Calcium mobilization stimulates *Dictyostelium discoideum* shear-flow-induced cell motility

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Summary

Application of hydrodynamic mild shear stress to adherent *Dictyostelium discoideum* vegetative cells triggers active actin cytoskeleton remodeling resulting in net cell movement along the flow. The average cell speed is strongly stimulated by external calcium (Ca²⁺, K₅₀%=22 μM), but the directionality of the movement is almost unaffected. This calcium concentration is ten times higher than the one promoting cell adhesion to glass surfaces (K₅₀%=2 μM). Addition of the calcium chelator EGTA or the Ca²⁺-channel blocker gadolinium (Gd³⁺) transiently stops cell movement. Monitoring the evolution of cell-surface contact area with time reveals that calcium stimulates cell speed by increasing the amplitude of both protrusion and retraction events at the cell edge, but not the frequency. As a consequence, with saturating external calcium concentrations, cells are sensitive to very low shear forces (20 pN; σ=0.1 Pa). Moreover, a null-mutant lacking the unique Gβ subunit does not respond to external Ca²⁺ changes (K₅₀%>1000 μM), although the directionality of the movement is comparable with that of wild-type cells. Furthermore, cells lacking the inositol 1,4,5-trisphosphate receptor (IP₃-receptor) exhibit a markedly reduced Ca²⁺ sensitivity. Thus, calcium release from internal stores and calcium entry through the plasma membrane modulate cell speed in response to shear stress.

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Key words: Calcium, Heterotrimeric G proteins, Motility, Hydrodynamic flow, Mechanosensitivity, *Dictyostelium discoideum*

Introduction

Amoeboid motility is a widespread cell propulsive mechanism involving a combination of protrusive and retractive activity (Bailly and Condeelis, 2002; Friedl et al., 2001). The motor force for protrusion is the actin dendritic polymerization underneath the plasma membrane (Borisy and Svitkina, 2000) while that for retraction is provided by myosin-2-mediated contraction of actin filaments (Clow and McNally, 1999). Two different protein machineries can therefore be recruited at the edges of the cell-substrate adhesion surface (‘adhesive belt’), with opposite mechanical effects.

At the molecular level, dendritic actin polymerization is controlled by small G proteins of the Rac/Cdc42 subfamily, which activate the Arp2/3 nucleator complex through WASP or VASP adaptor molecules (Krause et al., 2003; Welch and Mullins, 2002). Members of the myosin 1 family are also recruited and activated at the leading edge (Fukui et al., 1989). Small G proteins of the Rho subfamily stimulate growth of actin bundles and myosin 2 binding to actin through formin and Rho kinase (ROCK) activation (Gasteier et al., 2003). Efficient actin polymerization also requires barbed-end capping and uncapping activity (Curlier and Pantaloni, 1997; Pollard and Borisy, 2003) and the presence of profilin and cofilin to speed up the actin polymerization-depolymerization cycle (DesMarais et al., 2002; Wolven et al., 2000). The activity of many actin-binding proteins is regulated by phosphatidylinositol 4,5-bisphosphate (PIP2), whose turnover is very active at the leading edge (Sakasaka et al., 1997). In particular, gelsolin, an actin filament-severing and barbed end-capping molecule, binds to PIP2 and is released from the plasma membrane by micromolar concentrations of Ca²⁺ (Lin et al., 1997).

Recently, we have shown that application of hydrodynamic shear stress to *Dictyostelium discoideum* cells adhering to a flat glass surface triggers their movement in the direction of the flow (Décavé et al., 2003). Directionality of the movement requires the formation of a phosphatidylinositol 3,4,5-trisphosphate (PIP3) gradient through phosphoinositide 3-kinase (PI3K) activity. Since forces applied to the cell are lower or comparable with the ones internally exerted by the cell during random motility or chemotaxis, it is likely that mechanosensitivity is part of a physiological mechanism controlling the local movement of...
cell edges. Shear-flow-induced cell motility would therefore result from biasing the stress balance within the cell.

In this paper, we examine the role of calcium and heterotrimeric G proteins, two ubiquitous players of signaling pathways, in shear stress-induced motility. During chemotaxis, heterotrimeric G proteins play an essential role in determining the direction of cell movement (directional sensing) (Devreotes and Janetopoulos, 2003; Wu et al., 1995), whereas calcium entry through plasma membrane channels is associated with uropod contraction phases (Nebel and Fisher, 1997; Yamuna et al., 1996). Here we show that the presence of external calcium entry and calcium release from internal stores stimulate the amplitude of cell protrusions and retractions in contact with the substrate. During shear-flow-induced motility, these calcium fluxes stimulate cell speed, but marginally affect cell directionality. Using knockout mutants, we show that heterotrimeric G proteins are also involved in the control of cell speed, but not cell direction, probably through the activation of phospholipase C and the IP3-receptor.

Materials and Methods

Cell preparation and chemicals

*D. discoideum* Ax2, HM1038 (IP3-receptor null mutant), the parental cell line Robert Kay’s lab Ax2 (RK-Ax2), LW6 (Gβ-null mutant), the parental cell line DH1 and LW20 (Gβ-null mutant rescued with a Gβ-expressing plasmid) cells were grown at 21°C in axenic medium (Watts and Ashworth, 1970) in suspension on shakers rotating at 180 rpm (Ax2, RK-Ax2, HM1038) or in Petri dishes (DH1, LW6, LW20). The composition of the axenic medium is: 14.3 g l−1 Oxoid L34 peptone, 7.5 g l−1 Oxoid L21 Yeast Extract, 50 mM maltose, 5.33 mM Na2HPO4, 3.53 mM KH2PO4, and 0.34 mM dihydrostreptomycin. RK-Ax2 and HM1038 were obtained from the Dictyostelium Stock Center (http://www.dictybase.org/). Vegetative cells were harvested during exponential growth phase at a density of 2–4×10⁶ cells ml⁻¹, pelleted by centrifugation (1000 g, 4°C, 4 minutes), washed twice in Sörensen phosphate buffer (SB: 2 mM Na2HPO4, 14.5 mM KH2PO4, pH 6.2) or MES-Na buffer (20 mM morpholino ethane sulfonic acid, adjusted to pH 6.2 with NaOH) and used immediately. Glass plates or coverslips were cleaned with a ionic detergent and etched for 5 minutes with 14.5 M NaOH as previously described (Décavé et al., 2002). New glass plates or coverslips were used for each experiment.

