Dynamic instability of the intracellular pressure drives bleb-based motility

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Summary

We have demonstrated that the two- and three-dimensional motility of the human pathogenic parasite Entamoeba histolytica (Eh) depends on sustained instability of the intracellular hydrostatic pressure. This instability drives the cyclic generation and healing of membrane blebs, with typical protrusion velocities of 10–20 μm/second over a few hundred milliseconds and healing times of 10 seconds. The use of a novel micro-electroporation method to control the intracellular pressure enabled us to develop a qualitative model with three parameters: the rate of the myosin-driven internal pressure increase; the critical disjunction stress of membrane–cytoskeleton bonds; and the turnover time of the F-actin cortex. Although blebs occur randomly in space and irregularly in time, they can be forced to occur with a defined periodicity in confined geometries, thus confirming our model. Given the highly efficient bleb-based motility of Eh in vitro and in vivo, Eh cells represent a unique model for studying the physical and biological aspects of amoeboid versus mesenchymal motility in two- and three-dimensional environments.

Key words: Blebs, Cytoskeleton, Motility

Introduction

On the basis of extensive investigations in different cell types and in various contexts, two distinct modes of cell motility (mesenchymal and amoeboid motility) have been proposed (Friedl and Wolf, 2003; Sahai, 2007). These modes differ in many respects: cell morphology, the organization and dynamics of cell–substrate adhesion, the localization and activity of the actomyosin contractile machinery, and the distribution of the forces exerted on two-dimensional (2D) and three-dimensional (3D) substrates (Bray, 2001; Mierke et al., 2008). It has also been reported that some cells can undergo a transition between these modes (Friedl and Wolf, 2003; Friedl, 2004), and that the motility mode strongly influences migration efficiency (Carragher et al., 2006). It is therefore likely that cells optimize their motility by selecting distinct strategies, probably as a function of the state of their molecular ‘machinery’ and of their environment. This optimization could be relevant for metastatic invasion, leukocyte migration in lymph nodes or target tissues, and cell movements during embryonic development, where cells successively encounter different 3D contexts (Hugues et al., 2004; Raz and Reichman-Fried, 2006). For instance, during immune surveillance and the inflammatory response, leukocytes move through environments such as blood, mucus, epithelium and the lymphatic circulatory system (Hugues et al., 2004). We studied Entamoeba histolytica (Eh) cells, the causative agent of amoebiasis (dysentery) (Stanley, 2003). This pathogen passes through various environments (intestinal tissue layers, the portal vein and the liver microcirculation) as it invades and ultimately destroys the host intestine and produces liver abscesses (Stanley, 2001). Given this context, the objective of the present work was to elucidate the physical basis of Eh cell motility and elaborate a model of amoeboid behavior, with special focus on the role of the internal hydrostatic pressure.

In previous work using in vivo two-photon imaging, we observed that Eh migration in the liver during the infectious process is accompanied by very intense, cyclic production of spherical protrusions of the plasma membrane (Coudrier et al., 2005). In vitro, these protrusions can either retract or become stable and thus sustain the exploration of the local environment by the cell over several hours (Coudrier et al., 2005). In the absence of externally guided cell motility, there is no correlation between the directions of the protrusions, and efficient, random exploration of the substrate is observed. However, appropriate chemotactic gradients do orient and stabilize protrusions in particular directions (Blazquez et al., 2006; Zaki et al., 2006). It is known that myosin II is essential for Eh motility, both in vitro (Arhets et al., 1998) and in vivo (Coudrier et al., 2005). In the present work, we demonstrated that the Eh cell protrusions are blebs (Keller et al., 2002; Fackler and Grosse, 2008; Charras and Paluch, 2008). By studying the mechanism underlying their production, we further showed that the blebs directly actuate cell motility in a physiological context. Our observations suggest that Eh cells could serve as a valuable prototype for studying amoeboid motility in general.

Results

Spherical protrusions produced in 3D, 2D and liquid environments

Infection by Eh requires efficient motility through environments that vary in terms of their geometrical, mechanical and biochemical properties. In an initial report (Coudrier et al., 2005), we observed a strong correlation between the efficiency of the infectious process (as measured experimentally in animal models), in vivo cell motility and the active generation of cell protrusions. Here, we show that these protrusions are spherical and thus very distinct from the...
lamellipodia and filopodia involved in mesenchymal migration. Protrusion generation is similarly active in both 2D and 3D, as shown in the 3D liver parenchyma (supplementary material Movie 1) and on bare glass (supplementary material Movie 2). This suggests that the microenvironment in contact with the cell has a limited effect on protrusive activity. To further assess the role of cell–substrate contacts, amoebae were loaded at the interface between two fluids of differing density (a Percoll solution and culture medium). In this fluid environment, cells continue to actively produce round protrusions (supplementary material Movie 3). Similarly, when cells simply sediment through culture medium, active protrusions are still observed and persist once contact with the substrate is made (supplementary material Movie 4). These results clearly indicate that the protrusive activity is intrinsic to amoeba cells and is not induced by cell–substrate adhesion.

