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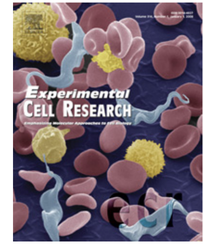

Research Article

Mechano-chemical signaling maintains the rapid movement of *Dictyostelium* cells

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ABSTRACT

The survival of *Dictyostelium* cells depends on their ability to efficiently chemotax, either towards food or to form multicellular aggregates. Although the involvement of Ca^{2+} signaling during chemotaxis is well known, it is not clear how this regulates cell movement. Previously, fish epithelial keratocytes have been shown to display transient increases in intracellular calcium ($[Ca^{2+}]_i$) that are mediated by stretch-activated calcium channels (SACs), which play a role in retraction of the cell body [J. Lee, A. Ishihara, G. Oxford, B. Johnson, and K. Jacobson, Regulation of cell movement is mediated by stretch-activated calcium channels. Nature, 1999. 400(6742): p. 382–6.]. To investigate the involvement of SACs in *Dictyostelium* movement we performed high resolution calcium imaging in wild-type (NC4A2) *Dictyostelium* cells to detect changes in $[Ca^{2+}]_i$. We observed small, brief, Ca^{2+} transients in randomly moving wild-type cells that were dependent on both intracellular and extracellular sources of calcium. Treatment of cells with the SAC blocker gadolinium (Gd^{3+}) inhibited transients and decreased cell speed, consistent with the involvement of SACs in regulating *Dictyostelium* motility. Additional support for SAC activity was given by the increase in frequency of Ca^{2+} transients when *Dictyostelium* cells were moving on a more adhesive substratum or when they were mechanically stretched. We conclude that mechano-chemical signaling via SACs plays a major role in maintaining the rapid movement of *Dictyostelium* cells.

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Introduction

Calcium controls many aspects of cell movement. Extracellular calcium is important for integrin-ligand binding and attachment of cells to their substrata [2,3]. Intracellular calcium $[Ca^{2+}]_i$ is involved in the reorganization of the actin cytoskeleton by modulating the activities of α-actinin [4], severin [5], and gelsolin [6]. Elevations in $[Ca^{2+}]_i$ increase cytoskeletal contractility via phosphorylation of myosin light chain kinase (MLCK) [7,8] or activation of a calcium-dependent protease like calpain [9,10]. Calcium also serves as a second messenger in many biochemical signaling pathways, including chemotaxis.

In moving cells, increases in $[Ca^{2+}]_i$ can occur either spatially in the form of a gradient or temporally as a transient rise in $[Ca^{2+}]_i$ [1,11]. For instance, newt eosinophils [11] maintain an increasing gradient of $[Ca^{2+}]_i$ from the leading edge to the cell rear that persists when the cell reorients in response to the repositioning of a chemoattractant source. Other highly motile cells, such as fish epithelial keratocytes and human neutrophils, display brief transient increases in $[Ca^{2+}]_i$, prior to

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retraction [1,12]. Previous work showed that Ca^{2+} transients are inhibited by the depletion of intracellular or extracellular calcium, which impairs retraction and forward translocation of the cell [1,12]. Calcium transients facilitate retraction by increasing cytoskeletal contractility [13,14], promoting adhesion disassembly [9,10,15,16], and by regulating actin binding proteins to prevent protrusion formation at the rear [6].

Various methods have been used to measure global elevations in $[Ca^{2+}]_i$ during movement of *Dictyostelium* cells chemotaxing towards folic acid or cAMP [17–22]. In general, a Ca^{2+} elevation displayed by a chemotaxing cell can range from ~50 to 160 nM from the baseline value, with a duration of ~20–60 s [21–28]. It has been shown that Ca^{2+} elevations are necessary for the orientation of extending pseudopods and movement toward a chemoattractant source [20,21,29,30]. For example, the addition of a Ca^{2+} chelator to *Dictyostelium* cells caused a decrease in the formation of pseudopods and impaired movement [30]. In addition, these elevations in $[Ca^{2+}]_i$ have been suggested to amplify the chemotactic signal [23,25].

A common feature of many mechano-sensitive cell types is the calcium-induced calcium release (CICR) from intracellular stores in response to applied force [1,31]. The calcium transients observed in keratocytes result from the activation of stretch-activated calcium channels (SACs) in response to increased cytoskeletal tension when retraction at the rear is impaired. Following calcium-induced retraction, cytoskeletal tension is decreased and cell movement resumes. Thus by acting as mechano-sensors SACs are believed to promote rapid, continuous cell movement.

Until now keratocytes have been the only cell type for which evidence of SAC mediated regulation of movement has been obtained. Interestingly, several studies indicate that *Dictyostelium* are mechano-sensitive. For example, in suspension *Dictyostelium* exhibit brief increases in $[Ca^{2+}]_i$ in response to a mechanical stimulus resulting from the rapid addition of buffer [27] and the rate of cell movement was shown to increase in the direction of shear flow [32]. Furthermore, it has been shown that elevations in Ca^{2+} occurring in chemotaxing *Dictyostelium* cells could be inhibited by trivalent cations or the depletion of extracellular Ca^{2+} [22]. This led us to hypothesize that these Ca^{2+} elevations result from the activation of SACs. In order to test this possibility, we measured changes in $[Ca^{2+}]_i$ using high resolution Ca^{2+} imaging in randomly moving and chemotaxing *Dictyostelium* cells loaded with Calcium Green-1 dextran. We found that *Dictyostelium* cells display Ca^{2+} transients that can be inhibited by gadolinium (Gd^{3+}) and are dependent on both intracellular and extracellular calcium. Our data provide evidence for the role of SACs in maintaining the rapid movement of *Dictyostelium* cells.

Materials and methods

Materials

Calcium Green-1 dextran (3 kDa), Calcium Calibration Buffer Kit #1, and calcimycin (A-23187, free acid) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). EGTA, thapsigargin, gadolinium hydrochloride III (Gd^{3+}), poly-L-lysine (70–150kDa), and folic acid were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA).

