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Research Article

Blebbing of *Dictyostelium* cells in response to chemoattractant

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ARTICLE INFORMATION

Article Chronology:
Received 25 January 2006
Revised version received
1 March 2006
Accepted 6 March 2006
Available online 19 April 2006

Keywords:
Cell motility
Dictyostelium
Blebbing
Chemoattractant
Hydrostatic pressure
Arp2/3 complex
Myosin-II

ABSTRACT

Stimulation of *Dictyostelium* cells with a high uniform concentration of the chemoattractant cyclic-AMP induces a series of morphological changes, including cell rounding and subsequent extension of pseudopodia in random directions. Here we report that cyclic-AMP also elicits blebs and analyse their mechanism of formation. The surface area and volume of cells remain constant during blebbing indicating that blebs form by the redistribution of cytoplasm and plasma membrane rather than the exocytosis of internal membrane coupled to a swelling of the cell. Blebbing occurs immediately after a rapid rise and fall in submembraneous F-actin, but the blebs themselves contain little F-actin as they expand. A mutant with a partially inactivated Arp2/3 complex has a greatly reduced rise in F-actin content, yet shows a large increase in blebbing. This suggests that bleb formation is not enhanced by the preceding actin dynamics, but is actually inhibited by them. In contrast, cells that lack myosin-II completely fail to bleb. We conclude that bleb expansion is likely to be driven by hydrostatic pressure produced by cortical contraction involving myosin-II. As blebs are induced by chemoattractant, we speculate that hydrostatic pressure is one of the forces driving pseudopod extension during movement up a gradient of cyclic-AMP.

Introduction

The mechanisms that power the movement of cells have yet to be fully resolved. Actin polymerisation is thought to produce force that extends the leading edge of most motile cell types [1] but whether this is the sole force driving protrusion is less certain. Indeed, other methods of extending pseudopodia have been put forward including diverse mechanisms such as lipid flow [2] and hydrostatic pressure [3–5]. Such models may not necessarily be mutually exclusive.

In addition, there are clear variations in the way different cell-types move. *Amoeba proteus* move by fountain streaming powered by contraction of a thick cortical layer made primarily of actin [4]. In this cell type, hydrostatic pressure provides the motive force and drives pseudopod extension [6]. In contrast, nematode sperm lack actin and no myosin-like motor has been identified. The movement of these cells, therefore, may well be powered solely by the action of major sperm protein (MSP), a globular protein that, like actin, readily polymerises and does so beneath the leading edge of the cell to project the membrane forward [7]. It seems likely that the processes powering the movement of other types of cells, including those of animals, fall somewhere between these two extremes of polymerisation-driven protrusion and hydrostatic pressure generated by contraction of the cell body.

Furthermore, cells of the same type appear to be able to move in a variety of ways under different circumstances. *Dictyostelium discoideum* amoebae, for instance, normally crawl in a similar manner to neutrophils and fibroblasts, but cells moving with a large rounded pseudopod devoid of actin have

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0014-4827/$ – see front matter.
doi:10.1016/j.yexcr.2006.03.007

been described at the slug stage [8] whilst paddle-shaped cells also arise during the differentiation of stalk cells developing in monolayers [9]. Deletion of the *amiB* gene yields *Dictyostelium* amoebae that move in a manner reminiscent of a keratocyte, with a broad flat lamellipod [10]. Such versatility in movement is not unique to *Dictyostelium*. Tumour cells can move in two ways, one utilising actin rich protrusions and another involving rounded bleb-like extensions, and can switch between the two modes of motility should one be blocked [11,12].

Thus, more than a single component, such as actin polymerisation at the cell front, may contribute to the force driving expansion of the leading edge. Methods are therefore required to dissect the various force-generating mechanisms that operate in a motile cell. The study of blebbing assists in this dissection as blebs represent a distinct form of plasma membrane extension. Bleb is a general term commonly applied to a cell extension that is rapidly extended and has a rounded morphology. However, blebs can differ in substantial ways. For instance, the blebs of *Dictyostelium* cells overexpressing dominantly active RacB are actin-rich processes, similar to modified pseudopodia [13]. Blebs of carcinosarcoma cells are characterised by a lack of F-actin beneath the plasma membrane as the bleb expands [14]. Such differences presumably reflect distinct mechanisms of bleb formation.

