Classification: Biological Sciences - Biophysics and Computational Biology

Title: How blebs and pseudopods cooperate during chemotaxis

Short Title: Localising cellular blebs by curvature

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Abstract

Two motors can drive extension of the leading edge of motile cells: actin polymerization, and myosin-driven contraction of the cortex, producing fluid pressure and the formation of blebs. *Dictyostelium* cells can move with both blebs and actin-driven pseudopods at the same time, and blebs, like pseudopods, can be orientated by chemotactic gradients. Here we ask how bleb sites are selected and how the two forms of projection cooperate. We show that membrane curvature is an important, yet overlooked, factor. *Dictyostelium* cells were observed moving under agarose, which efficiently induces blebbing, and the dynamics of membrane deformations analysed. Blebs preferentially originate from negatively curved regions, generated on the flanks of either extending pseudopods or blebs themselves. This is true of cells at different developmental stages, chemotaxing to either folate or cyclic-AMP, and moving with both blebs and pseudopods, or blebs only. A physical model of blebbing suggests that detachment of the cell membrane is facilitated in concave areas of the cell, where membrane tension produces an outward directed force, as opposed to pulling inwards in convex regions. Our findings assign a new role to membrane tension which can spatially couple blebs and pseudopods, thus contributing to clustering protrusions to the cell front.

Significance Statement

We show that blebs – pressure-driven extensions of the plasma membrane – preferentially form in concave regions of the plasma membrane of migrating *Dictyostelium* cells, and propose that the underlying physical reason is due membrane tension, which provides an outwards force at concavities. Blebs can transform into pseudopods by continued actin polymerisation and pseudopods can trigger blebs on their flanks. Thus the two forms of protrusion can cooperate during chemotaxis.

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Introduction

Crawling cells must restrict protrusions to a limited part of their periphery if they are to move efficiently, and when these cells chemotax, the location of projections must be further controlled by the chemotactic gradient (1-3). Cellular protrusions are of two main types: those driven by actin polymerization, such as pseudopods or lamellipods and those driven by fluid pressure, which are usually called blebs. Blebs form when the cell membrane locally detaches from the underlying cortex and is driven outwards by hydrostatic pressure, created by myosin-II driven contraction of the cortex (4,5). When blebs form, the cortex is left behind as an "F-actin scar", which depolymerizes, while a new actin cortex forms at the freshly exposed membrane.

Blebbing is important in cells migrating in 3-dimensional environments, such as during tumour invasion (6,7), zebrafish primordial germ cell migration (8,9) or migration of the pathogen *Entamoeba histolytica* in the liver (10). *Dictyostelium* amoebae can also move with blebs (11). In standard conditions on a two-dimensional surface under buffer, they move mainly with F-actin-driven pseudopods, but switch progressively to bleb-driven motility when faced with mechanical resistance to their movement (12). This can be conveniently applied by inducing the cells to migrate under an elastic overlay, such as agarose, which they must deform in order to progress (13). Blebbing is stimulated by acute treatment with the chemoattractant cyclic-AMP (14), and blebs can be chemotactically orientated by cyclic-AMP gradients (11,12).

Actin-driven pseudopods are preferentially formed up-gradient by chemotaxing cells, and they can be induced on the flanks of cells by applying a steep gradient of chemoattractant from a micropipette, showing, along with much other evidence, that the site of actin polymerization and pseudopod formation can be controlled by a signaling pathway from the chemotactic receptors (15). Despite intensive investigation, many aspects of this pathway remain to be established.

Since blebs, like pseudopods, are preferentially orientated to the front of moving *Dictyostelium* cells, and can be similarly orientated by gradients of cyclic-AMP, the questions arise of how are bleb sites selected and how is this selection influenced by chemotactic gradients? And how do blebs and pseudopods interact to produce sustained movement, rather than potentially interfering with each other?

There are several proposals for bleb site selection, including by local contraction of the cortex (8) or local weakening of it (16,17), and by local weakening of the attachment between cortex and membrane (18,19). In cells of the parasite *Entamoeba histolytica*, where blebs expand rapidly, there are however no visible signs of a weakening of the actin cortex before a bleb forms (10) and likewise blebs in *Dictyostelium* form without detectable weakening of the cortex.