**Relationship between motility and asymmetric and dynamic contacts**

Because *Eh* cells do not have specialized organelles for moving in liquids (e.g. flagella or cilia), they can only achieve net motion relative to their environment by interacting with a solid substrate. Although no net motion is observed in floating or sedimenting cells (supplementary material Movies 3 and 4), the latter start to move as soon as they contact with the substrate, which suggests an ability to transfer momentum.

To further investigate cell–substrate contact and its role in protrusive activity, we combined conventional phase-contrast imaging with the observation of adhesion patterns using reflection interference contrast microscopy (RICM) (Fig. 1; supplementary material Movie 5 and Fig. S3). Strikingly, the shape of *Eh* cells is quite distinct from their contacting surface; this is in contrast to fibroblasts and most adherent cells, for which adhesion strongly dictates the cell profile. Despite extended regions of tight contact (revealed by continuous zones of dark RICM signal), the production of protrusions leads to discontinuous contact zones away from the tight contact area (Fig. 1B). This observation indicates that protrusions are produced as elevated structures that can contact the substrate at a remote site. This is in agreement with observations of 3D protrusions in a liquid environment (supplementary material Movie 3) and with previous electron microscopy pictures (Gonzalez-Robles and Martinez-Palomo, 1983) showing that protrusions are triggered all around the cell surface (which has a typical size of 20 μm in all three dimensions).

The initial, discontinuous contacts then either nucleate and grow into larger surfaces (which eventually bridge with the main zone (supplementary material Fig. S3) or disappear if the protrusions retract. Hence, protrusion dynamics is coupled to dynamic spreading of adhesive contacts. The cell boundary seen in phase-contrast microscopy always extends beyond the dark RICM zone, indicating an ‘overhang’ type of geometry (sketched in Fig. 1D). Interestingly, this overhang is much greater in protruding regions than in retracting regions. This clear asymmetry can be interpreted as a contact angle difference, which is probably due to the plasma membrane and the associated cortex being peeled away in retracting zones.

For further investigation of the protrusion mechanism of *Eh* cells, it is important to note that cell–glass adhesion forces (which could in principle lead to an extensive contact area and a rather flat morphology) are dominated by the contraction-induced and elastic forces inside the cortex. These forces probably drive cell deformation in 2D and in 3D, as mentioned above. Nevertheless, the adhesion forces are strong enough to produce effective contacts and the momentum transfer required for the observed motility. In principle, the adhesive interactions are not specific on the molecular level, despite the surface expression of specific adhesion receptors (Coudrier et al., 2005). Indeed, similar motile behaviors have been observed on substrates with greater or lower hydrophobicity than bare glass (supplementary material Figs S1 and S2). These observations indicate that excessive adhesion could inhibit the production of *Eh* cell protrusions, as reported elsewhere (Friedl et al., 2001).

**Plasma membrane abruptly detaches from the cytoskeleton**

We next focused on the physical mechanism underlying protrusive activity by *Eh* cells moving on a glass surface. The results presented in the preceding section indicated that adhesion does not directly interfere with the intracellular processes that lead to protrusion production, which can be considered as operating independently of contact with the substrate. Phase-contrast video microscopy observations revealed deformations of the cell boundary on two distinct time scales: slow (10–30 seconds) overall changes (supplementary material Movie 6) and fast (0.1–5 seconds) local changes (supplementary material Movie 7). The slow, overall deformations exhibit a constant phase-contrast pattern, suggesting a stationary cell cortex structure. By contrast, fast deformations are protrusions that correspond locally to major changes in the phase-contrast signal; this indicates that the cortical structure is strongly modified. Interestingly, myosin inhibition produces conditions under which fast deformations are blocked and slow deformations...
this observation agrees with the fact that actin polymerization fronts are typically much slower (around 0.1 second) over which the structure of the actin cortex does not change. This observation suggests that the plasma membrane (supplementary material Movie 7). This observation strongly suggests that Eh cell protrusions are bona fide blebs, as indicated by their sphericity, the absence of submembrane structure and the hyaline-like appearance of the inner face. Protrusion production via membrane disjunction is analogous to the formation of apoptotic blebs by proteolytic cleavage of cytoskeleton–membrane links (Mills et al., 1998; Mills et al., 1999). Unlike necrotic blebs, Eh blebs are highly dynamic in nature; rapid expansion is followed by cytosol invasion and gradual association between the membrane and a new cortex. These events occur in a cyclic fashion.

