Exclusion of exogenous phosphatidylinositol-3,4,5-trisphosphate from neutrophil-polarizing pseudopodia: stabilization of the uropod and cell polarity

Wei Tian, Iraj Laffafian, Sharon Dewitt & Maurice B. Hallett*

Neutrophil Signalling Group, University Department of Surgery, University of Wales College of Medicine, Heath Park, Cardiff, UK

Although there is accumulating evidence that the generation and localization of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) have important functions in neutrophil polarization and chemotaxis, the mechanism of this linkage has yet to be established. Here, using exogenous fluorescent PtdIns(3,4,5)P₃ introduced into the inner leaflet of the neutrophil plasma membrane by a cationic carrier, we show that: first, PtdIns(3,4,5)P₃ uniformly delivered to the neutrophil plasma membrane is excluded from newly forming pseudopodia; second, PtdIns(3,4,5)P₃ translocates to and is immobilized at the pole opposite a stable polarizing pseudopod; third, asymmetric delivery of PtdIns(3,4,5)P₃ to the neutrophil triggers the generation of polarizing pseudopodia at the opposite pole; and finally, PtdIns(3,4,5)P₃ triggers repetitive Ca²⁺ signals, the onset of which precedes morphological polarization. These data suggest that translocation and immobilization of PtdIns(3,4,5)P₃ or a 3,*x*-phosphorylated metabolite in the uropod functions as an important polarization cue that defines neutrophil polarity and stabilizes the generation of pseudopodia at the opposite pole.

EMBO reports 4, 982–988 (2003)

doi:10.1038/sj.embor.embor947

INTRODUCTION

There is accumulating evidence that products of phosphatidylinositol-3-kinases (PI(3)Ks) have important functions in several aspects of neutrophil function. Neutrophil chemotaxis is impaired in mice deficient for the gene that encodes PI(3)K- γ (Sasaki *et al.*, 2000; Li *et al.*, 2000; Hirsch *et al.*, 2000; Stephens *et al.*, 2002), and there is evidence for functions of phosphatidylinositol-3-

phosphate in phagocytosis (Ellson *et al.*, 2001; Stephens *et al.*, 2002) and of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) in chemotaxis and cell polarization (Rickert *et al.*, 2000; Servant *et al.*, 2000). The evidence for a function of PtdIns(3,4,5)P₃ in neutrophil polarization comes from two main approaches: first, neutrophil chemotaxis is defective in mice deficient for the gene that encodes PI(3)K- γ (Sasaki *et al.*, 2000; Li *et al.*, 2000; Hirsch *et al.*, 2000). Second, green fluorescent protein (GFP)–PH-Akt (pleckstrin homology domain of Akt) constructs, which bind PtdIns(3,4,5)P₃ and other lipids, translocate to the leading edge of myeloid (neutrophil-like) cells during chemotaxis polarization (Rickert *et al.*, 2000; Servant *et al.*, 2000). However, the characteristics of this lipid product of PI(3)K in the neutrophil have not yet been defined. We have therefore determined the characteristics and consequences of introducing exogenous, fluorescently labelled PtdIns(3,4,5)P₃ into neutrophils.

RESULTS AND DISCUSSION

Membrane PtdIns(3,4,5)P₃ excluded from microdomains

Incubation with exogenous BODIPY–PtdIns(3,4,5)P₃ did not result in its incorporation into the outer leaflet of the plasma membrane or triggering of morphological changes in neutrophils. However, when a complex of the phospholipid with a cationic ‘shuttle-carrier’ or histone (Ozaki *et al.*, 2000) was formed first, PtdIns(3,4,5)P₃ readily crossed the plasma membrane, where the lipid was freed to insert into the inner face of the bilayer and other intracellular sites. Using this technique, fluorescently labelled PtdIns(3,4,5)P₃ introduced into the inner leaflet of the plasma membrane had a distinctive localization in the bilayer. Unlike other plasma-membrane markers (such as Dil), there were distinctive discontinuities in the distribution of PtdIns(3,4,5)P₃, with small regions of the plasma membrane from which PtdIns(3,4,5)P₃ was excluded (Fig. 1A). Phosphorylation of the inositol-head group of the lipid was required for this effect, as PtdIns (without head-group phosphorylation) or phosphatidic acid (PA; lacking the inositol-head group completely) were

Neutrophil Signalling Group, University Department of Surgery, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK

