyde (D) (corresponding correlation coefficients were 0.05 for C and 0.50 for D).

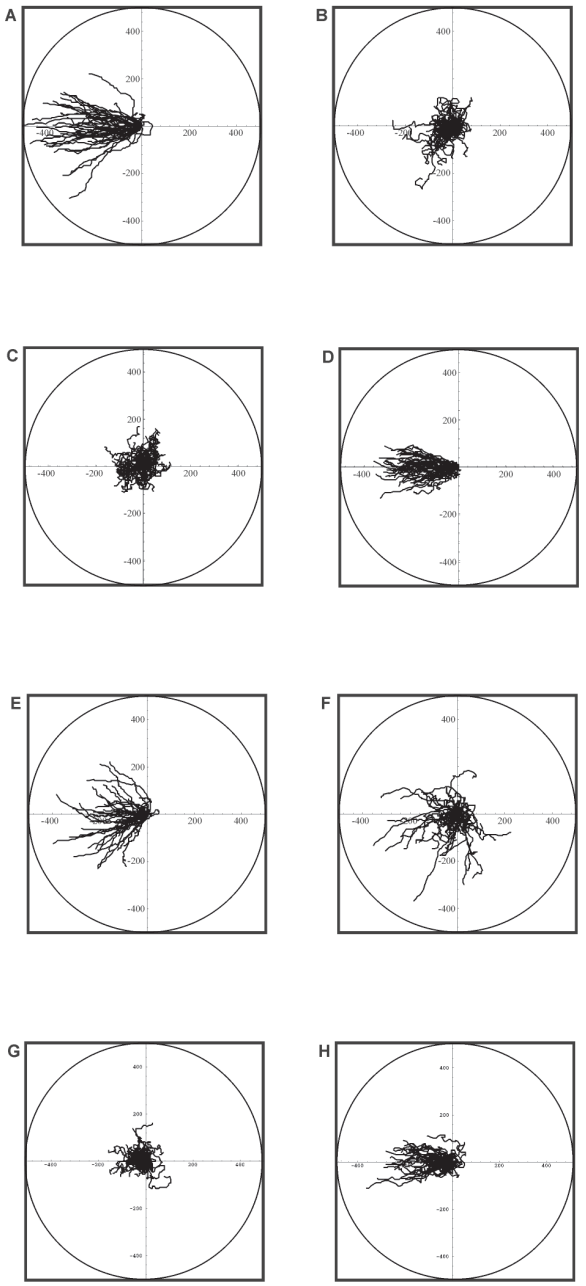

Fig. 4. The effects of folic acid (0.5 mM) (A, C, E, G) or cAMP (1M) (B, D, F, H), both in Chalkley's medium on cells directly taken from the growth medium (A, B), on cells which were starved in Chalkley's mineral salt solutions for 24 h (C, D), on cells which were starved in Chalkley's solutions in the presence of diluted 0.8 mM formaldehyde, directly after washing out the formaldehyde (E, F) and 6 h later when these cells were permitted to move actively without formaldehyde (G, H). Number of cells in each experiment, n=50.

Similar experiments have shown that cells incubated for 24 hours in 0.05 mM potassium permanganate and Chalkley's solution survived for 24 hours. Three hours after washing 77 % of cells resumed locomotion. Therefore the concentrations 0.8 mM of formaldehyde and 0.05 mM potassium permanganate were chosen for further experiments.

Cells treated with fixatives assumed a spherical shape and became detached from the substratum, freely floating within the medium. To examine whether the fixatives disturb and directly influence intracellular structures and their activity, or act primarily upon cell membranes, we performed experiments in which cell attachment to glass was artificially constrained (forced) by compressing the cells between two glass surfaces in a narrow chamber (KELLER & ZIMMERMANN 1985; KELLER *et al.* 1989, 2002; MALAWISTA & DE BOISFLEURY CHEVANCE 1997; MALAWISTA *et al.* 2000, 2002). Under such conditions 100% of the *D. discoideum* amoebae resumed locomotion within 3 hours in spite of the continuous presence of 0.8 mM formaldehyde or 0.05 mM potassium permanganate in Chalkley's solution. The cells artificially compressed in the presence of diluted fixatives moved along more straight trajectories than control cells under isotropic conditions (Fig. 3). By contrast, non-compressed cells in these solutions remained fully motionless (Fig. 2).