Cell cultures and coverslip preparation

All *Dictyostelium discoideum* cell lines were grown in 100 mm plastic Petri dishes with 10 ml HL-5 nutrient medium [33]. NC4A2 is a spontaneous axenic derivative of wild-type NC4 cells [34]. The *iplA*⁻ mutant (cell line HM1038) is an endoplasmic reticulum inositol (1,4,5)-triphosphate receptor (IP_3) mutant derived from wild-type cell line AX2 [23]. For all experiments, cells were placed in H5 buffer [25] with or without Ca^{2+} and added to glass Rappaport chambers. In cell stretching experiments, cells were placed on an elastic substratum made of 10% gelatin (Nabisco, Parsippany, NJ, USA) dissolved in low fluorescence axenic medium. In cell adhesion experiments, cells were placed on acid washed coverslips coated with a range of poly-L-lysine concentrations (0.0025 mg/ml, 4 mg/ml, and 10 mg/ml). Poly-L-lysine was added to the coverslips for 15 min and then washed with water and placed in 37 °C for 1 h before use.

Electroporation

Dictyostelium cells were harvested in HL-5 medium, washed twice in H-50 buffer [35] and the concentration of cells was adjusted to 5×10^6 cells/ml with H-50 buffer. A 100 μl of ice-cold *Dictyostelium* cell suspension containing 8 mg/ml Calcium Green-1 dextran (3 kDa) was placed in an electroporation cuvette. One pulse of 800V at 25 μF and 200 Ω was applied with an electroporator (Electro Cell Manipulator 630, Genetronics, BTX Instrument Division, CA, USA) such that the time constant was ~0.6 ms. Immediately after electroporation, the cell suspension was removed from the cuvette and added to 10 ml HL-5 medium for 1 h in the dark at room temperature. Approximately 100 μl of these cells was added to Rappaport chambers and allowed to adhere for 2 h, after which the medium was removed and replaced with 800 μl of H5 buffer containing 1 mM $CaCl_2$ before imaging.

Calcium imaging

Fluorescence images were collected on an inverted microscope (Eclipse TE 300; Nikon, Melville, NY, USA) using a Plan APO 100 x, 1.4 N.A. oil immersion objective. Fluorescence excitation was achieved using a Ludl high-speed filter wheel (Ludl electronic products, Hawthorne, NY, USA) equipped with a 492 nm excitation filter and a 32% neutral density filter (Chroma Technology, Brattleboro, VT, USA). Fluorescence emission was collected using a FITC filter set (Chroma Technology). Images were acquired using a back-illuminated, frame transfer CCD camera (Quantix 57, Roper Scientific, Tucson, AZ, USA) in 2×2 binning mode, using an exposure time of 0.5 s. Esee Analytical Imaging Software (ISee Imaging Systems, Raleigh, NC, USA) running on a Unix computer platform (SGI O^2, Silicon Graphics, Mountain View, CA, USA) was used for image acquisition and hardware control. Cells were treated *in situ* on the microscope stage, followed immediately by calcium imaging for 2–3 min. For chemotaxing cells, imaging began ~2–3 min after adding a source of chemoattractant. Images of Calcium Green-1 dextran loaded *Dictyostelium* cells were collected every 0.5 s for 2 min and stored on a time-lapse VCR. The taped images were then converted to tif files. To detect changes in $[Ca^{2+}]_i$, the average fluorescence intensity was measured within the region of the

cell body in sequential images. The background fluorescence was subtracted from these measurements to correct for any variations in the light source intensity (100W mercury arc lamp).

Chemotaxis assay

To detect changes in $[Ca^{2+}]_i$ during chemotaxis, *Dictyostelium* cells were loaded with the Ca^{2+} indicator and placed in Rappaport chambers in HL-5 medium for 2 h. The cells were then washed twice with Sǿrenson phosphate buffer [25], followed by addition of H5 buffer with 1 mM $CaCl_2$ to the chamber. A localized source of chemoattractant was provided using a micropipette (~1 μm tip diameter) filled with 25 mM folic acid that was positioned ~20 μm from the cell edge and ~20 μm above the substratum. Video tape recordings of cells chemotaxing towards the micropipette were made as described above in the Calcium imaging section.

Cell stretching assay

An external force was applied indirectly to single cells attached to a gelatin substratum by locally deforming it with two micropipettes. Cells loaded with Calcium Green-1 dextran were added to an elastic 10% gelatin substratum. Two glass micropipettes were made using a micropipette puller (P-97 Sutter Instrument Company, CA, USA) and lowered onto the substratum ~48 μm on each side of the cell using two micromanipulators (Harvard Apparatus, Holliston, MA, USA). To deform the substratum in the vicinity of the cell, one micropipette was held stationary while the other was pulled in the opposite direction for ~3 s. To observe the effects of blocking SACs, 100 μM Gd^{3+} was added to the cells 1 min prior to stretching.

Cell adhesion assay

To determine the relative adhesiveness of surfaces coated with different poly-lysine concentrations (0.0025 mg/ml, 4 mg/ml, and 10 mg/ml), local shear flow was applied to single cells using a micropipette. A micropipette with a ~6 μm tip diameter and filled with H5 buffer was positioned ~45 μm above the substratum and ~48 μm from the cell edge. Using a pressure injector (MPPI-2, Applied Scientific Instruments, OR, USA), 15–24 psi of pressurized air was applied to the buffer within the micropipette for 1 s. The flow rate was adjusted so that on uncoated glass coverslips 100% of cells were sheared off. The shear flow (F_s) produced was $\sim 4.45\times10^{+1}$ dynes/cm^2 estimated from this equation: $F_s=(v\times\nu)/d$, where v = flow rate, ν = viscosity (1×10^{-2} Poise) and d = distance of the pipette tip from the cell. Assuming that the surface area of the cell exposed to shear flow is 1/4 of a sphere with a diameter of 4 μm, we estimate the shear force acting on the cell to be $\sim 5.56\times10^{-4}$ dynes/cm^2. Video tape recordings were made during the application of shear flow and the number of cells sheared off the substratum out of the total number of cells tested was counted and expressed as a percentage. Since the poly-L-lysine concentration of 0.0025 mg/ml significantly increased cell adhesiveness, this was used in Ca^{2+} imaging experiments. To control for differences in pipette tip geometry we used the same micropipette for all experiments.