The surface of *Dictyostelium* cells can be observed to bleb dramatically after the application of a high and uniform concentration of cyclic-AMP. Here, we present a detailed analysis of this striking response to chemoattractant.

Materials and methods

Strain propagation and development

Strains were derived from the wild-type Ax2 and grown in shaken suspension or on tissue culture plates in axenic medium with vitamins (70 nM B12, 80 nM Biotin and 0.5 μM Riboflavin) at 22°C [15]. Aggregation competent amoebae were obtained by resuspending washed cells at 2×10^7 cells/ml in KK_2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, 2 mM $MgSO_4$) with shaking at 180 rpm at 22°C for 1 h and pulsed with 70–90 nM cyclic-AMP (final) every 6 min for a further 4 h.

Reporter and mutant strains

Cells were transformed with the reporter construct of monomeric RFP fused to the F-actin binding domain (ABD) of ABP-120 [16] by electroporation with 20 μg of plasmid DNA per 4×10^6 cells. Transformants were selected and maintained with axenic medium supplemented with 10 μg/ml blastocidin.

The Arp2-GFP mutant (strain HM2191) was produced by homologous recombination with a linearised vector composed, from 5′ to 3′, of the entire Arp2 gene, a TRGAG linker, the enhanced GFP sequence and 1.5 kb downstream sequence. Ax2 cells were transformed with this vector as described above. Fluorescent cells were isolated from a pool of transformants by FACS and without drug selection. Clones with the successful incorporation of the GFP tag were identified by PCR and confirmed by Western blotting using anti-Arp2 antibody (a kind gift of R. H. Insall). The myosin-II null mutant used was strain HS2206, the parent of which is Ax2.

Actin polymerisation in response to cyclic-AMP was measured using aggregation competent cells in suspension at 1.5×10^7 cells/ml. For each time-point, 3×10^6 cells were exposed to 1 μM cyclic-AMP and the fix/stain buffer (25 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8, 3% formaldehyde, 0.24% Triton X-100 and 0.6 μM TRITC-phalloidin) added after the appropriate time. The sample was then mixed at room temperature and the Triton insoluble cytoskeleton was pelleted by microfugation at maximum speed for 2 min. The supernatant was then aspirated off and the pellet washed in buffer (25 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8 and 0.1% saponin) for 30 min at room temperature. The cytoskeleton was pelleted as before and the TRITC-phalloidin extracted in 1 ml methanol by vigorous shaking for 15 min, after which the fluorescence in the sample was measured.

The blebbing assay

A simple assay, termed the blebbing assay, was developed in order to observe the morphological response of cells to the sudden addition of 1 μM cyclic-AMP. 4×10^5 aggregation competent cells in 150 μl KK_2 were allowed to settle in a well of a Labtek eight-chamber glass coverslip (Nalgene). DIC and fluorescent images were collected using a 60× oil immersion objective on a Nikon Eclipse TE300 inverted microscope fitted with the Biorad Radiance confocal system.

Final cyclic-AMP (1 μM) was introduced to the cells by gently pipetting 4 μM cyclic-AMP in 50 μl KK_2 into the glass chamber. This instantaneously mixes the cyclic-AMP to form a uniform 1 μM cyclic-AMP concentration, as shown by the rapid (less than 1 second) uniform dispersal of fluorescein added to the chamber in the same manner. Throughout this work, control Ax2 cells were used that express the mRFPmars actin binding domain (ABD) reporter which provides a temporal reference point since the reporter always redistributed to the cell perimeter 2 s after the addition of chemoattractant. Cells with the reporter bleb in a manner indistinguishable from Ax2 cells without the reporter (data not shown).

The timing of cyclic-AMP addition was recorded and an image of the cells taken every 2 s for a further 3 min, to produce a movie comprising one trial of the blebbing assay. Statistical analysis was carried out using paired or unpaired *t* test.

Surface area and volume measurements were obtained by taking sections every 0.25 μm from the bottom to the top of cells expressing the cyclic-AMP receptor (cAR1) tagged with GFP as they responded to 1 μM cyclic-AMP. The boundary of fluorescence in each section was then combined using Volocity software (Improvision) to generate a three-dimensional reconstruction of the surface of each cell from which the surface area and volume could be extracted (D. Traynor and RRK, unpublished observation). Other image analysis was performed using ImageJ software (NIH).