One clear interaction between blebs and pseudopods is where blebs give rise to pseudopods by continued actin polymerization: the F-actin-denuded membrane of a newly formed bleb is an excellent template for actin polymerization, which rapidly restores the cortex. In many cases, actin polymerization continues after the cortex has been restored, transforming the bleb into a pseudopod, and creating hybrid structures, which we called 'blebbopods' (12). Thus through this route of continued actin polymerization, blebs can determine where pseudopods form.

To study bleb formation and bleb-pseudopod interactions in detail we developed an accurate method for automatically analysing blebs in image time series. We describe here a second interaction, mediated by mechanical means, in which pseudopods (and potentially other F-actin projections) as well as other blebs can influence where blebs form by creating areas of local negative membrane curvature.

Results

To seek general rules governing bleb formation, we examined *Dictyostelium* cells in three situations. In each, wild-type Ax2 cells move on glass and are attracted under an agarose overlay, which provides mechanical resistance. We used: cells starved for 5-6 hours chemotaxing to cyclic-AMP under 0.7% agarose (Young's modulus E=39 kPa, 12), where both pseudopods and blebs form in equivalent numbers, along with some filopods; cells chemotaxing to cyclic-AMP under 2% agarose (E=295 kPa), where blebs but very few pseudopods form, and filopods are not detected at all (using the cAR1-GFP membrane marker); and finally, vegetative cells freshly harvested from growth medium and chemotaxing to folic acid under 2% agarose, where blebs and pseudopods form in roughly equal numbers, though chemotaxis is less efficient.

Characterisation and chemotactic orientation of cellular protrusions

Figure 1 shows the major types of protrusion made by cells migrating under 0.7% agarose towards cyclic-AMP. Cells are observed using ABD-GFP to mark F-actin, and with fluorescent dextran included in the agarose to reveal their outlines (Fig. 1a). F-actin-driven pseudopods (henceforth pseudopods) advance steadily with a continuous zone of F-actin beneath the plasma membrane, whereas blebs advance in a saltatory fashion, with very little F-actin beneath the membrane as they expand. The hybrid blebbopods start life as a bleb, and then transform into a pseudopod by continued actin polymerisation.

We enhanced our QuimP software (20,21) to semi-automatically determine the orientation of blebs and pseudopods made by cells moving in chemotactic gradients (for details see SI Text S1). As shown previously (12), polar plots of cellular projections under 0.7% agarose reveal that actin protrusions dominate the leading edge, while blebs also nucleate at the cell front, but preferentially on the flanks of the leading edge, forming a bimodal distribution. The distribution of blebbopods is a mix of that of blebs and pseudopods, suggesting that blebs formed at the cell front are more likely to elicit sustained actin polymerisation and so broaden the focus of actin driven protrusions.

We confirmed that the bimodal distribution of blebs also holds for the other two experimental conditions (Fig. 1h). Under 2% agarose, few pseudopods form and the orientation of blebs is more dispersed, but still bimodal. Cells also produce blebs preferentially towards folic acid in a bimodal manner, although the distribution of pseudopods is less well defined, consistent with the less efficient chemotaxis to this agent.

The tendency of blebs to form on the flanks of pseudopods, apparent under 0.7% agarose, suggests that the two forms of protrusion may interact. Because the flanks of pseudopods are characterised by negative curvature we hypothesised that this interaction might be controlled by membrane geometry.

To investigate this further we performed detailed mappings of pseudopods and blebs using a novel method - Electrostatic Contour Migration (21) – where charged virtual markers migrate in an electric field between successive contour segments to which opposite charges have been applied (Fig. 2a). Markers on the membrane can be assigned velocities (Fig. 2b) and marker tracks stitched together for any point on the cell contour (Fig. 2c). Changes in cell morphology and local curvature (see Methods) are visualised in 2D spatio-temporal maps, in which selected marker tracks show that our algorithm can closely follow deformations due to blebs, protrusions and contractions (Fig. 2c), enabling us to automatically characterise their velocity, curvature, and intensity.

The algorithm located 519 projections in 30 cells under 0.7% agarose with a false positive rate of 4.1% (determined by visual inspection and excluded from further analysis), and a sensitivity of 96.4% correctly identified projections.