Cell adhesion measurements

Cell adhesion to glass plates was measured in SB at different CaCl2 concentrations in the presence of the cytoskeleton depolymerizing agent N-(3-chlorophenyl)lisopropyl carbamate (CIPC) at a 2 μg ml⁻¹ final concentration from a 10 mg ml⁻¹ stock in DMSO, with a radial flow detachment assay (Décavé et al., 2002). Flow was applied during 15 minutes to complete the detachment kinetics. Results are given as critical shear stresses σ50% (in Pa) that detach 50% of the cells.

Titration of extracellular calcium concentration

Calcium Green 2 (CG2, Molecular Probes, Interchim France) was added at a 1 μM final concentration to the solution, whose calcium concentration had to be determined. Known amounts of calibrated calcium or EGTA solutions were then added and the CG2 fluorescence was measured, subtracting the autofluorescence background. The data were fitted with the following equation:

\[
\frac{F - F_{\text{EGTA}}}{F_{\text{Ca}} - F} = \frac{[\text{Ca}^{2+}]}{\kappa_d}.
\]

where \( F_{\text{Ca}} \) and \( F_{\text{EGTA}} \) are the maximum and minimum fluorescence levels (obtained at 1 mM CaCl2 or EGTA, respectively), \( \kappa_d \) the known CG2 dissociation constant (0.55 mM) and \( F \) the fluorescence level at the [Ca²⁺] total calcium concentration (Rooney et al., 1994). The total calcium concentration is the sum of the unknown and the added calcium concentrations.

Cell motility experimental setup

Motility assays were conducted in a transparent lateral flow chamber using glass plates as previously described (Décavé et al., 2003). To visualize cell morphological changes during motility at a higher magnification, a new lateral flow chamber was built, designed on a similar overall basis, but using 25×60 mm² coverslips (Erie). In both cases, shear stress was calculated from geometrical and hydrodynamical parameters as:

\[
\sigma = \frac{6D\eta}{le^2},
\]

where \( D \) is the flow rate, \( \eta \) the dynamic viscosity of the fluid, \( e \) the thickness of the flow chamber and \( l \) the width of the chamber.

Cell motility recording at low and high resolutions

To record shear-flow-induced motility at low resolution, cells were introduced into a glass plate flow chamber filled with SB or MES-Na buffer and allowed to adhere to the glass surface for 2 minutes. Unbound cells were removed by flowing buffer at a very low shear stress (0.1 Pa) from an upper tank. When needed, the fluid bathing the cells was replaced by flowing another solution for 3 minutes at low shear stress. Cell motility was triggered by the application of a constant larger shear stress and images were recorded every 15 seconds or 30 seconds during 10-15 minutes, depending on the expected cell speed. When indicated, calcium concentration was changed during the assay by exchanging the solutions flowing from different upper tanks using valves. Cells were observed at a 2.5× magnification under dark field illumination with a Zeiss EM405 inverted microscope. Images were taken by a SP-Eye cooled CCD video camera (Photonic Science, UK) controlled by the Image Pro Plus software (Media Cybernetics).

For high-resolution imaging using phase contrast, fluorescence or reflection interference contrast microscopy (RICM), cells were observed with a 60× oil objective on an inverted microscope (Olympus IX-71) under phase or episcopic illumination. Calcium Green 2 fluorescence was selected with a BGW cube (Olympus) and supplementary BG18 and BG28 excitation and emission filters (Melles Griot). Reflection Interference Contrast was obtained by selecting the 546 nm peak of the episcopic mercury lamp by a combination of interference and blue-green filters and illumination through an episcopic polarization cube. For all microscopic techniques, light intensity was reduced with neutral density filters, since high illumination intensities block cell movement.

To record phase contrast, CG2 fluorescence or RICM images during shear-flow-induced motility at high resolution, cells were introduced into a coverslip flow chamber in SB as described above. Typically, two kinds of experiments were performed. The first one consisted of applying shear stress to cells adapted to a given calcium concentration. The calcium concentration was raised to the indexed value at low shear stress, image recording was started (1 image per second) and a larger shear stress was applied 20 seconds later during 3 minutes. The second one consisted of varying the external calcium concentration around cells already submitted to shear-flow. A large shear stress was applied in SB, image recording was started (1 image per second) and the calcium concentration was raised during the experiment by flowing a 1 mM solution in SB from another upper tank. With valves located near the flow chamber, the calcium concentration was raised...
in less than 10 seconds. It is important to note that the fluid level in the different tanks was precisely adjusted to avoid any pressure changes, since bending of the coverslip slightly changed cell position relative to the objective. In addition, focusing was adjusted during the run by visual inspection. Images were taken by an intensified cooled CCD video camera (Photonic Science, UK) mounted on an Olympus IX-71 inverted microscope, controlled by the Image Pro Plus software. Since exposure to even moderate light levels is harmful to cells, samples were renewed after 45 minutes.

Intracellular calcium visualization

To visualize intracellular calcium concentration changes, Dictyostelium cells were resuspended in SB containing 100 μM CaCl$_2$ at 5×10$^5$ cells ml$^{-1}$ in a 60 μl total volume and electroporated in a 2 mm wide cuvette at 4°C (electrical settings: 3 μF, 750 V, time constant: 2 milliseconds, Biorad electropulse apparatus). Cells were recovered with 1 ml SB supplemented with 5 mM MgCl$_2$ and 0.1 mM CaCl$_2$, pelleted by centrifugation (1000 g, 4°C, 3 minutes), resuspended in 10 ml HL5 medium and incubated on a rotary shaker for 10 minutes at 21°C, after which they were introduced in a coverslip flow chamber as described above.