Calibration of Calcium Green-1 dextran

The concentration of intracellular calcium was calculated from the fluorescence intensity of the Ca^{2+} indicator using the following equation for a single wavelength indicator: $[Ca^{2+}]_i = K_d \times (F - F_{min}) / (F_{max} - F)$, where F = experimentally measured fluorescence intensity, F_{min} = fluorescence intensity of the cell in Ca^{2+} free medium, and F_{max} = fluorescence intensity of the cell in Ca^{2+} saturated medium [36]. K_d = 201 nM, is the dissociation constant of the Ca^{2+} indictor. We measured F_{max} by imaging the increases in $[Ca^{2+}]_i$ following addition of 10 μM calcimycin (A-21387). To obtain F_{min}, cell culture medium was removed while simultaneously adding a solution containing Ca^{2+} free H5 buffer, 20 μM EGTA and 0.01% Triton. Calibration curves were obtained by using a Calcium Calibration Buffer Kit #1 (Invitrogen Corporation, Carlsbad, CA, USA).

Data analysis

Pseudocolor images (Fig. 1A–J) were generated using Image J software (NIH). Diagrams of stacked cell outlines (Fig. 2A and B) were created using DIAS 3.0 software (The Dynamic Image Analysis System, Solltech, Oakdale, IA, USA) [37]. Morphometric analysis of rear retraction was determined using Image J software (NIH) by measuring the total displacement of the rear edge between the first and last cell image. Cell area was measured for 20 cells on uncoated glass coverslips and 20 cells on 0.0025 mg/ml poly-L-lysine coated coverslips using DIAS 3.0 software (Fig. 5B and C).

Results

Dictyostelium cells display Ca^{2+} transients during movement

In order to detect changes in $[Ca^{2+}]_i$, *Dictyostelium* amoebae were loaded with Calcium Green-1 dextran by electroporation and imaged during random motility on a glass substratum (Fig. 1A–J, see supplementary material, Movie 1). In this example, the cell displays 3 transients, during a 120 second period of observation. The average fluorescence intensity over the entire cell body area was measured in sequential images and used to calculate $[Ca^{2+}]_i$ as described in the Materials and methods section (Fig. 1K). A Ca^{2+} transient is defined here as a 15 to 25 nM increase in $[Ca^{2+}]_i$ from an average baseline value of ~120 nM (in this example it is 125 nM, Fig. 1K), with a duration of at least 2 s. In general, peak $[Ca^{2+}]_i$ levels ranged from ~142 to 671 nM with an average of 285 nM. On average, *Dictyostelium* cells generate 2.17±0.32 transients per minute (mean±s.e., n=29) however, the frequency of observed Ca^{2+} transients can vary from zero to more than six during an observation period of ~3 min. Calcium imaging experiments were repeated with cells moving towards a source of folate, as the chemoattractant (see supplementary material, Fig. 1 and Movie 2). Interestingly, these cells also displayed Ca^{2+} transients that lasted at least 2 s and peak $[Ca^{2+}]_i$ levels that ranged from ~144 to 621 nM with an average of 263 nM, which is comparable to randomly moving cells. Similarly, chemotaxing cells produced on average 1.78±0.24 transients per minute (mean±s.e., n=19, Fig. 3C) and the frequency of observed Ca^{2+} transients varied from zero to more than six in

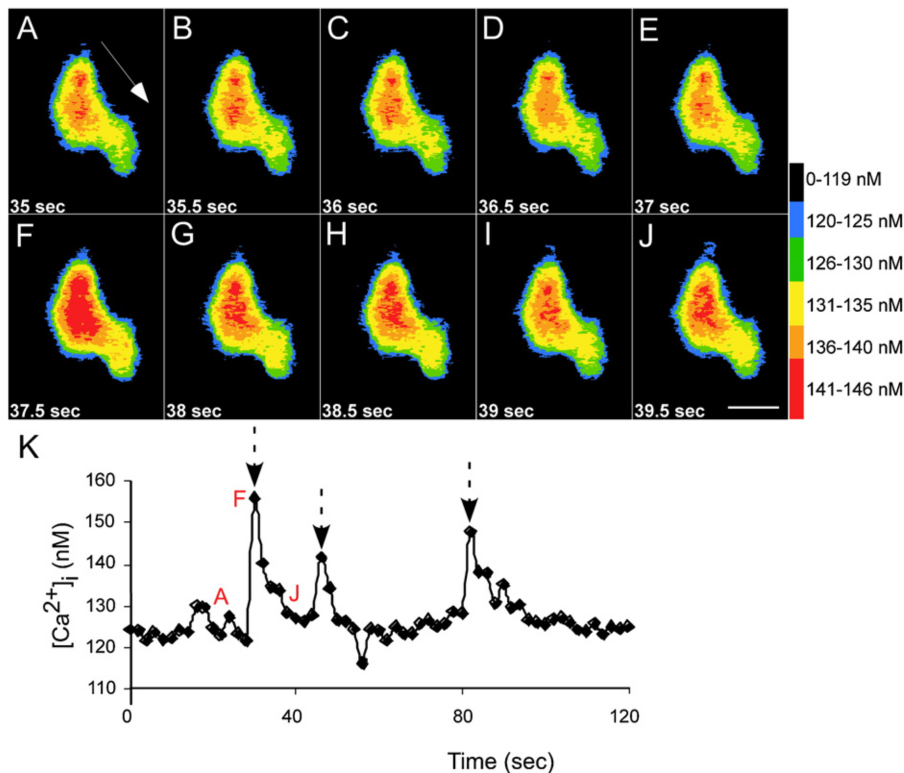

Fig. 1 – Ca^{2+} transients during *Dictyostelium* random movement. (A–J) A time series of fluorescence images obtained from a wild-type cell loaded with the calcium indicator moving in the general direction indicated (arrow). These images have been pseudocolored so that high $[Ca^{2+}]_i$ is represented by red and orange colors (136–146 nM), while areas of low $[Ca^{2+}]_i$ are represented by green and blue colors (120–130 nM). A transient (0.5 s) increase in $[Ca^{2+}]_i$ is shown by a brief increase in area of red (F), which returns to pre-transient values (G–J). Bar, 10 μm. (K) A plot of $[Ca^{2+}]_i$ versus time taken from average fluorescence intensity measurements from within the entire cell body. Three Ca^{2+} transients occur during a ~2 minute period of observation as indicated (arrows). The first transient in this plot corresponds to panels A–J.