Blebs preferentially nucleate at regions of negative membrane curvature: qualitative observations

To test the hypothesis that blebs form preferentially from areas of negative membrane curvature, we constructed velocity and curvature maps of cells moving towards cyclic-AMP under 0.7% agarose (Figs. 2c,d). As expected, blebs and pseudopods have distinctly different velocity signatures, with the short, sharp expansion of blebs contrasting with the slower more prolonged expansion of pseudopods. The curvature map shows that the two pseudopods (Fig. 2b; *p.1 – p.2*) each expand in areas of positive curvature, whereas consistent with the

hypothesis, the three blebs form in areas of negative curvature: *b.1* is for example created between two pseudopods, and *b.2* from the negative curvature created on the flank of *b.1*. Higher resolution shows the abrupt switch from negative to positive curvature as a bleb forms, and the induction of negative curvature on its flank (Fig. 2e).

Blebs preferentially nucleate at regions of negative membrane curvature: population data

To test the generality of the correlation between negatively curved membrane and bleb formation, we analysed populations of protrusions. Projections were manually classified as blebs or pseudopods depending on the presence of an actin scar and actin at the leading edge, and the relevant parameters extracted automatically.

A global view reveals a wide distribution in both speed and displacement for blebs and pseudopods under all conditions (Fig. 3, left column). Cells moving under 2% agarose are flatter (12) and bleb ten times more than those moving under 0.7% agarose (Table 1). In contrast, the rate at which pseudopods are produced remains unchanged, though at the higher agarose concentration they more often emerge directly from blebs, thus forming blebbopods.

During chemotaxis toward cyclic-AMP under 0.7% agarose, pseudopods clearly originate from regions of positive curvature (91% of cases), but at first sight, blebs do not appear so clearly directed to regions of negative curvature (63% of cases, Fig. 3a, middle column). For a more rigorous statistical analysis, we compared actual curvatures in regions where blebs originate to a test distribution based on random sampling of curvatures from the same cells and found that significantly more blebs form below -27 degrees than would be expected by chance. Likewise, the number of blebs in regions with more than +27 degrees of curvature is significantly reduced.

Blebs are more strikingly associated with areas of negative curvature in cells under 2% agarose, where the induction of negative curvature is almost entirely due to the blebs themselves (Fig. 3b). This argues that the correlation between blebbing and negative curvature is independent of the type of protrusion causing the negative curvature. Similarly, blebs in vegetative cells chemotaxing towards folic acid show a significant bias towards areas of negative curvature (Fig. 3c). Consideration of curvature also helps to understand why blebs are more common in the rear of cells under 2% than 0.7% agarose (Fig. 1h): SI Fig. S6 shows that

cells under 2% agarose have a less pointed rear. Because this is equivalent to a reduction of positive curvature, the chances of blebbing at the rear are increased.

Surprisingly, given the increased mechanical resistance, blebs are projected faster by developed cells under 2% than under 0.7% agarose (0.7%: 1.35 µm/sec, 2%: 1.88 µm/sec, P<0.001 two-sample Kolmogorov-Smirnov test), suggesting a greater motive force, presumably due to increased intracellular pressure. However, these faster blebs expand less far than their slower counterparts (0.7%: 1.14 µm, 2%: 0.97 µm, P<0.01). Fast (10 fps) imaging of cells under 2% agarose (SI Fig. S7), shows there is a 3% local area gain during bleb expansion, offset by a global loss of 2%, so that on average the cell area remains relatively constant, with a standard deviation of less than 1% (we take projected cell area because cells under 2% agarose are essentially flat and of uniform height (12); gobal contraction concomitant with blebbing has previously been described in (11)). Despite the differences in modes of movement observed under 0.7% and 2% agarose, cell speeds do not differ significantly (0.7%: 10.8 µm/min (±8.1), 2%: 7.6 µm/min (±4.6)), two-sample Kolmogorov-Smirnov test).

Blebbing in *Fundulus* deep cells

The literature contains many examples where blebs seem to originate preferentially from regions of negative curvature, but the possibility of a causal link has received little attention to date (10, Fig. 2B; 22, Fig. 10A; 23, Fig. 3A). *Fundulus* deep cells provide a beautiful example, which we have analysed with our software (24, data kindly provided by Rachel Fink, Mount Holyoke College, Video S8, SI Fig. S5b). Blebs form regularly from the front of these cells, and in the one shown, it can be seen that they arise mainly from flat or negatively curved areas, and not at all from strongly positively curved areas. It is also apparent that blebs do not form at the negatively curved waist separating the uropod from the main body of the cell, suggesting that membrane-cortex connections are reinforced in this area.