*Corresponding author. Tel: +44 29 20 742748; Fax: +44 29 20 761623; E-mail: hallettmb@cf.ac.uk

Received 28 October 2002; revised 11 June 2003; accepted 21 August 2003
Published online 26 September 2003

Fig. 1 | Restricted diffusion of phosphatidylinositol-3,4,5-trisphosphate in neutrophils. Confocal images are shown through the equatorial plane of spherical neutrophils loaded with (A) phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃; BODIPY FL C5,C6PtdIns(3,4,5)P₃), (B) phosphatidic acid (PA; BODIPY FL C5-HPA), (C) PtdIns(3,4)P₂ (BODIPY FL C5,C6PtdIns(3,4)P₂) and (D) PI(4,5)P₂ (BODIPY TMR-X C5,C6PtdIns(4,5)P₂). In the bottom panels, typical data are shown for (E) PtdIns(3,4,5)P₃ and (F) PA. The three confocal images (left panels in (E,F)) show the effect of laser photobleaching in the marked area (shown by a box). Cells are shown before photobleaching (upper left panels), immediately after photobleaching (middle left panels) and 120 s after photobleaching (bottom left panels), and the graphs (right panels in (E) and (F)) show the ratio (*R'*) of intensities in the marked zone and the opposite pole. A comparison of mobilities can be made from the extent of the spread of the bleaching outside the laser-scanning area and the return of fluorescence into this area. These data were typical of at least three other separate experiments in each case. The diameter of the neutrophils shown in this and subsequent figures was approximately 10 μ m.

distributed uniformly, without exclusion zones (Fig. 1B). Furthermore, phosphorylation at position 3 of the inositol head was essential for the exclusion effect as two phosphatidylinositol biphosphate (PtdInsP₂) molecules phosphorylated at the 3 position (PtdIns(3,4)P₂ and PtdIns(3,5)P₂) and delivered to the inner leaflet by shuttle-carrier also had discontinuities at the plasma membrane, whereas a PtdInsP₂ that is not phosphorylated at position 3 (PtdIns(4,5)P₂) did not (Fig. 1C,D). This effect did not depend on the nature of the fluor group in the 'fatty-acid chain', and was seen with both BODIPY FL and TMR analogues. As inositol phospholipids that are phosphorylated at position 3 and those that are not were both introduced into the cell by the same carrier, it was also concluded that the exclusion zones were not generated as a result of the carrier. Furthermore, exclusion zones were seen in the absence of carrier when BODIPY-PtdIns(3,4,5)P₃ was incorporated into the inner leaflet by microinjection.

Reduced mobility of PtdInsP₃ in the plasma membrane

From fluorescence recovery after photobleaching measurements, it was estimated that the mobility of introduced PtdIns(4,5)P₂ and PA had similar diffusion characteristics (diffusion constant

$D \approx 1 \mu\text{m}^2 \text{s}^{-1}$), with 100% of molecules free to diffuse (Fig. 1F). In marked contrast, the level of diffusion of PtdIns(3,4,5)P₃ was low, the molecule being unable to diffuse into exclusion-zone boundaries or photobleached regions (Fig. 1E). The mobile fraction of PtdIns(3,4,5)P₃, PtdIns(3,4)P₂ and PtdIns(3,5)P₂ at these sites was almost zero, suggesting that these inositol lipids that are phosphorylated at position 3 were immobilized in the inner leaflet of the bilayer. This reduced mobility was probably the result of association with a saturable binding site, as the mobile fraction increased with higher loading levels. These extremely low mobilities, perhaps resulting from tethering to immobilized components in the cytosol, provide an explanation for the inability of PtdIns(3,4,5)P₃ to move after an exclusion region had been established in the membrane.

PtdIns(3,4,5)P₃ exclusion from forming pseudopodia

Intracellular PtdIns(3,4,5)P₃, loaded as a membrane-permeant ester (PtdIns(3,4,5)P₃-AM) induces neutrophil polarization (Niggli, 2000). We also found that the frequency of random formation of small pseudopodia increased after carrier-loading with PtdIns(3,4,5)P₃ and that after 2–10 min this resulted in the production of a single, large pseudopod, which gave the neutrophils