~3 min. To determine whether Ca^{2+} transients are involved in retraction, we made plots of instantaneous retraction rate and $[Ca^{2+}]_i$ over time. We found that retractions almost always follow a Ca^{2+} transient with lag times ranging from ~10 to 15 s. In contrast, during periods when no transients are observed, the instantaneous retraction rate is very low (see supplementary material, Fig. 2). These observations suggest that calcium transients facilitate retraction at the rear.

Ca^{2+} transients are dependent on intracellular and extracellular calcium

To investigate the role of Ca^{2+} transients in *Dictyostelium* movement, intracellular calcium stores and extracellular calcium were depleted. Thapsigargin is known to inhibit the re-uptake of $[Ca^{2+}]_i$ to the endoplasmic reticulum and was used to deplete intracellular stores of calcium. Extracellular calcium was depleted using Ca^{2+} free medium or EGTA, and the effects of all treatments on the frequency of Ca^{2+} transients and retraction rate were recorded. Comparison of stacked cell outlines of an untreated wild-type cell displaying transients (Fig. 2A and C) and one treated with 10 μM thapsigargin (Fig. 2B and D), shows that inhibition of Ca^{2+} transients is associated with a significant decrease in the rate of retraction and forward movement (Fig. 2A and B). In this example, the transient frequency of the wild-type cell is ~3 per minute and the rate of retraction is ~3.06 μm/min. In contrast, the retraction rate decreases to ~1.03 μm/min when Ca^{2+} transients are inhibited following thapsigargin treatment. On average, the transient frequency of cells treated with thapsigargin is reduced to 0.59±0.17 per minute (mean±s.e., n=30) compared with a value of 2.17±0.32 per minute (mean±s.e., n=29, Fig. 2E) for control cells. The average rate of retraction is also decreased to ~1.60±0.22 μm/min (mean±s.e., n=30) in thapsigargin treated cells compared with ~2.31±0.21 μm/min (mean±s.e., n=29, Fig. 2F) in control cells. Another indication that a source of $[Ca^{2+}]_i$ is required for Ca^{2+} transients and that these are involved in retraction, comes from the Ca^{2+} imaging of *iplA*⁻ mutants, in which a gene similar to the IP_3 receptor of higher eukaryotes is disrupted. Therefore, without the IP_3-like receptor these cells cannot release internal stores of calcium. In *iplA*⁻ cells, the average frequency of Ca^{2+} transients is ~0.05±0.02 per minute (mean±s.e., n=30, Fig. 2E) and is associated with a very low retraction rate of ~0.90±0.10 μm/min (mean±s.e., n=30, Fig. 2F).

Depletion of extracellular calcium resulted in a significant decrease in the frequency of Ca^{2+} transients together with a reduced rate of retraction. In wild-type cells treated with Ca^{2+} free medium or 20 μM EGTA the average frequency of Ca^{2+} transients was reduced to ~0.40±0.14 per minute (mean±s.e., n=23) and ~0.09±0.04 per minute (mean±s.e., n=23, Fig. 2E), respectively.