On the molecular level, the actin cortex (seen in parallel time-lapse videos of phase contrast and F-actin fluorescence; Fig. 3) shows fast disjunction, with no change in the fluorescence of the initial structure. However, 4 seconds later, the fluorescence decays and the rim of the bleb becomes fluorescent (indicating the accumulation of F-actin).

The time courses of F-actin dynamics along the initial cortex and along the bleb rim (Fig. 4A) are shown as a series of kymographs (Fig. 4B,C) and are integrated over time (Fig. 4D,E). These semiquantitative data indicate that the typical times for depolymerization and distal repolymerization are similar (on the order of 5–10 seconds). This is in agreement with the notion that the F-actin cortex turns over constantly, with the balance between depolymerization and distal repolymerization rates at the plasma membrane possibly leading to a steady-state cortex in the absence of disruption (Fig. 4F) and simultaneous collapse/repolymerization in the event of disjunction.

The preceding observations are schematically summarized in Fig. 5A. Blebs drive the net motion of the cells via three sematic transitions occurring on different time-scales. From the initial state (a), the plasma membrane detaches from the cytoskeleton (typically within 0.1 seconds). From the second state (b), the cytoskeleton gradually depolymerizes and repolymerizes under the bleb membrane (c). Within 5–10 seconds, the ‘old’ cortex has vanished.
and the new cortex has fully matured (d). In this situation, contraction forces can deform the cell and eventually lead to a new disjunction.

Analysis of forces and proposal of a cyclic model

Most investigations of cell motility have focused on situations in which actin polymerization has a dominant role. In these situations the membrane has an ancillary role, which mainly consists of templating actin polymerization while remaining attached to the cortex. Here, given the disjunction, one must reconsider the forces exerted on the linkers by the membrane and the cytoskeleton (Fig. 5B) and their relationship with contractile activity.

The plasma membrane is subjected (Fig. 5B, blue arrows) to hydrostatic pressure from the outside fluid ($\pi_{\text{ext}}$) and the internal medium ($\pi_{\text{int}}$). It is also subjected to forces exerted by individual links, the surface density of which yields an effective pressure ($\pi_{\text{links}}$). Given the mechanical equilibrium of the links (i.e. as long as they hold firm), this pressure also equals the contractile pressure exerted upon the links by the cortex ($\pi_c$). The latter is produced by cortical contractile tension ($\gamma_c$) and curvature.

After membrane disjunction (Fig. 5B'), a different set of forces must be considered. The linker pressure ($\pi_{\text{links}}$) on the membrane and its opposing cytoskeletal counterpart disappear. This change corresponds to effective outward pressure of the same magnitude on the membrane, together with effective inward pressure on the cytoskeleton. We also consider that the outward hydrodynamic flux through the cortex leads to viscous resistance (corresponding to outward pressure on the cortex, $\pi_{\text{hydro}}$). Membrane disjunction implies the local disruption of a large number of non-covalent bonds (a few square micrometers), characterized at the individual level by finite on/off rates. When stressed, the off rate increases exponentially with the stress (Bell, 1978) and an ‘avalanche effect’ is expected above a critical pressure $\pi_{\text{links}}^*$ which will directly depend on the density and binding energy of the bond.

As discussed below, variations in the osmotic pressure on the membrane have been neglected because no water influx is observed during protrusion production and resorption. In principle, two additional forces should be considered. First, the membrane is subjected to surface tension that could (along with curvature) slow down or stop bleb expansion. This was suggested by our observation that blebs often exhibit larger front velocities in concave regions (data not shown). This aspect has been considered in a theoretical publication (Brugues et al., 2010) but cannot be easily taken into account in the experiments presented herein. Second, cell–substrate contact generates surface forces at the membrane and primarily prevents local bleb formation. These forces do not impede blebbing in contact-free zones.

On the basis of the above mechanical analysis, we propose a cyclic model (Fig. 5C) in which cortical contraction builds up the inside pressure difference across the plasma membrane to the point where it exceeds the critical disjunction pressure ($\pi_{\text{links}}^*$) that the linker distribution can withstand. When the membrane detaches, the new cortex has fully matured (d). In this situation, contraction forces can deform the cell and eventually lead to a new disjunction.
Concomitantly, the excess internal pressure $\pi_{\text{int}}$ relaxes locally. Next, the local pressure drop of $\pi_{\text{int}}$ propagates throughout the cell and leads to small amplitude cytosolic motions. As the bleb stabilizes through actin polymerization and contraction, the pressure rises again until the next disjunction transition. Hence, the resulting cycle is essentially characterized by instability of the intracellular pressure. According to our model, the time between bleb formation events is determined by the rate of stress accumulation through contraction and by the tensile strength and density of the linkers. The resulting instability will be periodic if these biological control parameters remain constant. We have investigated two key predictions of this model: the respective roles of contractility and pressure, and the periodicity.