A simple physical model

To make our ideas explicit, we made a simple physical model relating membrane curvature to blebbing propensity. In this we assume that the plasma membrane is attached to the cortex by evenly-spaced linkers, and that fluid pressure on the membrane is everywhere the same. On the time-scale of bleb nucleation the cortex is considered to be fixed. Membrane tension

produces a force whose direction depends on curvature: in regions of positive curvature it is directed inwards (Fig. 4a) and in regions of negative curvature outwards (Fig. 4b). Similar considerations apply to membrane bending, but its contribution is an order of magnitude less. In these conditions a bleb will form anywhere where the net outward force on the membrane due to fluid pressure and membrane tension is strong enough to stretch and eventually break linkers.

Fig. 4c shows a snapshot of a simulation where blebs originate from the negatively curved neck region of a protrusion (SI Video S9, for details see SI Text S1). Fig. 4d shows that the force acting on linkers is directed outwards in the position where the two blebs form; the values in the piconewton range are only indicative of the expected order of magnitude. The corresponding curvature map (Fig. 4e) shows that blebs themselves generate negative curvature on their flanks. That this mechanism can actually spawn new blebs is apparent with real cells under 2% agarose, which sometimes extend consecutive blebs in a chain reaction because of successively induced concavities (Figs. 4f,g, SI Video S3). The characteristic jumps in curvature (from blue to red) seen in the model (Fig. 4e) agree well with the patterns marked by the short traces of blebs in the experimental curvature map (Fig. 4g). It is notable that daughter blebs in the real cell do not form at the apex of the earlier bleb, where the F-actin cortex is at its thinnest, but the membrane curvature is positive, but at the flank: thus curvature is likely the dominant factor in this case. The propagation of a bleb around a cell in 'circus movement' may have a similar explanation, in that the bleb continuously expands into areas of negative membrane curvature (25,26).

Discussion

Bleb driven motility is a feature of cells moving in tissues and other mechanically resistive environments, and in *Dictyostelium* it seems to be provoked by the mechanical resistance itself (12). Here we have addressed the questions of how the site of blebbing is selected and how blebs can cooperate with pseudopods when both are present, and thus ultimately of how a cell moving with blebs is steered.

In each of the three physiological situations examined, bleb formation is correlated with areas of moderate-to-strong negative curvature, and decreased in areas of positive curvature. This is particularly striking in cells under 2% agarose, where chains of blebs often form, with each new bleb triggered from the concavity formed at the flank of the preceding one. The correlation does not depend on the chemoattractant used or the nature of the projection causing curvature – bleb or pseudopod – consistent with the idea that it is a purely physical interaction. The underlying reason is readily apparent: membrane tension provides an inward force opposing bleb formation in convex areas and an outward one, favouring it, in concave areas.

To test whether membrane curvature can alone predict where blebs will form, we asked whether blebs always form in the region of the cell with the greatest negative (or least positive) curvature (SI Fig. S8). Analysed in this way, the data confirms the strong correlation between negative curvature and bleb formation, but also shows that blebs do not necessarily form in the areas of highest negative curvature. Similarly, our analysis of *Fundulus* deep cell movement shows that although blebs form from areas of negative curvature, they do not form in the concave waist between the cell body and uropod.

These observations show that other factors apart from membrane curvature must also regulate bleb site selection. Such factors could include the density of membrane-cortical linkers and the local concentration of PIP2 in the membrane, as this can modulate the attachment of these linkers (18,27). Consistent with this, in melanoma cells, it has been argued that a high level of the membrane–cytoskeleton linker ezrin inhibits blebbing from the uropod (28).

Since concavities often form on the flanks of pseudopods, pseudopods can trigger adjacent blebs and provide indirect chemotactic steering to them. However, when cells re-orientate

towards a moving cyclic-AMP source, they follow a distinct series of events in which actin microspikes first form in the new direction of the gradient, followed by blebs, and pseudopods only later (12). In this case, blebs are not triggered by a preceding pseudopod, but could be by the actin microspikes, which can also create local negative membrane curvature. Filopods could also play a role in triggering blebs, but not an essential one, since they are not detected in strongly blebbing cells under 2% agarose. Conversely, blebs can determine where pseudopods form, through continued actin polymerisation and the formation of blebbopods. Thus each type of projection can trigger formation of the other, helping to focus protrusive activity to the front of the cell. But since cells can move chemotactically primarily with either pseudopods (vegetative cells under buffer) or blebs (developed cells under 2% agarose) it seems likely that in addition to their chemotactic cooperation, blebs and pseudopods also have independent chemotactic steering.