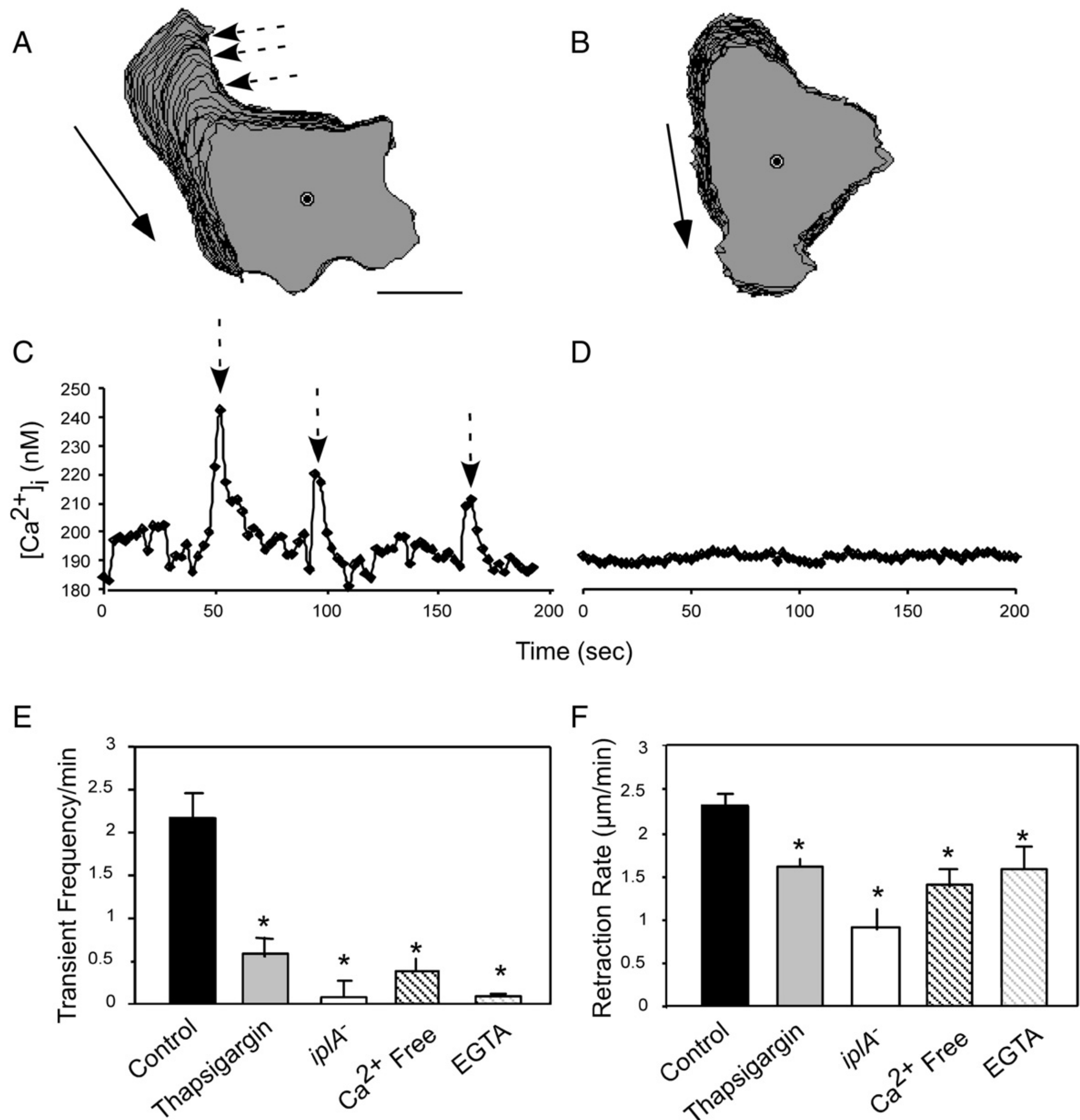

Fig. 2 – Effects of depleting intracellular or extracellular calcium on the frequency of Ca^{2+} transients and retraction rate in moving *Dictyostelium* cells. (A–B) Stacked cell outlines over a ~3 minute period of observation of a wild-type cell (A) and a thapsigargin treated cell, in which retraction is inhibited (B), moving in the general direction indicated (arrow). Bar, 10 μm. (C–D) Plots of $[Ca^{2+}]_i$ over time, for the cells shown in panels A and B, respectively. The control cell shows three Ca^{2+} transients as indicated (A and C, arrows) and is able to retract at the rear. Calcium transients are abolished in the treated cell (B and D) and retraction rate is decreased. (E–F) Summary histograms of the average frequency of transients per minute (E) and the associated retraction rate (F) for wild-type cells (black bar, *n*=29), cells treated with 10 μM thapsigargin (gray bar, *n*=30), *iplA*⁻ cells (open bar, *n*=30), cells placed in Ca^{2+} free medium (black striped bar, *n*=23), or treated with EGTA (gray striped bar, *n*=23). Controls for each treatment are not shown since all control values are not significantly different, using ANOVA. Asterisks indicate statistically significant differences compared with control cells for each treatment ($P<0.05$), using Student's *t*-test, assuming unequal variances.

Similarly, the rate of retraction is decreased to ~1.38±0.19 μm/min (mean±s.e., *n*=23) when cells are placed in Ca^{2+} free medium and to ~1.58±0.27 μm/min (mean±s.e., *n*=23, Fig. 2F) following treatment with 20 μM EGTA. Similar results have previously been found for keratocytes, chemotaxing neutrophils and *Dictyostelium*, where extracellular Ca^{2+} was found to be essential for increases in $[Ca^{2+}]_i$ [1,12,22]. Together our data demonstrate that Ca^{2+} transients depend on both intracellular and extracellular sources of Ca^{2+}, which in turn are important for retraction.

Blocking SACs inhibits Ca^{2+} transients in randomly moving and chemotaxing cells

The fact that Ca^{2+} transients and retraction were inhibited by the absence of intracellular or extracellular calcium raises the question of whether SACs are involved in the mechanochemical regulation of *Dictyostelium* movement. To address this question, we imaged $[Ca^{2+}]_i$ in randomly moving and chemotaxing *Dictyostelium* cells treated with gadolinium (Gd^{3+}),