Fig. 2 | Exclusion of phosphatidylinositol-3,4,5-trisphosphate from polarizing pseudopodia. (A) Formation and withdrawal of an abortive polarizing pseudopod (indicated by an asterisk). (B) Distribution of loaded phosphatidylinositol-3,4,5-trisphosphate. (C) Intensity (I) of fluorescence measured in the two zones indicated on the first fluorescent image (B). (D) Ratio (R) ($I_1 - BG$)/($I_2 - BG$), where I_x is the intensity in zone x and BG is the background signal. (E) A fluorescent sequence is shown in (a–d) in which an initially abortive pseudopod develops into a permanent polarizing pseudopod (e). The intensity of fluorescence and ratio from this sequence are shown in (F) and (G), as described in (C) and (D), respectively. The complete data set from which (Ea–Ee) were taken can be viewed as supplementary information online.

their polarized morphology. Abortive polarizing pseudopodia formed and retracted during this period (Fig. 2A). As this occurred, PtdIns(3,4,5)P₃ was temporarily excluded from the site of pseudopod formation, returning to that region of the cell as the pseudopod relaxed (Fig. 2B). The loss of fluorescence at these sites is unlikely to be due to an irreversible bleaching phenomenon, as the loss of fluorescence at one pole was accompanied by increased fluorescence at the other (Fig. 2C,D), consistent with the movement of fluorescent molecules around the cell. When stable polarizing pseudopodia formed, often at the same location as a previous abortive pseudopod, a similar movement of fluorescent PtdIns(3,4,5)P₃ was seen (Fig. 2E–G; and see supplementary information online), producing a permanent major exclusion zone for exogenous PtdIns(3,4,5)P₃ (Fig. 2F,G) that was seen in both perpendicular and horizontal confocal sections (Fig. 3B). This exclusion resulted in the translocation to and immobilization

of PtdIns(3,4,5)P₃ at the uropods of polarized and chemotactic neutrophils (Fig. 3C). Although non-uropodal PtdIns(3,4,5)P₃ was released by Triton X-100 (0.5%), uropodal PtdIns(3,4,5)P₃ was resistant to this treatment (Fig. 3D,E), suggesting that it might be bound to cytoskeletal components that were not dissociated by this treatment.

To test whether the BODIPY–PtdIns(3,4,5)P₃-induced polarizing pseudopod was based on the actin cytoskeleton, cytochalasin B, an actin polymerization inhibitor, was used. When pre-treated with cytochalasin B (5 μ g ml^{−1}) before loading with BODIPY–PtdIns(3,4,5)P₃, no polarizing pseudopodia formed. After cell morphological polarization was initiated, but before full retraction of PtdIns(3,4,5)P₃ into the uropod (Fig. 3C), cytochalasin B caused withdrawal of the polarizing pseudopod (Fig. 3A) without an accompanying release of BODIPY–PtdIns(3,4,5)P₃, which remained immobilized and polarized (Fig. 3A). Thus, the formation

Fig. 3 | Translocation of phosphatidylinositol-3,4,5-trisphosphate. (A) Images (Aa) and (Ab) show a field of neutrophils before (a) and soon after (b) polarization was evident, with newly formed polarizing pseudopodia indicated by asterisks in (b). (Ac) Images of cells after addition of cytochalasin B (CB; 5 $\mu\text{g ml}^{-1}$), with the retracted pseudopodia marked with double asterisks. The images in the lower row correspond to those directly above them in the upper row, and show the distribution of BODIPY-phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) in the same cells. (B) Confocal section in the horizontal (xy; left panel) and perpendicular (xz; right panel) planes showing BODIPY-PtdIns(3,4,5)P₃ distribution in a newly polarized neutrophil. (C) A time sequence taken at 60-second intervals, showing the progressive movement of BODIPY-PtdIns(3,4,5)P₃ into a single region, the uropod, of a highly polarized neutrophil. (D,E) Neutrophils before (D) and after (E) the addition of Triton X-100 (0.5%; the same cells are shown in (D) and (E)). Two cells have defined uropods with accumulated BODIPY-PtdIns(3,4,5)P₃ (indicated by asterisks), which are resistant to detergent extraction. The third cell has a polarized distribution of BODIPY-PtdIns(3,4,5)P₃ (marked by arrows), which is dispersed by Triton X-100 (0.5%).

of the polarizing pseudopod was dependent on actin polymerization, but the tethering PtdIns(3,4,5)P₃ was not sensitive to cytochalasin B. This latter evidence might not rule out the cytoskeleton as the immobilization tether because, unlike F-actin in pseudopodia, the cortical actin network is largely resistant to depolymerization by cytochalasins (Sheterline *et al.*, 1986, 1989). The immobility of PtdIns(3,4,5)P₃ may result from binding to PH-domain-containing cytoskeleton-associated proteins. Such a protein has recently been identified as an unconventional myosin, myosin X, in macrophages (Cox *et al.*, 2002). It is possible that this protein is present in other myeloid cells, including neutrophils, and that it may translocate to the rear of the polarized neutrophil with myosin II (Eddy *et al.*, 2000) and moesin (Seveau *et al.*, 2000).