It is notable that blebs are projected faster than actin-driven pseudopods in each condition we investigated, suggesting that they are impelled by a greater force; this greater force provides a rationale for why blebs often predominate in resistive conditions. The global propensity of cells to form blebs is regulated by cortical contractility, which is mediated through myosin-II. Cells might thus adapt to resistive conditions by increasing their expression of myosin-II, as seen in *Dictyostelium* development, or by acute increases in myosin-II activity (29).

Our observations add to the range of cellular processes that can be controlled by membrane tension. As well as blebbing, these include endocytosis and in the field of cell motility, the long-range suppression of pseudopods by tension produced by a dominant pseudopod (30), all emphasising the role of mechanics in controlling cell physiology (31).

Methods

Cells and microscopy

The axenic strain Ax2 of *Dictyostelium discoideum,* used throughout (Kay laboratory strain; DBS0235521 at http://dictybase.org), was grown on HL5 medium (ForMedium). All experiments were at 22°C. Ax2 was transformed with markers for F-actin (GFP–ABD120, 32), or the plasma membrane/filopods (cAR1-GFP, 33).

Cells for folate chemotaxis experiments were diluted 10-fold from growth medium into KK_2 buffer (16.5 mM KH_2PO_4, 3.9 mM K_2HPO_4, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, pH 6.1), plated and the medium replaced with fresh KK_2 after 10 min. Cells for cyclic-AMP chemotaxis experiments were washed free of growth medium and starved for 5.5 hr with pulses of cyclic-AMP (around 90 nM final) added every 6 min after the first hour. Imaging was performed under KK_2: cells were placed into wells cut in an agarose (SeaKem GTG; Lonza) overlay on Lab-Tek coverslips (Thermo Fisher Scientific) and attracted under the agarose towards an adjacent well, containing either 4 µM cyclic-AMP or 1 mM folic acid (12). Rhodamine-B-isothiocyanate-dextran (RITC-Dx), 0.5 mg/ml, was included in the agarose for negative staining, as indicated. Dual channel images were acquired at 2-10 fps using either a Zeiss 710 laser scanning confocal microscope (Zeiss, Germany) with a 63x oil-immersion objective or a spinning-disk confocal microscope (Ultraview; PerkinElmer) with 100x oil-immersion objective.

Image processing and protrusion analysis

GFP-ABD120 images were background corrected and intensity normalised. For the purpose of segmentation, RITC-Dx images were inverted and a Gaussian convolution (σ=1 pixel) applied to smooth high frequency noise. Cells were segmented and analysed using the QuimP11 software (http://www.warwick.ac.uk/quimp) and passed through our automatic protrusion detection algorithm (see SI Text S1 for an overview of the QuimP11 software).

Quantifying projection peak velocity

Maxima in protrusion velocity profiles suffer from membrane fluctuations and segmentation noise. To alleviate this, we compute the associated displacement profile and fit a sigmoid curve of the form $f(x) = a(1 - exp(-\frac{x^c}{b})) + d$ to it, which allows for asymmetrical acceleration and deceleration and is differentiable. Peak velocity is determined as the maximal gradient,

$\max\left(\frac{df(x)}{dx}\right)$ (SI Fig. S4). A sample of peak velocities computed from automatically extracted projection paths were verified using manually obtained projection paths and found to be in good agreement (SI Fig. S4e).

Computing projection rates

Given a sequence of *N* cell outlines with *K* detected projections, the projection rate *PR* is computed as the number of observed projections per micron, per minute, $PR = \frac{K}{IP}$, where $IP = \sum_{i=1}^{N}(T_{i+1} - T_i)\frac{P_i + P_{i+1}}{2}$ is the integrated perimeter, *T* is time, and *P* the cell perimeter.

Defining curvature and Chi-squared tests

Curvature in the range (-180,180) was determined by summing up the angles formed between markers on the cell contour and their neighbours over 1 micron. Zero is flat, positive values are convex, and negative values are concave. Curvature at bleb nucleation was computed by interpolating between closest markers in the frame immediately prior to an expansion by 0.2 µm. Significance testing employed a test distribution of randomly sampled curvatures (> 4000) drawn from actual cell contours at times when blebs appear. Comparisons using Chi-squared tests were performed binning the data into 6 classes with boundaries [-Inf,-45,-27,-9,9 ,27,Inf]. A Bonferroni correction for multiple comparisons was applied to final *P* values.