Quantitative analysis of cell motility

Individual cell tracks were reconstituted from low-resolution movies as described previously (Décavé et al., 2003). Cells that came into contact with other cells or that detached during the recording were excluded from the following analysis. For a given cell, the instant velocity is given by:

$$v_i(t_i) = \frac{\bar{t}_{i+1} - \bar{t}_{i-1}}{t_{i+1} - t_{i-1}}, \quad (3)$$

where $\bar{t}_i$ is the cell centroid position at time $t_i$. Speed and directionality are defined as the modulus of cell velocity and the cosine of the angle $\theta_i$ between the cell velocity and the direction of the flow, respectively, averaged over 30-40 cells:

$$\text{speed} = \langle |v_i| \rangle, \quad (4)$$

$$\text{directionality} = \langle \cos \theta_i \rangle. \quad (5)$$

Dynamics of cell-substrate contact areas

After recording RICM images, a background image obtained in the absence of cells was subtracted and the resulting images were segmented, using the Image Pro Plus software (IPP, MediaCybernetics). This generates a black and white movie showing the contact area evolution with time, where the cell-substrate contact areas appear white (pixel value 255) over a dark background (pixel value 0). The cell speed is defined as the mean velocity modulus of the center of mass of the contact area. Gain and loss zones are defined as the areas where the pixel value increases from 0 to 255, or decreases from 255 to 0, respectively, between two frames. The steady zone is the cell area where the pixel value stays constant. Mathematically, these areas are obtained by computing at each pixel the following function:

$$F_i = \frac{p_{i+1} - p_{i-1}}{6} + 127 + \frac{p_i}{255}, \quad (6)$$

where $p_i$ is the pixel value at time $t_i$. The gained, lost or steady areas correspond to $F_i=170$, 86 or 128, respectively, and the background to 127. The running average over three frames (from $t_i$ to $t_{i+3}$) eliminates the pixel noise associated to the numerical definition of the contact area edges. The gain and loss growth rates are defined as the cell area gained or lost by unit time.

$$(\frac{dA}{dt})_{\text{gain}} = \frac{\text{gained area}}{t_{i+3} - t_i}, \quad (\frac{dA}{dt})_{\text{loss}} = \frac{\text{lost area}}{t_{i+3} - t_i}. \quad (7)$$

A Visual Basic IPP macro was written to perform these operations, generate a colored movie and send the values of the gain and loss growth rates along time to an Excel datasheet. This program is available upon request.

The gained and lost area growth rates are then plotted as a function of time and smoothed with a Savitzky and Golay algorithm. A 7- or 11-point algorithm is used for gain and loss growth rates, respectively. The position and values of the peaks and valleys are extracted. Peaks whose height is less than 0.2 μm$^{-1}$ s$^{-1}$ over that of a neighbor valley are rejected because the existence of the peak is not significant compared with the pixel noise. Finally, for each recording, peak height and frequency are computed. The peak height is defined as the average value of all peaks. The peak frequency is defined as the inverse of the average time separating two successive peaks.

Results

External calcium entry is required for efficient shear-flow-induced cell motility

Application of shear-flow triggers a cellular response in D. discoideum cells resulting in a net cell movement in the direction of the flow (Décavé et al., 2003). Analysis of the movement showed that both the modulus and orientation of cell velocity was affected by shear stress. Furthermore, PI3K activity was required for cells to move in the direction of the flow, but not for cells to move quicker in response to the flow. Speed and directionality of cell movement are therefore independently regulated. More recently, we noticed experimental variations in cell velocities whose origin was tracked down to the Sörensen phosphate buffer solution used for the experiments. In this medium, calcium concentration is not controlled and depends on water purity (3-6 μM for distilled water). We found that external calcium concentration is a key parameter to stimulate shear-flow-induced cell motility.

Fig. 1 shows cell responses to shear-flow at different external calcium concentrations. Raising the calcium concentration from <10 μM up to 1 mM moderately increases cell speed in the absence of the flow, but stimulates it fivefold in the presence of a 2.4 Pa shear stress (Fig. 1A, see also supplementary material, Movies 1 and 2) also marginally enhanced under these conditions (Fig. 1B). Conversely, lowering calcium concentration to about 0.4 μM by chelation with 100 μM EGTA instantaneously decreases cell speed (Fig. 1C). This effect is reversible since cell movement resumes when the external calcium concentration is increased again (Fig. 1C). In the continuous presence of 100 μM EGTA, cell motility spontaneously recovers after 5 minutes (data not shown), suggesting that cells adapt to low external calcium concentrations. In addition to its effect on cell motility, Ca$^{2+}$ chelation also induces significant cell detachment from the substrate, which will be quantified below. Addition of 100 μM Gd$^{3+}$, a known inhibitor of plasma membrane Ca$^{2+}$-channels, reversibly and competitively stops cell movement (Fig. 1D). No spontaneous recovery is observed in this case. The possible role of magnesium, sodium and potassium ions was also addressed using alternative buffer formulation (MES-Na or MES-K buffers supplemented with MgCl$_2$ or CaCl$_2$, see
Finally, extracellular pH was varied from 5 to 7 using MES and phosphate buffers. No change in cell speed nor directionality was observed between pH 6 and 7, ruling out a significant role of transmembrane pH gradients in cell motility (supplementary material, Table S1). Altogether, these results show that calcium channels are active at the plasma membrane and that calcium entry stimulates cell motility for cells submitted to shear stress.