Amoebae incubated in the presence of formaldehyde or potassium permanganate, either in suspension or weakly attached to a glass surface, remained fully motionless for 24 hours. Unstarved amoebae taken from growth medium reacted chemotactively to folic acid but did not react to cAMP (Fig. 4A, B). After starvation in mineral salt solution for 6 or 24 hours the amoebae became sensitive to cAMP and did not react to folic acid (Fig. 4C, D). In contrast, amoebae starved for 24 hours in the solution supplemented with diluted fixatives maintained chemotactic responses to FA and did not react to cAMP (Fig. 4E, F). These cells required several hours after removal of fixatives to start reacting to cAMP (Fig. 4G, H). Similar behaviour of *D. discoideum* amoebae was observed after incubation in the presence of diluted potassium permanganate.

Discussion

In recent years research on cell motility has mainly concentrated on the molecular mechanisms operating in signaling pathways and on mechanochemical processes occurring in the cytoskeleton and on the specific proteins involved (KESBEKE *et al.* 1990; JACOBELLI *et al.* 2004). The extracellular factors which influence cell motile responses have received relatively little study. This communication has examined the induction of signaling pathways which influence the reception and selection of specific and non-specific extracellular signals.

Spontaneous cell movement takes place in the absence of any particular stimulus from the environment, whereas directed cell movements occur

in response to environmental stimuli. They depend upon the sensitivity of specialized receptors in cell membranes corresponding to sense organs in metazoan organisms. It is commonly recognized that the specific receptors and ion channels localized in cell membranes play a decisive role in the direction of cell movement (NEBL & FISHER 1997; SALLUSTO *et al.* 1998; ARKOWITZ 1999; KRISHNAN & IGLESIAS 2004). The general properties of lipid bilayers and protein-lipid interactions, modified by the cell fixatives formaldehyde and potassium permanganate, have strongly suggested that cell surface dynamic properties are instrumental in the process of cell motility. Thus, inhibition of cell movement was achieved with cell fixatives, which rather nonspecifically and in different ways stabilize the dynamic properties of the cell membrane. Both fixatives inhibited cell movement reversibly at concentrations which did not significantly influence cell viability. The modification of cell membranes was found to inhibit cell movement, to cause cell rounding and detachment of cell from substrata. In the presence of fixatives, the cells remained viable and accumulated neutral red (cf. LASAROW *et al.* 1992).

To check whether the fixatives affected primarily the cell membrane or acted directly upon signaling pathways or the actin cytoskeleton, we adapted a method commonly used to study movement of normally non-adherent cells and preventing passive cell translocations. Locomotion of non-adherent cells was studied in narrow slide-coverslip preparations (depth: 5-8μm) prepared as described by KELLER (KELLER & ZIMMERMANN 1985, 1986; KELLER *et al.* 1989, 2002; ZIMMERMANN & KELLER 1992, 1993; FEDIER & KELLER 1997; FEDIER *et al.* 1999; KELLER 2000) and MALAWISTA (MALAWISTA & DE BOISFLEURY CHEVANCE 1997; MALAWISTA *et al.* 2000, 2002, 2003; MONTGOMERY *et al.* 2004). The method has been described as locomotion of cells in "thin slide--coverslip preparations" (KELLER & ZIMMERMANN 1985, 1986; KELLER *et al.* 1989), in "close quarters" (MALAWISTA *et al.* 2000) or "chimneying of cells" (MALAWISTA & DE BOISFLEURY CHEVANCE 1997; MALAWISTA *et al.* 2000, 2003). Under such conditions artificially attached and compressed cells resumed movement in the presence of fixatives, thereby showing that the fixatives impaired cell movement by acting at the cell membrane rather than upon signaling pathways or the actin cytoskeleton. This suggests that such experiments may be useful for examining whether other specific factors act upon the cell surface or more directly interfere with intracellular processes of other cell systems. Such modulation of cell surface properties for the regulation of cell functions was elegantly demonstrated by BURGER (1971) who has shown that coating of the neoplastic cancer cell surface with monovalent concanavalin A reversibly restored contact inhibition of cell movement and growth. Nevertheless, non-motile cells when compressed in thin preparations did not only resume locomotion in the presence of diluted fixatives but moved along more straight trajectories. For cells moving under such conditions a closer correlation of the final cell displacement and length of cell trajectories was observed than for cells moving under isotropic conditions in thicker preparations. Such scatter correlation diagrams as shown in Fig. 3C, D illustrate the homo/heterogeneity of cell populations and the tendency of cells to translocate along torturous or straight trajectories (cf. WALIGÓRSKA *et al.* 2007).The observations reported may therefore suggest that cell compression by close approximation of slide and coverslip causes not only better contact of cells with the solid substratum (i.e. the "chimneying" effect discussed by KELLER (KELLER & ZIMMERMANN 1985; KELLER 2000) and MALAWISTA (MALAWISTA & DE BOISFLEURY CHEVANCE 1997; MALAWISTA *et al.* 2000, 2003) but also modifies cell stiffness and tensegrity causing more permanent cell polarization (cf. INGBER & FOLKMAN 1989; INGBER 2003).