Respective roles of pressure effects and contraction

We first determined to what extent bleb formation was sensitive to external perturbations of the inside–outside pressure difference. Using micropipette aspiration (supplementary material Movie 9), we observed that the membrane region exposed to low external pressure (~500 Pa) exhibited active blebbing, whereas the opposite end of the cell stopped making blebs. This strongly suggests that the inside–outside pressure difference is a key parameter in triggering membrane disjunction. In agreement with several previous reports (Arhets et al., 1998; Paluch et al., 2005; Charras et al., 2005), the inhibition of actomyosin contraction with ML-7 and Y27632 was found to block bleb formation (supplementary material Fig. S4B,D). To elucidate the respective roles of contraction and the pressure increase (both of which appear to be required for blebbing), we designed an experiment in which the inside–outside pressure difference was cancelled while the contractile machinery remained intact.

Electroporation is known to transiently permeate the cell membrane and can therefore be used in conjunction with a micropipette to cancel the hydrostatic pressure difference at will on the subcellular scale and with good temporal resolution. $Eh$ cells were held in tight contact with the micropipette tip. The electrical resistance was in the order of 25–30 MΩ. In this situation, the cells produced blebs that immediately disappeared at the onset of electrical pulse trains (see supplementary material Movie 10). As long as pulses were maintained, the cells shrank (Fig. 6 and supplementary material Fig. S5). As soon as the pulses ceased, blebbing resumed within a few seconds. In addition, electroporation-induced shrinkage was absent when either of two actomyosin inhibitors (ML-7 and Y27632) were added to the medium (see supplementary material Movie 11). Taken together, these results indicate that the contraction machinery is not affected by electroporation but cannot alone induce blebs when the hydrostatic pressure difference is cancelled. Meanwhile, the fact that a normally membrane-impermeant dye (propidium iodide) penetrated into the cell suggests that pores are formed. Interestingly, dye influx systematically occurred through a single permeation point (see supplementary material Movie 12) located outside of the micropipette. Indeed, the effect of electroporation, as revealed by propidium iodide experiments (see supplementary material Movie 12), is to create pores at a single point, through which endoplasmic material immediately leaks out and through which the dye enters a few seconds later, before it equilibrates throughout the cell (supplementary material Fig. 12). The effect of pores is to cancel, at least locally, the contraction-induced pressure difference. The fact that the electroporation-induced local pressure relaxation is immediately followed by a global effect on bleb inhibition strongly suggests that membrane disruption and blebbing result from a direct pressure on the membrane and from linker rupture, and also that the local pressure drop immediately propagates throughout the cell.

Blebbing can be forced into a periodic mode

Assuming that the rate of contraction and linker strength and density are constant and uniform, our model predicts that blebs...
Fig. 6. Electroporation stops blebbing but not contraction. Eh cells (n=5) were held by a micropipette that delivers trains of electrical pulses (see Materials and Methods). Upper panel shows the change in the inside-outside pressure difference over time. Letters a–d indicate the time-points at which the images below were taken. (a) When no pulse is applied (t=0 seconds), the cell blebs as it does under normal conditions (Fig. 2 and supplementary material Movie 2). (b,c) As soon as electrical pulses are applied (0<t<10 seconds), blebbing stops and the cell shrinks. (d) Blebbing resumes as soon as electrical pulses cease. Arrows point to blebs.

will be produced periodically. In cell-on-glass, blebs occurred one by one. There was neither time overlap (supplementary material Fig. S6) nor a well-defined period. Indeed, the blebs were heterogeneous in terms of duration and size (supplementary material Fig. S7) and the fluctuations over time were so large that no statistically meaningful period could be distinguished. According to our model, this temporal irregularity suggests that the control parameters are not constant over time and/or are not uniform in space. The simplest explanation is that the local pressure on the linkers (πc) depends on the local membrane curvature, i.e. \( \pi_c = 2 \frac{E_h}{R} \) (Fig. 5B). Curvature is indeed strongly non-uniform in Eh cells (Fig. 2). Interestingly, when the cell is forced to adopt a more regular shape in micropipette aspiration experiments, a clear periodicity appears (Fig. 7). The period (8±2 seconds, n=46 blebs) matches that of the polymerization/depolymerization measured above. This clearly shows that blebbing frequency fluctuations are dominated by geometric effects and that the control parameters are constant. Our micropipette experiments have been extensively described in a detailed theoretical analysis of various dynamic modes for cortical instability (Brugues et al., 2010).