Using cell speed as a measure of cell response to shear stress and raising or lowering the external Ca\(^{2+}\) concentration in the bathing fluid, we can plot the characteristic dose-response curve (Fig. 2A). To avoid any precipitation between calcium and phosphate in SB, these experiments were conducted in the presence of 20 mM MES-Na buffer (pH 6.2). Data are fitted with a single hyperbolic curve, suggesting that Ca\(^{2+}\) interacts mainly with a single class of targets (K\(_{50}\)=22 µM). The direct effect of calcium on passive cell adhesion to glass was also investigated, measuring \(\sigma_{50}\) as a function of Ca\(^{2+}\) concentration in a radial flow chamber. This parameter is the shear stress at which half of the cells detach and is directly related to the interaction energy between adhesion protein complexes and the substrate (Décavé et al., 2002). Radial flow detachment assays were conducted in the presence of CIPC, to uncouple passive adhesion from cytoskeleton dynamics (Décavé et al., 2002; Décavé et al., 2003). Fig. 2B shows that D. discoideum binding to glass in the presence of SB or MES-Na buffer is sensitive to calcium concentration, but the ‘apparent affinity’ for \(\sigma_{50}\) is ten times smaller than for cell speed (K\(_{50}\)=2.5 µM). The calcium target affecting cell adhesion is therefore distinct from that affecting cell motility.

Cell motility was then examined as a function of applied shear stress, at either low (5 µM) or high (1 mM) calcium concentration. We previously reported that above 1 Pa, shear stress invariably triggers an increase in cell speed and directional motility with the flow (Décavé et al., 2003). Enhancement of cell speed with more calcium and longer recording periods allow observing additional cell behaviors at intermediate shear stresses. Typical results are exemplified on Fig. 3A,B, where cell speed (panel A) and directionality (panel B) are shown as a function of time. Shear stresses under 0.1 Pa trigger a hardly detectable motile response. Application of intermediate shear stresses (between 0.1 and 0.6 Pa) gives rise to a complex cell response. On short time (<2 minutes), cell speed increases and cell movement is oriented along the flow. On longer time (>2 minutes), cell speed decreases slightly and cells tend to move against the flow (see also supplementary material, Movie 3). Higher shear stresses (>0.6 Pa) only trigger cell movement in the direction of the flow, but a decrease in cell speed and directionality is noticeable after 2 minutes. Cell motile response, therefore, can be divided into an immediate and adapted phase. During the immediate phase, cells move in the direction of the flow, while in the adapted phase, they move against or along the flow depending on the shear stress value. These results are quantitatively summarized in Fig. 3C,D, where cell speed and directionality in the adapted phase are...
plotted as a function of shear stress under low or high calcium concentrations. Again it is clear that calcium does not affect cell directional orientations but makes cells faster in response to shear flow. Half-maximum speed is reached at 0.5 Pa and 3.5 Pa at 1 mM and 5 μM calcium concentrations, respectively.

An increase of cell speed is also observed when calcium concentration is raised under constant shear stress (Fig. 4A,B). As calcium enters the flow chamber, cell speed first slightly decreases during about 1 minute, then it increases for 3-5 minutes then it returns to its low calcium concentration level in about 15 minutes. Again, almost no change is observed on cell directionality. This transient acceleration is concomitant with a striking change in cell morphology (Fig. 4C,D, see also supplementary material, Movie 4). Dictyostelium cells examined 2 minutes after calcium rise are elongated, with one or a few large round protrusions at the front edge (Fig. 4C, white arrowheads) and a triangular retraction zone at the rear edge, very much looking as chemotaxing neutrophils (Xu et al., 2003). Furthermore, cell movement appears very smooth, with almost no side protrusions. In many occurrences, the front edge of the cell rhythmically moves, with a 9-12 seconds time period (see supplementary material, Movie 4, bottom cell and top right cell). Cells observed before the calcium rise or 15 minutes later present several smaller protrusions extending from the front edge (Fig. 4D, black arrowheads), without any structure clearly associated with the retracting parts. These results show that plasma membrane calcium channels are open in motile cells, and that their number or activity are regulated in response to shear stress and to external calcium concentration changes.

Cell edge dynamic is stimulated by the presence of external calcium

To understand the origin of the increased cell motility with calcium, cell-substrate contact areas were imaged by RICM during shear-flow-induced motility. Because of the time needed to focus on single cells, these experiments were done several minutes after exposing the cells to shear flow. At each calcium concentration, series of movies were obtained, where the dark areas correspond to zones of close proximity between the cell and the substrate (see supplementary material, Movies 5-7).

The previous analysis of the movies consisted of determining the position of the front and rear edges of the cell along the direction of the movement (Décavé et al., 2003). The front edge moves through fast extensions (bursts), separated by immobility phases whereas the rear edge moves more smoothly. Such analysis conducted on recorded movies shows that burst length increases with the external calcium concentration (data not shown). However, at calcium concentrations higher than 100 μM, many cell extensions correspond to the formation of new contact zones rather than to the lateral extension of existing ones. A new image analysis procedure was therefore designed, so that gained and lost contact areas between successive image frames are detected (see Materials and Methods). A color model of the cell-substrate contact areas is generated, where the gained areas are blue, the lost ones red and the steady areas green (see supplementary material, Movies 5-7).

To focus on single cells, these experiments were done several minutes after exposing the cells to shear flow. At each calcium concentration, series of movies were obtained, where the dark areas correspond to zones of close proximity between the cell and the substrate (see supplementary material, Movies 5-7). The areas gained and lost between successive frames, divided by the time interval, give the gained and lost area growth rates, respectively. They are plotted on Fig. 5A as a function of time, for individual cells and the substrate (see supplementary material, Movies 8-10). The areas gained and lost between successive frames, divided by the time interval, give the gained and lost area growth rates, respectively. They are plotted on Fig. 5A as a function of time, for individual cells at different calcium concentrations. At 5 μM calcium, the gained area growth rate is irregular, with large peaks separated by periods of reduced activity. Large peaks correspond to bursts detected in the previous analysis. As for the lost area growth rate, almost no organization appears over a steady 1 μm² s⁻¹ loss rate. At larger calcium concentrations, the gain and loss growth rates appear much more regular, with peaks of high activity repeating at 9-11 seconds time intervals. Representative examples are shown, that were recorded at 100 and 300 μM calcium concentrations (Fig. 5A). Periodic occurrence of protrusions and retractions is especially noticeable on Movie 10 (see supplementary material) (Fig. 5A).
Fig. 3. Force sensitivity is modulated by the external calcium concentration. (A,B) Ax2 cells were allowed to adhere to glass at a low (5-10 μM) calcium concentration in MES-Na buffer, after which the calcium concentration was raised to 1 mM at low shear stress. The flow was then stopped and video recording started (t=0 minutes). After 2 minutes, a constant shear stress was applied (arrow): ▲, σ=0.1 Pa; □, σ=0.5 Pa; circles, σ=1 Pa. The instant cell velocity modulus (A) and directionality (B) are plotted as a function of time. (C,D) Average cell speed (C) and directionality (D) are plotted as a function of the applied shear stress at a 1 mM (▲) or 5 μM (□) calcium concentration.