The changes in receptors during cell differentiation occur not only during maturation of *D. discoideum* amoebae, but are often observed during cell differentiation, in immune cell systems during sensitization to specific antigens or under the effect of cytokines (SALLUSTO *et al.* 1998; SERVANT *et al.* 1999).

The observations reported here have shown that changes in recognition of specific chemoattractants in *D. discoideum* amoebae require not only cell starvation (TILLINGHAST & NEWELL 1987; MILNE & COUKELL 1991; REYMOND *et al.* 1995; NEBL & FISHER 1997), but also cell movement or attachment to solid substratum. This may be a more general phenomenon for other cell types. Cell polarization and shape changes which occur during cell locomotion are accompanied by changes in the location of cell surface components, including receptors (JOHANSSON *et al.* 1993; SERVANT *et al.* 1999; PRIGOZHINA & WATERMAN-STORER 2004). Indeed, in anchorage dependent cells, their attachment to substratum causes cell activation, entrance of cells inhibited in the G1 phase of the cell cycle to the S phase, stimulation of cell respiration, glycolysis, and fagocytosis (KOROHODA 1972). Such processes apparently depend upon attachment of cells to substratum. initiating signaling pathways, changes in cell shape, growth and differentiation. Similarly, cell movement is reported to influence membrane recycling and the exchange of plasma membranes with intracellular membranes, as postulated for amoebae by PANTIN (1924) and GOLDACRE

(1964), and for tissue cells by HARRIS (1973) and BRETSCHER (1976, 1996a, 1996b). Quantitative studies with amoebae have shown that this process is slow and requires hours for full recycling of the cell membrane, sufficient for cells to move a distance many times greater than the cell length. In migrating cells, membrane internalization takes place at the cell uroid (in amoebae) (HAUSMANN *et al.* 1972; HAUSMANN & STOCKEM 1972; STOCKEM 1972) or in fibroblasts at the tail and perinuclear regions (HARRIS 1973; BRETCHER 1996a). Near the leading fronts of extending surface protrusions, intracellular membranes are incorporated which originate from the internalized cell membrane and from secreting vacuoles formed in Golgi apparatuses (STOCKEM 1972).

The basic mechanism responsible for changes in chemoattractant response by *D. discoideum* amoebae is likely to relate to receptor expression, apparently regulated by cell adhesion and motility, both related to starvation and maturation (VAN HAASTERT & KIEN 1983; MILNE & COUKELL 1991; REYMOND *et al.* 1995; TANAKA *et al.* 1998). We therefore propose that these observations of changes in the dynamic properties of cell membranes can facilitate further studies on the mechanisms of chemotaxis, and on membrane turn-over and recycling during cell locomotion.

In conclusion the results presented in this communication show that:

i) cells can be reversibly immobilized with diluted fixatives without interfering with cell viability;

ii) cells can be induced to move in the presence of fixatives by an artificial increase of cell attachment and shape changes by gently compressing the cells between glass surfaces;

iii) *D. discoideum* amoebae after starvation in the presence of diluted fixatives on the removal of fixatives continued to respond chemotactively to folic acid and required a further few hours of starvation in the absence of fixatives to become sensitive to cAMP.

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