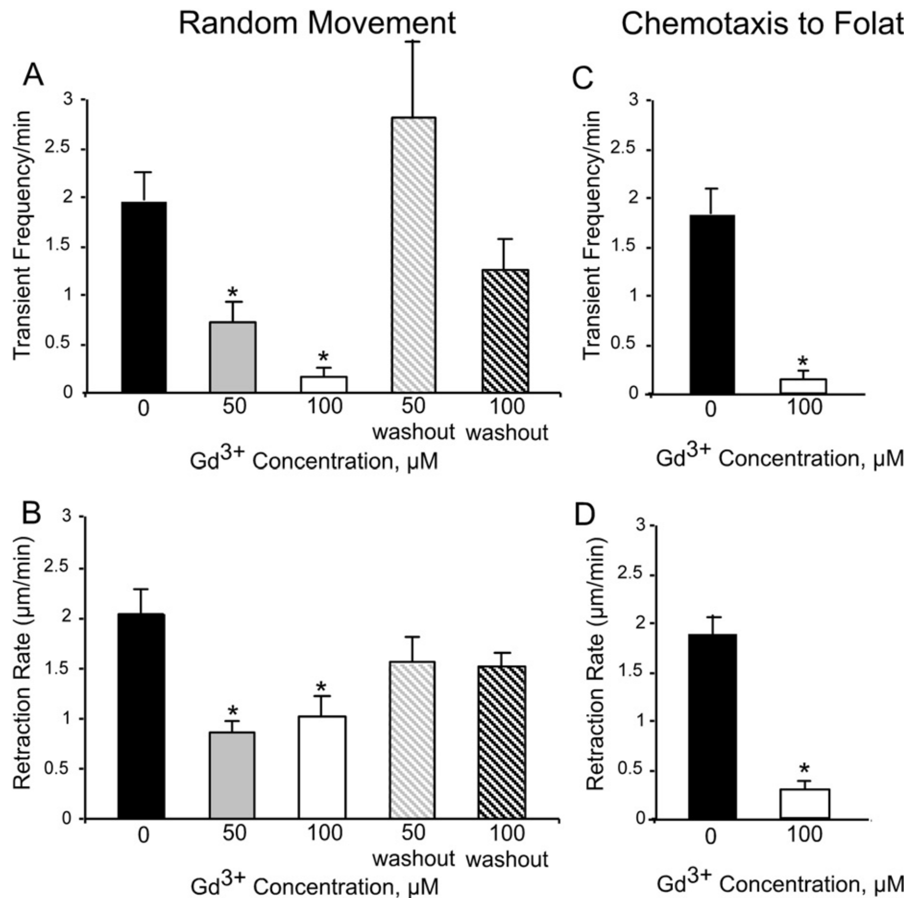

Fig. 3 – Effects of gadolinium on transient frequency and retraction rate in randomly moving and chemotaxing *Dictyostelium*. (A–B) Histograms of the average transients per minute and retraction rate, respectively, in control cells (black bar, *n*=28), cells treated with 50 μM Gd^{3+} (gray bar, *n*=28), 100 μM Gd^{3+} (open bar, *n*=28), following wash-out of 50 μM Gd^{3+} (gray striped bar, *n*=16), and following wash-out of 100 μM Gd^{3+} (black striped bar, *n*=16) during random movement. (C–D) Histograms of the average transients per minute and retraction rate, respectively, in control cells (black bar, *n*=18) and cells treated with 100 μM Gd^{3+} (open bar, *n*=18) during chemotaxis. Controls for each treatment are not shown since all control values are not significantly different, using ANOVA. Asterisks indicate statistically significant differences compared with control cells for each treatment ($P<0.05$), using Student's *t*-test, assuming unequal variances.

which is commonly used to block SACs [38]. The number of randomly moving cells exhibiting Ca^{2+} transients decreased with increasing concentrations of Gd^{3+} (Fig. 3A). Cells treated with 50 μM Gd^{3+} showed a significant decrease in the number of transients ~0.71±0.22 per minute (mean±s.e., *n*=28) compared with the transient frequency of ~1.96±0.28 per minute (mean ±s.e., *n*=28, Fig. 3A) for control cells. Addition of 100 μM Gd^{3+} produced a further dramatic decrease in the frequency of transients to ~0.14±0.11 per minute (mean±s.e., *n*=28, Fig. 3A). The effect of Gd^{3+} was reversible as shown by the increase in transient frequency to pretreatment values following wash-out of 50 μM and 100 μM Gd^{3+} (Fig. 3A). This dose-dependent, reversible inhibition of transient frequency by Gd^{3+} is consistent with the blockage of increasing numbers of SACs. These effects of Gd^{3+} treatment and wash-out were paralleled by decreases in retraction rate to ~0.85±0.12 μm/min (mean±s.e., *n*=28) and ~1.00±0.22 μm/min (mean±s.e., *n*=28) following addition of 50 μM and 100 μM Gd^{3+}, respectively, compared with the value of ~2.03±0.24 μm/min (mean±s.e., *n*=28, Fig. 3B) for control cells. Retraction rate increased to ~1.45±0.18 μm/min (mean± s.e., *n*=16, Fig. 3B) following wash-out of Gd^{3+}.

Treatment of chemotaxing cells with 100 μM Gd^{3+} reduced transient frequency to ~0.12±0.09 per minute (mean±s.e., *n*=18) compared with the control value of ~1.78±0.24 per minute (mean±s.e., *n*=18, Fig. 3C), which is identical to what was observed in randomly moving cells. Similarly, retraction rate is also decreased to ~0.31±0.07 μm/min (mean±s.e., *n*=18) compared with the value for control chemotaxing cells of ~1.92±0.18 μm/min (mean±s.e., *n*=18, Fig. 3D). These results provide a strong indication that Ca^{2+} transients result from the activation of SACs in both randomly moving and chemotaxing *Dictyostelium*.

Cell stretching can induce a Ca^{2+} transient

To test whether a mechanical stimulus can trigger a Ca^{2+} transient, cells were stretched without direct physical contact. *Dictyostelium* cells pre-loaded with Ca^{2+} indicator were plated

onto a 10% gelatin substratum. Two micropipettes were used to apply the indirect stretch; one micropipette (white asterisk in Fig. 4A) was held stationary on one side of the cell, while the other (white triangle in Fig. 4A) was used to deform the substratum by pulling in the opposite direction for ~3 s (Fig. 4A–C). In 30 out of 34 cells, a Ca^{2+} transient occurred within 1 s after stretch was applied, whose duration (~2 to 3 s) and magnitude were similar to Ca^{2+} transients in non-stretched cells (Fig. 4D). To control for changes in cell shape and thickness that may occur during stretching, this experiment was performed with cells pre-loaded with a Ca^{2+} insensitive Texas Red-conjugated dextran. No change in fluorescence intensity was observed when these cells were stretched (0 out of 18 cells) confirming that the changes in fluorescent intensity of the Ca^{2+} indicator were due to transient increases in $[Ca^{2+}]_i$, not fluctuations in cell body thickness.

When 100 μM Gd^{3+} was added to the cells prior to stretching, the majority (15 out of 17) of cells showed no stretch-induced increase in fluorescence intensity (Fig. 4E). Gadolinium treatment was also reversible as shown by the stretch-induced increase in fluorescence intensity that was observed following the wash-out out of Gd^{3+} (6 out of 9 cells). Therefore, these data confirm that Ca^{2+} transients result directly from a mechanical stimulus, most likely through the activation of SACs.

The frequency of Ca^{2+} transients is proportional to substratum adhesiveness

It has been suggested that when cells are plated on a stickier substratum, they may be subjected to larger, more frequent mechanical stimuli as they attempt to move, because cell detachment will require more force to break stronger adhesions [1]. To investigate whether changes in cell adhesiveness affect the frequency of Ca^{2+} transients, we first determined the relative adhesiveness of *Dictyostelium* cells attached to various concentrations of poly-L-lysine coated coverslips by performing single cell shear flow experiments. The number of cells detached by shear flow decreased as the concentration of poly-L-lysine increased, indicating that these cells are more tightly adhered to the substratum compared with controls (Fig. 5A). In addition, the cross-sectional area of cells on 0.0025 mg/ml poly-L-lysine was 54% larger than cells attached to uncoated glass (n=20, Fig. 5B and C). Calcium imaging experiments were performed on glass coverslips coated with 0.0025 mg/ml poly-L-lysine since this led to a significant increase in adhesiveness compared to uncoated glass. Transient frequency increased by ~3 times (5.61±0.41 per minute, mean±s.e., n=20) compared with control cells moving on uncoated glass (2.03±0.38 per minute, mean±s.e., n=20, Fig. 5D). However, the duration of