The average cell speed and directionality are determined from the steady-state portion of the motility response to shear stress (from 7 to 15 minutes in Fig. 3A,B). Data are collected from several independent experiments performed in Sörensen or MES-Na buffer.

300 μM calcium: 90-150 seconds left panel, 30-90 seconds right panel. Periodic protrusive activity is also noticeable at low calcium concentration on the graph. A similar analysis was performed on ten cell recordings and, for each of them, the average height of the peaks and the average time between successive peaks were calculated. These parameters are plotted on Fig. 5B,C as a function of the average cell speed during the recording. A good correlation is found between the gain and loss peak height and cell speed, whereas peak frequency is constant, whatever the calcium concentration. The frequency of protrusions is slightly larger (0.11±0.01 Hz) than that of retractions (0.08±0.01 Hz).†

The cell speed increase due to external calcium therefore results from a stimulation of cell spreading and retraction activities. This stimulation acts on the protrusion and retraction area growth rates but not on the frequency of these events.

As an attempt to visualize possible internal calcium concentration variations during cell movement, cells were loaded with the calcium indicator CG2. Spreading of these CG2-loaded cells to glass absolutely required addition of at least 100 μM calcium in the external medium and full reconstitution of movement was obtained only after allowing cells to recover in growth medium (see Materials and Methods). The calcium requirement for spreading again proves the existence of a calcium influx coupled to the deformation of cell cortex. The same effect was observed with other calcium indicators at a similar concentration. Within the cells, CG2 fluorescence accumulated in vesicles of different sizes in addition to a uniform, probably cytosolic distribution. Neither gross variation of the uniform fluorescent background nor a clear intracellular gradient was observed in cell undergoing fast shear-flow-induced motility. Periodic protrusive and reactive activities associated to cell movement therefore do not correspond to large intracellular calcium fluctuations.

Interestingly, in most cells, a fluorescent zone was observed at the cell rear, whose intensity rhythmically fluctuated (see supplementary material, Movie 11). With a period of about a minute, fluorescence steadily increased then suddenly decreased. Alternating phase contrast and fluorescence illuminations showed that this oscillation originates from the contractile vacuole activity, which presumably pumps CG2 out of the cell through a calcium-rich compartment as discussed previously (Rooney et al., 1994).

Heterotrimeric G proteins are involved in calcium stimulation of shear-flow-induced cell motility

To test for the involvement of heterotrimeric G proteins in shear-flow-induced Dictyostelium cell motility, a mutant cell line was used, LW6, where the single gene coding for the β-subunit of this protein family was removed by homologous recombination (Lilly et al., 1993; Wu et al., 1995). At 5 μM calcium concentration, Gβ-null cells exhibit directional motility in response to shear stress, comparable with that of the parental DH1 cell line. Similarly, lowering calcium concentration with EGTA blocks cell motility and induces its detachment (data not shown). Increasing calcium concentration...
however does not speed up cell movement. This is quantitatively shown in Fig. 6A, where cell speed is plotted as a function of the external calcium concentration. As shown before, results are fitted with a single class of Ca^{2+} sites whose affinity is not measurable for Gβ-null cells (Gβ3) cells (K_{S0}>1000 \mu M). Expression of a Gβ gene in Gβ-null cells partially restores Ca^{2+} sensitivity to shear-flow-induced motility (K_{S0}=150 \mu M). The speed of Gβ-null cells is also much less sensitive to external shear stress than the rescued ones in the presence of millimolar calcium (Fig. 6C). However, directional sensing is marginally affected by the lack of the heterotrimeric G protein β-subunit, since clear orientation in the direction of the flow occurs at shear stresses higher than 1.7 Pa for Gβ-null cells and 1.5 Pa for the rescued ones (Fig. 6D). Furthermore, when calcium concentration is raised from the micromolar to millimolar levels under constant shear flow, no change in cell speed is recorded for Gβ-null cells, whereas the rescued cells accelerate, albeit more slowly than Ax2 wild-type cells (Fig. 6B). These results suggest that calcium channels are closed in Gβ-null cells under shear flow.

Cell-substrate contact area was also imaged for Gβ-null cells under shear flow at different calcium concentrations and its dynamics was compared with that of the rescued cells. Protrusion and retraction dynamics are analyzed as explained above and shown in Fig. 7. Whatever the calcium concentration, the growth rate activity of Gβ-null cells is low and comparable with that of wild-type cells at 5 \mu M calcium (Fig. 7A and see supplementary material, Movie 12). By contrast, the gained or lost area growth rates of Gβ-null cells and the parental RK-Ax2 cells. Cells from both strains move in the direction of the flow and are sensitive to external calcium concentrations but IP3-receptor null cells require more calcium to reach the same speed than wild-type cells (Fig. 8A). The mobilization of internal Ca^{2+} stores is therefore a major contributor to cell speed under shear stress.