In neutrophil-related myeloid cells, the PtdIns(3,4,5)P₃-binding construct, GFP-PH-Akt, shows that endogenously produced PtdIns(3,4,5)P₃ accumulates at the front of polarized myeloid cells (Rickert *et al.*, 2000; Weiner *et al.*, 2002; Stephens *et al.*, 2002), with no PtdIns(3,4,5)P₃ being detectable at the uropod. However, in T cells a similar approach detected PtdIns(3,4,5)P₃ at both the front (at the immunological synapse) and rear of the cell (Costello *et al.*, 2002). There are several possible reasons for the discrepancy in location of GFP-PH-Akt-tagged PtdIns(3,4,5)P₃ and exogenous PtdIns(3,4,5)P₃. The first is that the exogenous

PtdIns(3,4,5)P₃ was rapidly dephosphorylated. In T cells, endogenous PtdIns(3,4,5)P₃ was broken down in 2–3 min to metabolites that are not recognized by GFP-PH-PKB (protein kinase B; Costello *et al.*, 2002). It is possible that, in neutrophils, exogenous PtdIns(3,4,5)P₃ is also rapidly dephosphorylated to another product (possibly PtdIns-3-P), which located to rear of the neutrophil but was not able to bind to the GFP-PH-Akt construct. However, thin-layer chromatography (TLC) analysis of extracted fluorescent lipid, showed that exogenous PtdIns(3,4,5)P₃ was converted to PtdInsP₂ (with no PtdIns(3)P being detectable), with a half-time of about 5 min (see supplementary information online). At the time that the fluorescent signal started to become polarized, at least 40–80% of the fluor was still present in PtdIns(3,4,5)P₃. However, as two of the exogenously added phosphatase products of PtdIns(3,4,5)P₃, namely PtdIns(3,4)P₂ and PtdIns(3,5)P₂, also translocated to the rear of the neutrophil, either of these lipid metabolites (but not PtdIns(4,5)P₂) could contribute to the phenomenon. It should be noted that the immobility of exogenous BODIPY-PtdIns(3,4,5)P₃ (detectable before significant dephosphorylation) is consistent with the immobility of PH-GFP binding in a myeloid cell line (Marshall *et al.*, 2001). The second possibility for the difference in distribution of exogenous and GFP-PH-Akt-tagged endogenous PtdIns(3,4,5)P₃ might be that the tight binding between PtdIns(3,4,5)P₃ and the

cytoskeletal components excluded binding to GFP-PH-Akt. 'Clearance' of PtdIns(3,4,5)P₃ by this mechanism may be more significant under the experimental conditions used here, namely a uniformly (and artificially) elevated level of PtdIns(3,4,5)P₃, than under other conditions. In fixed neutrophils, an antibody to PtdIns(3,4,5)P₃ was able to detect endogenously formed PtdIns(3,4,5)P₃ in exogenous PtdInsP₃-induced pseudopodia and at the front of fully polarized neutrophils (see supplementary information online). However, the antibody failed to detect the exogenous PtdInsP₃ either in the uropod or away from forming pseudopodia. This phenomenon was also reported by Weiner *et al.* (2002) and was consistent with tight binding between PtdIns(3,4,5)P₃ and cytoskeletal components, precluding binding to either GFP-PH-Akt or antibody.