Discussion

Bleb formation is observed in apoptotic cells (Mills et al., 1998; Sebbagh et al., 2005; Leverrier and Ridley, 2001; Sebbagh et al., 2001), migrating cells (Blaser et al., 2006; Yoshida and Soldati, 2006), non-migrating cells (Keller and Eggli, 1998; Gutjahr et al., 2005; Charras et al., 2005; Paluch et al., 2005) and in cells before they fully adhere to a flat substrate (Norman et al., 2010). Nevertheless, the only previously reported situation in which motility is entirely driven by blebs is primordial germ cell (PGC) migration in zebrafish (Blaser et al., 2006; Raz and Reichenman-Fried, 2006). The key message of the present work is that Eh cell migration (as with PGCs) is solely driven by blebbing, at least under the circumstances investigated here. However, unlike zebrafish PGCs (which are immobile in vitro), Eh cells exhibit bleb-based motility both in vitro and in vivo and therefore appear to constitute a unique model for investigating the mechanism of bleb formation and its relationship with motility in a relatively normal physiological context. We took advantage of this unique feature and used RICM to qualitatively elucidate the relationship between bleb formation and retraction, cell–substrate contact dynamics and net cell motion.

The present data and our previous observations on in vivo infection-related Eh cell motility (Coudrier et al., 2005) together strongly suggest that blebbing-based cell motion is efficient compared to the well-known mesenchymal migration mode. We observed extremely high bleb front velocities: up to two orders of magnitude higher than the average speed for mesenchymal cells (Bray, 2001). Nevertheless, these velocities are transient and do not directly reflect average motility speed. Given that the Eh cell blebs are produce isotropically and lead to random motion (supplementary material Movie 13), the efficiency of motility is better reflected by the mean square displacement (supplementary material Fig. S8), which is typically in the order of 0.1–10 \( \mu m^2/second \). One of the most remarkable features of Eh bleb-based motility is the very high blebbing frequency, which makes it possible for the cell to change direction several times per minute.

Comments on the blebbing model

In the initial state in the cycle (Fig. 5Aa), the cell is in a static situation in which the increasing stress does not translate into significant deformation. The internal pressure is uniform and one would expect to have a uniform distribution of the disjunction probability if the linker distribution and curvature are uniform. However, it should be borne in mind that linkers at the membrane cytoskeleton interface can be very dynamic (Coscoy et al., 2002); hence, density fluctuations can trigger local changes in the disruption pressure \( \pi_{link} \). In a few reports, collapse of the cortex is seen as the first event. However, this situation has only been seen...
Biochemical control of the dynamic instability

Our model is founded on three key parameters: (1) the rate at which pressure (due to actomyosin contraction and curvature) builds up, (2) the critical disjunction pressure (resisted by membrane–cytoskeleton linkers) and (3) the turnover rate of the actin cortex. The rate of pressure build-up depends on both the level of mechano-enzymatic activity and the cortex density. Therefore, in the presence of a structurally stable cortex, the time needed to reach the disjunction pressure is expected to vary inversely with the reciprocal of the contraction rate. However, the actin cortex is far from being a static structure (Pantaloni et al., 2001; Pollard and Borisy, 2003) and its turnover will certainly interfere with the pressure build-up. Indeed, each time a microfilament is lost through turnover, the accumulated stress disappears and is subtracted from the network stress. One therefore expects the tensile stress to reach a steady-state level that is modulated by the contraction rate and the actin turnover rate. This steady state will not be reached if disjunction occurs faster than stress saturation. In this context, the kinetic competition between contraction and polymerization rates (along with the critical disjunction pressure) should lead to different dynamic modes, with or without disjunction (supplementary material Fig. S11). Although a quantitative, theoretical investigation of these dynamic modes has been carried out (Brugues et al., 2010), the qualitative model developed here should help us to understand how blebbing is biochemically controlled.

Efficiency of motility and external mechanical control

Our RICM experiments showed that *E. histolytica* cells even adhere to a bare glass slide, probably due to the relatively nonspecific nature of the molecular adhesive machinery of the parasite. If blebs are to produce motion in a viscoelastic environment, the sum of the net momentum successively transmitted to the substrate (first during bleb emission and then during the maturation/contraction steps) must be non-zero. During bleb emission, the opposing forces exerted on the bleb arise from either viscous drag in the liquid phase or from friction between the membrane and the substrate. Both types of forces are effectively counteracted by adhesion of the rest of the cell to the substrate, as suggested by the fact that the centre of mass does move forward during that phase. As the bleb matures, cortex polymerization and the formation of links with adhesion receptors result in a rather uniform solid-like friction. Upon further contraction, the centre of mass is therefore not expected to move. Qualitatively, the liquid-like structure of the bleb breaks the symmetry of the friction forces in space and time and thus powers net motion.