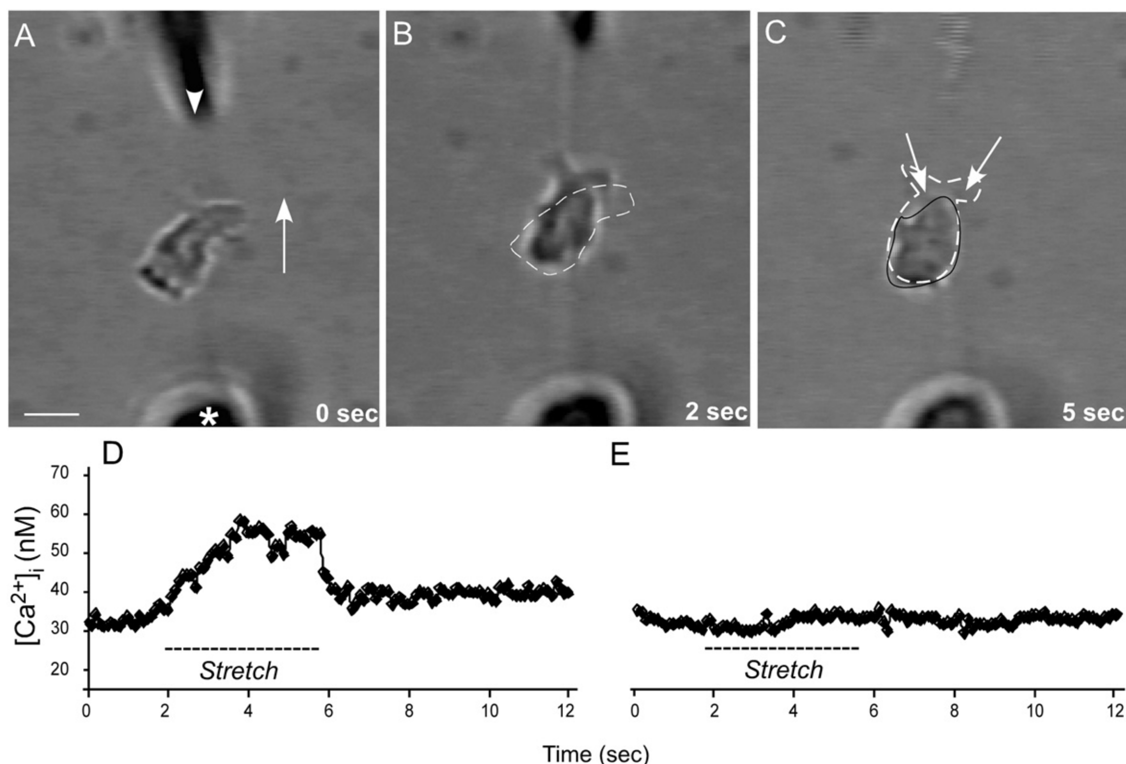

Fig. 4 – Induction of a Ca^{2+} transient by indirect cell stretching. (A) A micropipette (white asterisk) is held stationary on one side of the cell, while another smaller micropipette (white triangle) is pulled in the direction indicated (arrow) to deform the substratum and stretch the cell for ~3 s (A–C). The cell outline in (B) is traced from the image of the unstretched cell in (A) to show the amount of cell deformation. The cell outline in (C) is traced from the stretched cell in (B) to show where retraction occurs (arrows). Bar, 10 μm. (D–E) Plots of changes in $[Ca^{2+}]_i$ over time prior, during, and after stretching. In (D) an increase in $[Ca^{2+}]_i$ is seen a few seconds after the stretch occurs, remains high until the substratum is released, and then $[Ca^{2+}]_i$ returns to pre-stretch values. In (E) treatment with 100 μM Gd^{3+} prior to stretching, abolishes the stretch-induced increase in $[Ca^{2+}]_i$.

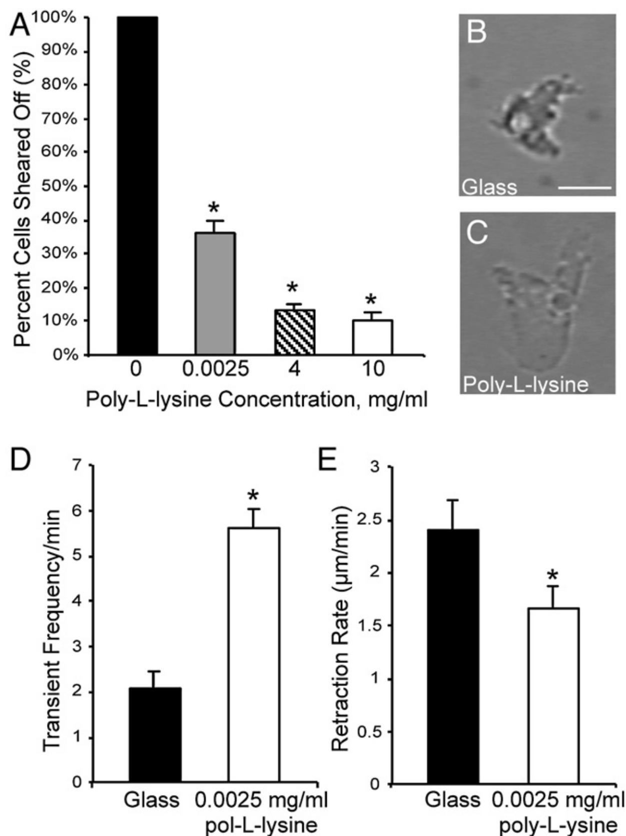

Fig. 5 – A more adhesive surface increases the frequency of Ca^{2+} transients. (A) Histogram showing the percentage of cells detached from the substratum by fluid shear flow, while attached to uncoated glass (black bar), 0.0025 mg/ml poly-L-lysine (gray bar), 4 mg/ml poly-L-lysine (striped bar) and 10 mg/ml poly-L-lysine (open bar). The 0.0025 mg/ml poly-L-lysine coating significantly decreased the number of cells detached by fluid shear and was used for Ca^{2+} imaging experiments. (B) A phase contrast image of a cell attached to uncoated glass and (C) a cell attached to 0.0025 mg/ml poly-L-lysine showing a 54% increase in spread area compared with (B). Bar, 10 μm. (D) Histogram of the average transients per minute and (E) retraction rate for cells moving on uncoated glass (black bar) and 0.0025 mg/ml poly-L-lysine (open bar). Asterisks in panel A indicate statistically significant differences compared with untreated cells ($P<0.05$), using ANOVA, assuming unequal variances. Asterisks in panels D and E indicate statistically significant differences compared with untreated cells ($P<0.05$), using Student's *t*-test, assuming unequal variances.