A Dictyostelium IP3-receptor like protein amplifies calcium stimulation of shear-flow-induced cell motility

In higher eukaryotic cells, heterotrimeric G proteins activate phospholipase C β-isoform, which cleaves membrane-bound PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). In turn, soluble IP_3 triggers intracellular calcium release from the endoplasmic reticulum by binding to a specific IP3-receptor. In Dictyostelium, a single PLC isoform is known, which belongs to the δ-isofom family and is activated by calcium (Cubitt and Firtel, 1992; Drayer et al., 1994). In addition, an IP3-receptor-like gene was found (iplA), whose disruption produces null cells in which Ca^{2+} entry in response to chemoattractants is greatly reduced (Traynor et al., 2000). Nevertheless, IP3-receptor null cells are still able to chemotax toward cAMP, and cAMP or cGMP productions in response to a cAMP pulse are normal.

We measured shear-flow-induced motility of IP3-receptor null cells and the parental RK-Ax2 cells. Cells from both strains move in the direction of the flow and are sensitive to external calcium concentrations but IP3-receptor null cells requires more calcium to reach the same speed than wild-type cells (Fig. 8A). The mobilization of internal Ca^{2+} stores is therefore a major contributor to cell speed under shear stress.
In addition, the directionality of the movement of the mutant cells is significantly lower at low calcium concentration (Fig. 8B). Furthermore, although IP3-receptor null cells accelerate in response to an increase of the external calcium concentration, they do so much more slowly than the parental RK-Ax2 cells (Fig. 8C) and their movement is less directional (Fig. 8D). The dynamics of cell substrate contact area of IP3-receptor null cells is similar to that of wild-type cells, although at a given calcium concentration, protrusion and retraction growth rates peak at smaller values (data not shown). The simplest interpretation is that calcium influx is much reduced in IP3-receptor null cells, as reported for the cAMP or folate response of these cells (Traynor et al., 2000). Another possibility is that calcium influx has less effect inside IP3-receptor null cells. The underlying mechanistic assumption is in the former case, that internal calcium release triggers external calcium entry (capacitive calcium uptake) or in the latter case, that external calcium entry triggers internal calcium release (calcium-induced calcium release).

In conclusion, the presence of heterotrimeric G proteins, the IP3-receptor-like protein and an external calcium concentration above 30 μM are required for rapid actin cytoskeleton reorganization, resulting in efficient cell motility.

Discussion

This work provides evidence for the involvement of Ca2+ and heterotrimeric G protein signaling in adhering cells submitted to shear flow. More precisely, G protein activation is necessary for cells to modulate their speed in response to shear stress, through activation of plasma membrane calcium channels and IP3-mediated internal calcium store release. Vegetative cells lacking G proteins indeed behave similarly to wild-type cells exposed to shear stress in the presence of low (e.g. 5 μM) calcium concentrations, although they possess the machinery to uptake calcium in response to folic acid (Nebl et al., 2002). Cells lacking the IP3-receptor require higher external Ca2+ concentrations to reach the same speed as wild-type cells. However, neither G protein activation nor Ca2+ entry is necessary for directional sensing. On the contrary, PI3K activity is necessary for directional sensing, but not for the stimulation of speed by shear stress (Décavé et al., 2003). It follows that, for the mechanosensing response, heterotrimeric G proteins do not activate PI3K. This suggests that exposure to shear stress involves two signaling pathways in Dictyostelium cells, one which activates G proteins, is sensitive to calcium and controls cell speed, and another one which results in the production of a PIP3 gradient and defines cell orientation (Fig. 9). Interestingly, PIP3 appears to be at the crossroad between the direction and speed pathways, since it is the source of both PIP3 and IP3. In this respect, it is indicative that IP3-receptor null cells exhibit both speed and orientation defects.
Different effects are observed, depending on the calcium concentration level. (1) At low concentration (<10 μM), calcium is necessary for Dictyostelium binding to glass. In addition, competition between cell-cell and cell-glass adhesion is observed in the flow chamber at high cell concentration (data not shown). This suggests that cell-cell contact and cell-glass adhesion share the same molecular receptor, which could be DdCAD-1 (also known as contact site B or gp24), a protein belonging to the large Ca^{2+}-dependent cadherin family (Chadwick et al., 1984; Knecht et al., 1987; Wong et al., 1996). Interestingly, DdCAD-1 interacts with catenin (Coates and Harwood, 2001; Grimson et al., 2000) and talin (Niewohner et al., 1997) homologs. The physiological significance of shear-flow-mediated motility could therefore be related to the formation and disruption of homotypic cell contacts. (2) At higher extracellular calcium concentration (between 10 μM and 100 μM), calcium enters the cell and speeds up both membrane spreading and retraction. Our results show that calcium influx through the plasma membrane occurs during cell motility. This is indicated by the effect of the competitive

![Fig. 6](image_url)

Cells devoid of the β subunit of the heterotrimeric G protein family are insensitive to an increase of the external calcium concentration. (A) Average speed of LW6 Gβ-null cells (GβΔ, ○) or LW20 Gβ-rescued cells (Gβ-rescued GβΔ, ▲) is plotted as a function of the free calcium concentration in the flowing fluid (σ=2.4 Pa). The solid line corresponds to a fit with an apparent calcium affinity of 150 μM and a minimum and maximum speed of 3.5 and 13 μm minute⁻¹, respectively. (B) LW20 (Gβ-rescued GβΔ, ▲) and LW6 (GβΔ, ○) cells were allowed to adhere to glass at a low (5 μM) calcium concentration, then a constant shear stress (2.4 Pa) was applied and video recording started (t=0 minutes). After 2 minutes, the calcium concentration was raised to 1 mM at the same shear stress. Instant cell speed is plotted as a function of time. Note that at low calcium concentrations, LW6 cell speed is slightly, but significantly higher than that of LW20 cells. (C,D) Average cell speed (C) and directionality (D) are plotted as a function of the applied shear stress at a 1 mM calcium concentration either for LW20 (Gβ-rescued GβΔ, triangles) or LW6 (GβΔ, ○) cells. Average cell speed and directionality are determined from the steady-state portion of the motility response to shear stress.