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is an attractive concept that should help us to understand how cells optimize migration strategies in various ‘soft’ and ‘stiff’ microenvironments and enhance our knowledge of cellular behavior during embryogenesis, parasite infection, tumorigenesis and immune cell migration (Hughes et al., 2004; Coudrier et al., 2005; Raz and Reichman-Fried, 2006; Beadle et al., 2008).

Materials and Methods
Preparation of Entamoeba histolytica cells and drug treatments
E. histolytica HM1-IMSS (Diamond, 1961) wild-type strain was grown and resuspended in TY-S3 medium before the experiments. For experiments in non-adhering conditions, cells were kept in suspension at the interface between a low density medium (culture medium) and a high density medium (Percoll, Sigma) corrected with calcium chloride to obtain the usual osmolarity of cell cultures (300 mM). Propidium iodide (Sigma) was used for checking the membrane integrity during electron microscopy. Cells were fixed with 10% formaldehyde and postfixed with 1% osmium tetroxide. The observation chamber was created by glass beads (Polysciences). To this end, cells were protected from the flow by a thin metal mesh (Saulas) maintained 400 μm above the floor of the observation chamber by glass beads (Polysciences).

Scaling argument for the pressure velocity relationship
The scaling argument used to relate the expansion velocity to the pressure that drives it reads as follows: The Stokes equation between pressure gradient (through the cortex) and the viscous stress is ΔP=ηΔv, where ΔP is the pressure difference, η the cytosol viscosity and Δv the mesh size. The pressure gradient is coupled to the pressure difference through the cortex by ΔP=h·ΔP, where h is the thickness of the cortex. Velocity gradients within the cortex are dictated by the surface tension of the cell boundary and speed is ΔP=Δv·h·η. With η=10−3 Pa.s, Δv=10−6 m, the value found for the pressure that drives the bleb is 1–10 Pa.

Live actin imaging of cytoskeleton
The actin cytoskeleton was scanned by fluorescence video microscopy using the LifeAct peptide (MGVADLKKFESIIK) (Riedl et al., 2008) fused to GFP through a N-terminus ATG site (bold nucleotides) for direct cloning into the pcDNA3-GFP vector.

Live imaging
Most observations were made with an inverted IX-70 Olympus microscope using LaCon PComicini observation chamber in "open" configuration. The chamber was completely filled with medium and closed, to maintain anoxic conditions during hours. Phase-contrast observation movies were recorded with a firewire AVT Guppy F-080B CCD camera with the BTV Pro acquisition software at a 15 Hz frame rate. Protrusions expand in 3D, but we chose to use 2D wide-field imaging to sustain a high acquisition rate. Because we used low magnification imaging, the depth of field was large enough to capture all protrusion. Fluorescence imaging was performed with a CoolSNAP HQ2 (Roper Scientific) CCD camera with Micro-Manager software. Fluorescence and phase contrast (or DIC/phase contrast) were acquired quasi-synchronously by sequential illumination (LEDs for bright field alternated with HBO Mercury lamp), using a home-made LabView program.

Micropipette experiments
Sample chambers were assembled as two clean glass coverslips glued with vacuum grease and fixed with nail polish to a 1-mm thick aluminum support. Once filled with cells and medium, the chamber was sealed with mineral oil to prevent water evaporation and limit oxygen entry. The chamber was placed on the stage of an inverted microscope (Axiovert 200, Zeiss), equipped with 60× Olympus UPlanFl immersion oil objective (1.25 NA) and a 0.8 NA air condenser. Temperature was regulated by a home-made water circulation around the objective, and fluid temperature was regulated by a thermostated circulator. A homemade micromanipulator was used to move micropipettes and capillaries of about 2 μm tip diameter were connected to a mobile water tank. Phase contrast images were collected by an analog CCD camera (XC-ST30C, Sony). Micropipette-based electroporation was performed with the 800A Axoparator. Pulse trains of alternating polarity were used to avoid a permanent current bias and hydrolysis. Electroporation conditions are defined by the pulse amplitude (V0), the pulse duration (τ), the pulse repetition frequency (f) and the train duration (T). Typical values of these parameters are: V0=±6 V, τ=1 ms, f=100 Hz, and T=10 seconds. The typical resistance of micropipettes was 20 Ω.