Acknowledgements

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Figure legends

Figure 1: Characteristics of the projections produced by *Dictyostelium* cells under an agarose overlay. The cells express an F-actin marker (green), and RITC-dextran, included in the agarose, shows cell outlines (red). **(a)** Typical bleb: arrows – freshly polymerised actin of the reconstituting bleb cortex; starred arrow – residual cortex. DIC (grey) shows that blebs are organelle free. **(b)** Typical pseudopod: F-actin is associated with the membrane at all times. **(c)** Two adjacent blebs produced in less than 1 sec: initially there is little F-actin (first bleb, 1 sec), which builds up quickly (2 sec), while the actin scar breaks down, becoming visibly ruptured (4 sec). **(d)** Blebbopods: fast, bleb-like initial extension is followed by slower extension driven by actin polymerisation. **(e-g)** Kymographs of protrusion along yellow lines shown in *b-d*, highlighting the continuous association of actin during pseudopod extension, sudden membrane detachment in blebbing, and two-stage extension in blebbopods. **(h)** Chemotactic orientation of projections (green dot is up-gradient). Pseudopods dominate the leading edge of cells chemotaxing to cyclic-AMP under 0.7% agarose while blebs have a bimodal orientation (30 cells; N^{pseud}=304; N^{blebs}=194, Hartigan's dip test: $P<0.05$). Distribution of blebbopods is a mix of that of blebs and pseudopods, N^{bp}=49. Blebs are also bimodal on cells chemotaxing to cyclic-AMP under 2% agarose (13 cells, N^{blebs}=530, Hartigan's dip test: $P<0.01$) or folate (10 cells, N^{blebs}=212, Hartigan's dip test: $P<0.01$). Bleb distributions differ between 0.7% and 2% agarose (Kuiper two-sample test, $P<0.001$) and mode peaks appear closer together under 2% agarose. In all cases projections align with the gradient (zero mean), though cells chemotaxing to folate are significantly less directed (circular one-sample t-test).

Figure 2: Tracking protrusions suggests that blebs originate in areas of negative curvature. **(a)** Principle of the tracking method, which is based on migration of charged markers in an electrostatic field. A sector is defined by the intersection of successive cell contours and here sector Y_T is positively charged, Y_{T+1} negatively charged, forming an electrostatic field in which positively charged markers migrate from Y_T to Y_{T+1}. The method minimises the total path integral and thus the energy required to deform Y_T to yield Y_{T+1}; as field lines never cross, it creates a unique and smooth mapping. **(b)** Tracking applied to pairs of successive frames (0.5 sec intervals) showing example blebs (*b.1-b.3*), protrusions (*p.1-p.2*), and a contraction (c.1). Colours indicate node speed (red: high, green: low). Positions on the membrane can be followed over extended periods by interpolation of mappings. **(c)** Spatio-temporal velocity map.

The fastest moving points within the example regions labelled in (b) are automatically traced through the entire sequence (black traces) and clearly follow patterns of cellular deformations (black frames, see SI Fig. S3 for complete tracking). **(d)** Corresponding curvature map. It is apparent that the selected blebs originate in areas of negative curvature and the pseudopods in areas of positive curvature **(e)** Zoomed curvature map demonstrates *b.1* originates from a region of negative curvature and induces negative curvature from which bleb *b.2* nucleates.

Figure 3: Population studies show blebs preferentially nucleate in areas of negative curvature. **(a)** Cells moving towards cyclic-AMP under 0.7% agarose (30 cells, 144 blebs, 304 pseudopods); **(b)** cells moving towards cyclic-AMP under 2% agarose (13 cells, 454 blebs, 37 pseudopods); **(c)** cells moving towards folic acid under 2% agarose (10 cells, 118 blebs, 231 pseudopods). For each condition, blebs and pseudopods are characterised by their peak projection speed and total displacement (excluding blebbopods), and the curvature of the membrane from which they originate (blebbopods included as blebs). In **(a)** and **(c)** there is a significant difference in distribution of blebs and pseudopods with curvature (Kolmogorov-Smirnov test P < 0.001, Mann-Whitney-Wilcoxon test P < 0.001; insufficient pseudopods in **(b)** to test). To test the significance of the increased frequency of blebs at negative curvature and decreased frequency at positive curvature, the data was split into 6 classes of curvature (chosen to maintain required minimum frequencies for testing), and chi-squared tests made between observed and expected bleb distributions (stars). In addition, blebbing is 10-fold increased in cells chemotaxing to cyclic-AMP under 2% compared to 0.7% agarose, and the blebs expand faster (0.7% = 1.35 ±0.92 μm/sec, 2% = 1.88 ±0.99 μm/sec)