the Ca^{2+} transients in these cells was 2.22±0.11 per minute (mean±s.e., $n=20$), which was the same as control cells (2.20± 0.14 per minute, mean±s.e., $n=20$, data not shown). The retraction rate of cells on poly-L-lysine was reduced to ~165± 0.22 μm/min (mean±s.e., $n=20$) compared with the value for control cells of ~2.39±0.27 μm/min (mean±s.e., $n=20$, Fig. 5E). These data support the idea that the increased number of transients observed on more adhesive surfaces is due to increased SAC activity.

Discussion

Using high resolution Ca^{2+} imaging, we provide evidence for the activity of SACs in regulating the movement of randomly moving and chemotaxing *Dictyostelium* cells. *Dictyostelium* exhibit small, brief (~2 s) Ca^{2+} transients that are important for facilitating retraction and maintaining rapid movement. We show that Ca^{2+} transients are dependent on intracellular and extracellular calcium. The involvement of SACs is indicated by the inhibition of Ca^{2+} transients in a dose-dependent, reversible manner by the SAC blocker, gadolinium. Further support for this is given by the occurrence of Ca^{2+} transients in response to cell stretching and the increase in transient frequency seen in cells attached to a more adhesive substratum.

The similarity in the size and frequency of Ca^{2+} transients in *Dictyostelium* cells compared with fish epithelial keratocytes suggests that they result from the activation of SACs. For example, in *Dictyostelium* the transient frequency (2.17± 0.32 per minute, mean±s.e., $n=29$, Fig. 2E) is comparable to that observed in rapidly moving keratocytes (0.62 to 1.69 transients per minute) [1]. Furthermore, the $[Ca^{2+}]_i$ increase in *Dictyostelium* ranged from ~142 to 671 nM, with a baseline value of ~120 nM, while in keratocytes the size of Ca^{2+} transients ranged from ~219 to 683 nM, with a baseline value of ~138 nM.

Since the Ca^{2+} transients we observed in chemotaxing *Dictyostelium* were of the same duration, frequency, and magnitude as in randomly moving cells, we believe that these are distinct from the elevations in $[Ca^{2+}]_i$ previously observed during chemotaxis [21–28]. For example, the duration of Ca^{2+} transients displayed by chemotaxing cells in our study was ~2 s compared with ~20 s in fura-2 loaded *Dictyostelium* cells responding to cAMP [21] and up to ~40 s in *Dictyostelium* expressing aequorin [22]. Furthermore, treatment of chemotaxing cells with Gd^{3+} inhibited SAC mediated Ca^{2+} transients, yet the protruding edge could still change direction when the source of chemoattractant was repositioned, similar to previous findings [21,22,29,30]. Therefore, we suggest that two separate Ca^{2+} signaling mechanisms operate in *Dictyostelium*, one that is associated with the chemotactic response, while the other results from the activation of SACs. It is possible that these two signaling pathways are encoded by the differences in frequency and magnitude of the rises in $[Ca^{2+}]_i$, as previously found for spiral neurons and β lymphocytes [39,40].

The fact that Ca^{2+} transients in *Dictyostelium* can be induced by a mechanical stimulus, and can be inhibited by Gd^{3+}, is in common with many other mechano-sensitive cell types in which SACs are known to function. Endothelial cells, for instance, respond to shear stress [41] and direct force [42] by displaying Ca^{2+} transients that can be blocked by Gd^{3+}. Calcium transients also occur in embryonic heart cells [31] and vascular smooth muscle cells [43] when they are stretched. Another feature that *Dictyostelium* shares with other mechanosensitive cell types is the dependence of Ca^{2+} transients on external and internal sources of Ca^{2+}, consistent with calcium-induced calcium release [1,31]. In relation to this, both external and internal sources of calcium were shown to be required for the mechanosensory response of *Dictyostelium* cells to shear flow [32].

The role of Ca^{2+} in the regulation of *Dictyostelium* movement is apparent from the decreased rate of retraction when Ca^{2+} transients are inhibited. The inability of cells to retract in the absence of Ca^{2+} is in stark contrast to protrusion at the leading edge (data not shown), which can occur without Ca^{2+} or retraction, clearly implicating the dependence of retraction on Ca^{2+} transients. In support of this, it has been previously observed that EGTA inhibits retraction during *Dictyostelium* chemotaxis, but protrusion toward the cAMP gradient is unaffected [30,44]. In addition, other ions, such as magnesium, sodium, and potassium, were previously tested in *Dictyostelium* cells exposed to shear flow, however, only Ca^{2+} was found to be specifically required for cell movement [32]. The dependence of retraction on Ca^{2+} transients in *Dictyostelium* cells was also observed in our study when cells were tightly adhered to poly-L-lysine surfaces. These cells had difficulty in retracting but displayed many more Ca^{2+} transients, as previously shown for fibroblastic-like keratocytes and neutrophils plated on more adhesive surfaces [1,12].

We propose that in *Dictyostelium* cells, SACs provide a positive feedback mechanism that is important for maintaining rapid movement. Interestingly, neutrophils have been found to display multiple Ca^{2+} transients that require a source of intracellular and extracellular calcium [12]. In addition, multiple Ca^{2+} transients are necessary for retraction on poly-L-lysine coated surfaces. These findings, together with our own, suggest that mechano-sensing may be a general mechanism for maintaining rapid movement in fast moving cell types.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2008.02.001.

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