Image analysis
Several Matlab routines were specifically developed for image analysis. Because we wanted to use a constant contrast imaging, the use of constant parameters (threshold, gradient, etc.) was not accurate enough. We developed instead an autocorrelation algorithm that is embedded in the program that directly processes movies. Fluorescence images of Fig. 4 could not be processed by the autocorrelation method, and edges were manually determined frame by frame. Kymograms were constructed as follows: for each time point, we represented the average radial distribution of the fluorescence intensity obtained by averaging over the contour.

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Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/XX/XXXX/DC1

References


Figure S1: The hydrophobicity of the substrate does not influence cell movements.

The motility of Eh cells is shown in terms of the mean square displacement (msd). The msd is computed from the position of the surface's geometric centre inside the cell boundary. Various hydrophobic substrates were used: bare glass (A); glass slides grafted with perfluorosilane C10 (B); glass with adsorbed C-18 alkane (C). These three plots show similar cell behaviour, indicating that the dynamics underlying the movement of the centre of mass is not influence by interaction with the substrate. Importantly, and in contrast to the msd plots shown on Figure S8 at longer time-scales, the present plot reveals non-Brownian behaviour (discussed below).

The msd shown on the three plots in Figure S1 can be fitted to $\langle \delta r^2 \rangle = \alpha \tau^\beta$. Fits of the whole curve typically yielded values of $\beta \approx 1.7 - 1.8$. However, at short times, $\beta = 2$ and the prefactor ranged from 0.1 to 1 $\mu m^2/s^2$. This corresponds to a ballistic regime for the centre of mass's underlying random walk which, at longer times, displays the Brownian behaviour shown in the Figure S8. The time scale on which the ballistic regime changes to a Brownian one is typically around 10 seconds, as seen in Figure S1.
Figure S10: The cortical cytoskeleton recoils upon membrane disjunction.
A bleb is shown inside a micropipette. Membrane disjunction and forward movement are clearly correlated with backward motion of the cortical cytoskeleton behind the membrane (green dashed line). This recoil is fast and has a high amplitude (2µm within 0.2 s). This amplitude steadily increases as the bleb front continues to move forward, suggesting that a contraction-induced recoil force overcomes the stress induced by the hydraulic resistance and the cytosolic flow through the cortex. The fact that membrane disjunction immediately correlates with a cortical recoil indicates that the force exerted by the linkers (lost during the disjunction) is larger than the hydraulic stress (triggered by the disjunction) that would otherwise push the cortex forward. We infer that the stress generated by the cytoskeleton’s hydraulic resistance is negligible, when compared with the stress associated with the contractile activity and the linkers. When considered together with the data shown in Figure S9, these findings argue against the involvement of poroelasticity in blebbing.

Figure S11: Schematic representation of the different dynamic modes expected for cytoskeleton-membrane association.
The excess internal hydrostatic pressure ($\pi_{\text{int}} - \pi_{\text{ext}}$) is shown over time. This pressure difference increases at a rate $\rho$ which is controlled by myosin activity. Assuming that the actin turnover on a time scale $\tau_{\text{actin}}$ prevents the accumulation of contractile stress at longer time scales, the pressure difference should saturate for $\tau > \tau_{\text{actin}}$. In our model, membrane disjunction requires the pressure difference to exceed the linker resistance: $\pi_{\text{links}*} < \pi_{\text{int}} - \pi_{\text{ext}}$. This condition will only be met if $\rho\tau_{\text{actin}} > \pi_{\text{links}*}$. Two examples (with and without disruption) are shown. In the latter case, the internal pressure is expected to reach a steady-state value, with no cortical instability. Any biochemical regulation with an overall or local impact on one of these three parameters ($\rho, \tau_{\text{actin}}$ or $\pi_{\text{links}*}$) should, in principle, contribute to the modulation of bleb-based motility.
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Figure S12: Time-series of fluorescence images extracted from the video S12.

The initial transmission image (A) is followed by the propidium iodide fluorescence image taken immediately at the onset of the electroporation pulse sequence (t = 2 sec). The latter shows a point-like fluorescence signal that initially expands outwards (see movie S12). This outward expansion (t = 8 sec, C) indicates that PI-visible endoplasmic material is indeed expelled by electro-pores located at the initial puff. Later on (t > 14 sec), the dye penetrates, as shown by the development of intracellular fluorescence (t = 16 sec, D). This intracellular fluorescence forms a diffuse pattern from the same initial point, and a clear membrane staining gradually appears (t = 20 sec, E), thus showing that we do have both extracellular and intracellular signals. Eventually, the whole cell is rather uniformly labelled (t = 120 sec, F) because the dye distribution equilibrates.
Figure S2: Enhanced hydrophilicity does not modify cell movements. 
*Eh* cell on a hydrophilic (PEG-grafted) substrate. Successive images correspond to a 10-second time intervals. Blebbing is similar to that seen on untreated surfaces (video not shown).