Figure 4. Proposed mechanism for bleb nucleation. Red: cell membrane. Green: cortex. Black: tethers. **(a)** In regions of positive curvature intracellular pressure is opposed by membrane tension and tethering. **(b)** In regions of negative curvature tension is inverted thus putting more pull on tethers. During detachment, a surplus of membrane becomes available for expansion. **(c)** Model simulation (SI Video S9 and SI Text S1 for model details). A protrusion, formed from initially circular geometry, induces concavities at its flanks from which blebs form spontaneously. **(d)** Space-time map of the net force due to membrane tension and bending acting on tethers. Force is high at the tip of the simulated pseudopod and directed inward preventing bleb formation. In the neck region membrane tension is of similar magnitude but is directed outwards and no longer opposes fluid pressure. This causes linkers to rupture and the high pulling forces on the basal bleb linkers (red streaks) promote unzipping of further linkers. Stopping bleb expansion is discussed in SI Text S1. **(e)** Curvature map of simulated blebbing

shows a distinct pattern of negative curvature being flipped to positive curvature at the bleb site, and induced negative curvature at bleb flanks. This simple model gives a feedback mechanism in which F-actin driven pseudopods promote blebbing at their flanks. **(f-g)** A bleb cascade (SI Video S3). A cell chemotaxing to cyclic-AMP (source at bottom) under 2% agarose produces a cascade of blebs at the leading edge in which the first (0.0 sec) produces negative curvature to spawn flanking blebs (0.5 sec) which in turn spawn further blebs along the cell sides (1.0 – 2.5 sec) consistent with the model proposed in *a-e*.

a

b 0 sec / 6 sec / 12 sec / 18 sec / 24 sec

c 3 µm / 0 sec / 1 sec / 2 sec / 3 sec / 4 sec

d 0 sec / 4 µm / 2 sec / 4 sec / 7 sec / 10 sec

e distance(µm) 0 → 6.5 / time (s) 0 / 24

f 0 → 4.7 / time (s) 0 / 4

g 0 → 7.1 / time (s) 0 / 10

h Ax2 Dev. 0.7% agar

Pseudopodia 80 40

Blebs 20 10

Blebbopodia 6 4 2

Ax2 Dev. 2.0% agar
Blebs 40 20

Ax2 Veg 2.0% agar
Blebs 20 10

a

γ_T

γ_{T+1}

b

2.6 s   12.7 s   30.2 s   49.0 s

c.1

5 µm

b.1   p.1   p.2   b.2   p.3

c

time (s)

0.0   10.4   21.9   31.1   41.6

b.1

p.1

p.2

c.1

b.2

p.3

normalised cell outline

0.0   0.5   1.0

expansion

retraction

speed (µm/sec)

5

0

-1

d

b.1

p.2

p.1

b.2

c.1

p.3

normalised cell outline

0.0   0.5   1.0

convex

concave

curvature (deg)

180

0

-180

e

b.1

b.2

a   Ax2 Dev. 0.7% agar

b   Ax2 Dev. 2.0% agar

c   Ax2 Veg. 2.0% agar

a

b
hydrostatic pressure
membrane tension
tether

c
A
B
5 μm
t = 0.7 sec

d
time (sec)
Force / node (pN)
0.6
0
-0.2
A ▶ cell outline (node number) ◀ B

e
time (sec)
curvature (deg)
49
-49
A ▶ cell outline (node number) ◀ B

f
5 μm
17
0.0 sec
18
19
0.5 sec
20
1.0 sec
21
1.5 sec
22
23
2.0 sec
25
24
2.5 sec

g
0
17
24 23
18
19 20
21 22
25
time (s)
speed (μm/sec)
4
0
-1
38

0
time (s)
curvature (deg)
72
-72
38
normalised cell outline