![Fig. 7](image_url)

Protrusions and retractions are less active during shear-flow-induced cell motility in cells devoid of the beta subunit of the heterotrimeric G protein family. LW6 or LW20 cells undergoing shear-flow-induced motility (σ=2.4 Pa) in the presence of the indicated calcium concentration were imaged at high resolution under RICM and morphology of cell-substrate contact areas was analyzed as described in Materials and Methods. (A) Growth rates of gained (left) and lost (right) contact areas are represented as a function of time, for a single Gβ-null cell at a 1 mM calcium concentration. (B,C) Mean peak height and frequency are plotted as a function of individual cell speed. Δ, ▲ protrusions; ○, ● retractions. Filled symbols, Gβ-rescued cells (LW20); open symbols, Gβ-null cells (LW6). In B, the dotted line is a linear least square fit of the LW20 data points (slope 4.9 μm; ordinate at origin 0.7 μm² second⁻¹). Standard deviations for protrusion and retraction peak height are 0.9 and 0.7 μm² second⁻¹ for Gβ-null cells and 0.7 and 0.5 μm² second⁻¹ for Gβ-rescued cells, respectively. In C, the dotted lines represent the mean values of the retraction and protrusion frequencies.
Fig. 8. Shear-flow-induced motility of IP3-receptor null Dictyostelium cells. (A,B) Average speed (A) and directionality (B) of IP3-receptor null cells (IP3-ReceptorΔ, circles) or parental RK-Ax2 cells (wild type, triangles) are plotted as a function of the free calcium concentration in the flowing fluid (σ=2.4 Pa). The solid line corresponds to a fit with an apparent calcium affinity of 120 μM and a minimum and maximum speed of 5 and 32 μm minute–1, respectively. The dotted line is a guide for the eye. (C,D) IP3-receptor null cells (IP3-ReceptorΔ/H17034) or parental Ax2-RK cells (wild type, /H17009) were allowed to adhere to glass at a low (5 μM) calcium concentration, then a constant shear-flow (2.4 Pa) was applied and video recording started (t=0 minutes). After 3 minutes, the calcium concentration was raised to 1 mM under the same shear stress. (C) Cell speed is plotted as a function of time. The lines are hand-drawn. (D) Cell directionality is measured during the 1-3 minutes (white bars) and 8-12 minutes (black bars) portions of the recordings, corresponding to a 5 μM and 1 mM extracellular concentration, respectively.

Fig. 9. (A) Model of Dictyostelium signaling pathways involved in shear stress-induced motility. Experimental evidence supporting a role for PI3K in the directionality response is presented elsewhere (Décavé et al., 2003). A link between mechanical stress and heterotrimeric G protein activation is shown in the present work since Gβ-invalidation reduces cell speed in response to shear stress (Fig. 6C). In both cases, molecular details are unknown. Heterotrimeric G protein mediated Ca2+ entry (arrows 1 and 2) is supported by the effect of Gd3+ ions (Fig. 1D) and Gβ-invalidation (Fig. 6B). Ca2+-induced PLCδ activation (arrow 3) is shown in (Cubitt and Firtel, 1992). Direct biochemical evidence for IP3-mediated calcium release (arrows 4 and 5) is given elsewhere (Schaloske et al., 2000). Our work suggests Dictyostelium IP3-receptor-like protein as a possible mediator (Fig. 8). The link between intracellular calcium and cell speed is shown in our work and (Van Duijn and Van Haastert, 1992). Note that calcium pumping activities, which are essential to restore low cytosolic calcium concentrations, are omitted. (B,C) Protrusive and retractile activities of cell-substrate contact area as a function of cell speed. Data are gathered from Fig. 5 (Ax2 cells), Fig. 7 (LW6 and LW20 cells) and from data obtained with IP3-receptor null cells (not shown). In B, the solid line is a linear least square fit of all data points (slope 9.2 μm; ordinate at origin 0.84 μm² second–1). In C, solid and dotted lines are linear least square fits of protrusion and retraction frequencies (slope –0.03 and –0.02 μm–1, respectively; ordinate at origin 0.13 and 0.09 Hz, respectively).
calcium blocker Gd$^{3+}$ and the cell morphology changes and acceleration observed when the calcium concentration is raised under constant shear flow.

The calcium response of wild-type cells is however extremely slow, compared with the time expected for calcium diffusion within the cells. Furthermore, cells lacking the IP$_3$-receptor require 20 times more calcium to reach the same speed and respond even more slowly to external calcium changes. This suggests that in wild-type cells, the main calcium flux comes from internal stores and that external calcium entry stimulates internal calcium release, as observed in many cells. A possible candidate for this process is PLC$_8$, which is indeed activated by calcium in vegetative cells (Cubitt and Firtel, 1992). It has been shown in vitro that IP$_3$ triggers Ca$^{2+}$ release from Dictyostelium intracellular compartments (Schaloske et al., 2000). In this way, intracellular calcium release would amplify the small calcium entry from the plasma membrane. Without IP$_3$-receptors, residual sensitivity to external calcium is however still possible, whereas it is abolished in G$\beta$-null cells. Heterotrimeric G proteins would control plasma membrane calcium channels in vegetative motile cells. A tentative scheme combining the biochemical knowledge of Dictyostelium signaling pathways and our new results is depicted in Fig. 9.

Using fluorescent markers, no gross change in cytoplasmic calcium concentration is experimentally observed. Calcium variations are indeed well buffered within Dictyostelium cells and therefore, calcium concentration changes are necessarily restricted to the border of plasma membrane or internal calcium stores. A localized calcium increase would facilitate the turnover of actin polymerization-depolymerization, for instance by activating proteins such as gelsolin, whose activity is modulated by calcium at a 15-25 $\mu$M affinity (Ditsch and Wegner, 1995; Kinosian et al., 1998). Calcium is therefore involved in the fast remodeling of actin structures rather than in cell polarization mechanisms.