Figure S3: Dynamics of adhesion during bleb expansion. 
The first three images are RICM snapshots of cellular adhesion during bleb expansion. The last image shows a merged RGB representation (with the individual colours corresponding to the 1st, 2nd and 3rd images, respectively). Bleb emission leads to an initial, discontinuous contact zone (red) that appears away from the main contact area (image 2). The initial discontinuous contact grows into a larger surface area (yellow) that bridges with the main zone (image 3), whereas contact is lost some distance away from the bleb (blue).
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Figure S4: Cell motions are dramatically reduced by inhibition of myosin and actin turnover.
Each of panels A, B and C shows 4 cells. For each cell, an initial phase-contrast image (in red) is superimposed on the subsequent, successive contours of the cell (taken every second, for 30 seconds). Contours are shown relative to the cell’s centre of mass in order to display deformations and not any displacements that may result. Deformations are shown under normal conditions (panel A) and in the presence of myosin inhibitors (50 µM ML7 and 40 µM Y27632; panel B) and an actin turnover inhibitor (200 nM jasplakinolide; panel C). Panels D and E show the mean square displacement (msd) for one cell from panels B and C, respectively, before (blue curve) and after (red curve) drug treatment. The msd values are shown on a logarithmic scale and error bars are represented by the distance between the curves and the triangles. Myosin inhibition clearly inhibits cell deformation and the motility is significantly impaired. Actin turnover dramatically reduces cell motility and reduces deformations (although to a lesser extent than myosin inhibition does).
Figure S5: Electroporation suppresses blebbing, while ongoing contraction shrinks the cell.
These Figures are snapshots from a video S10 showing the effect of electroporation (see the legend to video S10). The time is indicated as negative prior to electroporation, which starts at t=0s and stops at t=10s. Red arrows indicate blebs occurring before the electroporation. During electroporation, the cell clearly shrinks (due to its contractile machinery, which triggers blebs again once the electrical pulses are removed).
Figure S7: Blebbing on bare glass does not exhibit clear, long-term periodicity.
The graph represents the blebbing over time by a single cell. Blebs (o) are indicated by stars (*) when the disjunction is very fast. Membrane deformations occurring after cortical collapse are indicated as "flows" (⊿). The bleb lifetime is indicated by blue bars. Blebs are successively marked on the vertical axis. This representative graph (one of 8 separate experiments) shows a chaotic blebbing pattern, including short- and long-lived blebs with no clear periodicity.
Figure S8: On a long time scale, Eh cells undergo efficient, diffusive motions. The graph indicates the mean square displacement (msd) of Eh cells as a function of time. A total of 472 trajectories are shown in terms of their msd versus time. To illustrate the efficiency of the diffusive motion of Eh cells we show the msd values for typical directed mesenchymal motions with a velocity of 30 µm/min (△) and 3 µm/min (▽). The motility of Eh cells (when expressed as an msd) is clearly more efficient than directed motility over the time scale considered here (τ < 1 hour).

Figure S9: Evidence to suggest that the hydrostatic pressure equilibrates instantaneously throughout the whole cell. Four differential interference contrast images are shown. The first three images were taken prior to the bleb formation (A), immediately afterwards (B) and 0.3 s later afterwards (C). Image D is the RGB sum of images A, B and C (coded in blue, green and red, respectively). Local spots of colour therefore indicate motion and show that during the disjunction, granules located away from the bleb start moving with it immediately. This is a strong indication that a pressure gradient immediately spreads throughout the cell and argues against the involvement of cytoskeletal and/or cytoplasmic poroelasticity.
Figure S8: On a long time scale, Eh cells undergo efficient, diffusive motions. The graph indicates the mean square displacement (msd) of Eh cells as a function of time. A total of 472 trajectories are shown in terms of their msd versus time. To illustrate the efficiency of the diffusive motion of Eh cells we show the msd values for typical directed mesenchymal motions with a velocity of 30 µm/min (△) and 3 µm/min (▽). The motility of Eh cells (when expressed as an msd) is clearly more efficient than directed motility over the time scale considered here (τ < 1 hour).

Figure S9: Evidence to suggest that the hydrostatic pressure equilibrates instantaneously throughout the whole cell. Four differential interference contrast images are shown. The first three images were taken prior to the bleb formation (A), immediately afterwards (B) and 0.3 s later afterwards (C). Image D is the RGB sum of images A, B and C (coded in blue, green and red, respectively). Local spots of colour therefore indicate motion and show that during the disjunction, granules located away from the bleb start moving with it immediately. This is a strong indication that a pressure gradient immediately spreads throughout the cell and argues against the involvement of cytoskeletal and/or cytoplasmic poroelasticity.