A correlation between intracellular calcium levels and chemotactic motility has already been observed in neutrophils (Mandeville et al., 1995). Furthermore, internal calcium concentrations periodically fluctuate in moving keratocytes and application of shear stress by stretching a flexible substrate underneath the cell induces a calcium transient (Lee et al., 1999). Recently, it was reported that ultrafast Ca$^{2+}$ waves travel through fMLP-stimulated neutrophils (Kindzelski and Petty, 2003). In Dictyostelium cells, cAMP receptor activation stimulates calcium entry (Nebl et al., 2002; Schaloske et al., 2000; Yamura et al., 1996), but eliminating capacitive calcium entry by knocking out the Dictyostelium IP$_3$-receptor-like gene does not affect chemotaxis speed and directionality (Traynor et al., 2000). It should, however, be noted that in the reported cAMP chemotaxis experiments, Dictyostelium speed was close to that observed during random motility, suggesting that stimulation was not at its maximal. In addition, introduction of EGTA calcium buffer stops net cell movement but does not prevent cell orientation in the direction of a cAMP gradient, revealed by protrusion elongation in this direction (Unterweger and Schlatterer, 1995; Van Duijn and Van Haastert, 1992). Intracellular loading with BAPTA, a calcium buffer which has a similar Ca$^{2+}$ dissociation constant to EGTA but an about 150-fold faster rate of Ca$^{2+}$ binding (Tsien, 1980), prevents both motility and oriented pseudopod emission (Schlatterer and Malchow, 1993; Unterweger and Schlatterer, 1995). It seems, therefore, likely that during chemotaxis as well, calcium controls cell speed, but not directionality. At this stage of our knowledge, chemotaxis and shear-flow-induced motility signaling pathways differ in two respects. (1) Heterotrimeric G proteins are essential for cAMP and folate acid signal transduction but are dispensable for mechanosensitivity. (2) The phg2 kinase is required for vegetative cell exploratory or shear-flow-induced motility but not during multicellular development, hence for cAMP chemotaxis (Gebbie et al., 2004).

The new image analysis procedure developed in this work points out to the existence of oscillations in the cell edge movement, at a 9 and 12 seconds period for protrusions and retractions, respectively. The amplitude of these oscillations increases with cell speed, but their frequency is almost constant. The linear relationship between gained or lost area growth rate and cell speed is easily interpreted. Some protrusions and retractions of the cell edge are effective since they result in net cell movement. Others do not and their activity is independent of cell speed. We can thus write the area growth rate as the sum of two terms:

$$dA/dt = (dA/dt)_0 + L \times v,$$

where $(dA/dt)_0$ is the ineffective cell protrusive or retractive activity and $L$ is a length, which relates cell speed to the growth rate of the cell-substrate contact area. This is the length along which gained or lost areas are in the direction of cell movement. In a perfectly aligned cell, $L$ is the width of protrusions or retractions. It is noteworthy that all data obtained with all four cell lines used (Ax2, LW6, LW20 and IP$_3$-receptor null cells) can be put on the same graphs (Fig. 9B,C), giving $(dA/dt)_0=0.84\pm0.14 \mu\text{m}^2 \text{s}^{-1}$ and $L=9.2\pm0.7 \mu\text{m}$ under the reported RICM experimental conditions. $(dA/dt)_0$ is comparable with the smallest recorded peaks. $L$ is about a quarter of the cell-substrate contact perimeter. This makes sense, because this is the typical length of the cell contour facing or opposite to the flow. Thus for wild-type cells, the protrusion and retraction activities become more and more effective as the calcium concentration increases. As for the peak frequencies, they slightly decrease with cell speed. The oscillation period, therefore, does not contribute significantly to cell speed changes.

Finally, this work extends our previous report of directional mecano-sensitivity: at low shear stress (0.5 Pa), cells tend to oppose to the force exerted by the flow whereas they tend to move in the direction of the force at higher shear stress. Dictyostelium indeed recapitulates the behavior of various cell lines: keratocytes, for instance, move in the direction of the force (Verkhovsky et al., 1999) in contrast to fibroblasts, which oppose detachment by reinforcing focal contacts (Riveline et al., 2001). It should be noted that at a shear stress giving rise to zero directionality (about 0.6-1.2 Pa), cell behavior is not random, but clearly bi-directional (see supplementary material, Movie 3). Bi-directionality of cell movement is indicative of a complex relationship between cell edge velocity and membrane tension. At each cell edge, two alternative macromolecular assemblies can indeed be recruited, that allow
spreading or retraction. These machineries are both mechanically and biochemically incompatible at the same site (Meli and Firtel, 2003; Xu et al., 2003). Our results show that membrane tension, which is modulated by shear-flow within a single cell, affects the state of the complex actin cytoskeleton dynamics.

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References


Supplementary Material

Table S1. Ionic composition of the bathing fluid and SFICM

Ax2 cells were introduced in the shear flow chamber in Sörensen buffer, then the buffer was exchanged for the indicated ones at low shear stress and after 3 min, high shear stress was applied (σ = 2.4 Pa). SFICM was recorded and measured as explained in Materials and Methods. The first four solutions test for the involvement of Na⁺, K⁺, Ca²⁺ and Mg²⁺ in SFICM. The last fours show that H⁺ marginally influences SFICM. It should be noted that cells detach from the surface at pH larger than 7.5.

<table>
<thead>
<tr>
<th>Bathing solution</th>
<th>Speed (µm/min)</th>
<th>Directionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM MES-NaOH, pH 6.5</td>
<td>10 ± 2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>20 mM MES-KOH, pH 6.5</td>
<td>10.3 ± 0.8</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>20 mM MES-NaOH, pH 6.5 + 1 mM CaCl₂</td>
<td>26 ± 2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>20 mM MES-NaOH, pH 6.5 + 1 mM MgCl₂</td>
<td>10 ± 2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>20 mM MES-NaOH, pH 5.0</td>
<td>3.5 ± 0.5</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>20 mM MES-NaOH, pH 5.5</td>
<td>4.7 ± 0.7</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>20 mM MES-NaOH, pH 6.0</td>
<td>10 ± 2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>20 mM MES-NaOH, pH 7.0</td>
<td>10 ± 2</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>