Uropod formation by PtdIns(3,4,5)P₃

A notable feature of the region of the membrane in which BODIPY-PtdIns(3,4,5)P₃ accumulated during polarization was its unresponsiveness and apparent immobility. Pseudopodia did not form in the PtdIns(3,4,5)P₃-containing regions of the cell membrane when challenged by f-met-leu-phe, or in response to the presentation of opsonized particulate stimuli (Dewitt & Hallett, 2002), whereas areas of the plasma membrane in the same cell that were devoid of exogenous PtdIns(3,4,5)P₃ were able to form pseudopodia (Fig. 4D). This unresponsiveness and immobility is reminiscent of the uropod, which is usually a small region at the extreme rear of the cell. It is therefore possible that before restriction of PtdIns(3,4,5)P₃ to the uropod, the exogenously added PtdIns(3,4,5)P₃ generated an artificial and enlarged 'uropod-like'

Fig. 4 | Effects of phosphatidylinositol-3,4,5-trisphosphate asymmetry on morphology and Ca²⁺ signalling. The effect of asymmetric phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) delivery is shown in (A), in which the sequence of images (taken at 20-second intervals) shows (a) the initially non-polarized neutrophil before ejection of PtdIns(3,4,5)P₃, (b) immediately after formation of the polarizing pseudopod, (c) after full development of the polarizing pseudopod (with the cell edge nearer the micropipette still showing a similar curvature to that seen before the stimulus), and (d) finally developing motility and (e) moving away from the micropipette. The whole sequence can be viewed as supplementary information online. In (B), the images show cytosolic free Ca²⁺ changes in a fura2-loaded neutrophil, pseudo-coloured according to the scale shown, as PtdIns(3,4,5)P₃ was ejected from the micropipette (indicated by arrows). Both the cytosolic free-Ca²⁺ concentration and the morphological changes are evident. The graph in (C) shows typical repetitive Ca²⁺ signalling from a neutrophil in response to asymmetric PtdIns(3,4,5)P₃ delivery. The lower traces show data obtained in the presence of the Ca²⁺-channel-blocking ion Ni²⁺ (1 mM) and LY294002 (LY; 50 µM). These data are representative of at least three separate experiments. (D) Images showing the asymmetric distribution of BODIPY-PtdIns(3,4,5)P₃ in a neutrophil, with the position of two C3bi-opsonized zymosan particles indicated by asterisks. The last three images show that the lower particle (double asterisk), which was firmly attached to a region of the cell membrane labelled with BODIPY-PtdIns(3,4,5)P₃, was not phagocytosed, whereas when the upper particle (single asterisk) was brought into contact with the cell in a region without BODIPY-PtdIns(3,4,5)P₃, phagocytosis occurred immediately. The images were taken at ten-second intervals and show the formation and closure of the phagocytic cup around the upper particle. This effect was observed in at least three other cells.

surface. PtdIns(3,4,5)P₃ immobilized to the sub-cortical cytoskeleton may be 'forced' away from the site of formation of pseudopodia by newly formed actin filaments, as has been shown for DiIc₁₆-staining, detergent-resistant membrane domains (Seveau *et al.*, 2001). In this case, the movement of PtdIns(3,4,5)P₃ to a particular pole (to ultimately form a tight uropod) would stabilize cell polarity by ensuring that pseudopodia could not form on all parts of the cell. To test this, PtdIns(3,4,5)P₃ was delivered asymmetrically to non-polarized neutrophils using a micropipette, producing a localized increase in PtdIns(3,4,5)P₃ at one pole of the cell (Fig. 4A). Under these conditions, asymmetrically introduced PtdIns(3,4,5)P₃ (or BODIPY–PtdIns(3,4,5)P₃), but not PtdIns(4,5)P₂, caused neutrophils to polarize rapidly, with the stable pseudopodia forming only at the pole opposite the source of the PtdIns(3,4,5)P₃ (Fig. 4A). The pole nearer to the source of the PtdIns(3,4,5)P₃ failed to form pseudopodia, was inert, and often maintained its original curvature (Fig 4A; and see supplementary information online).

PtdIns(3,4,5)P₃-induced Ca²⁺ signalling before polarization

As there is a known link between PtdIns(3,4,5)P₃ and Ca²⁺ signalling in myeloid cells (Vossebeld *et al.*, 1997; Ching *et al.*, 2001), cytosolic Ca²⁺ signalling during exogenous PtdIns(3,4,5)P₃ elevation was monitored. Asymmetric challenge with PtdIns(3,4,5)P₃ stimulated repetitive global Ca²⁺ signalling events (Fig. 4C), the first of which preceded the formation of the polarizing pseudopod (Fig. 4B). Both the Ca²⁺ signalling and the polarization were inhibited by Ni²⁺ (Fig. 4C), an ion that blocks plasma membrane Ca²⁺-channels in neutrophils (Davies & Hallett, 1996). Surprisingly, the Ca²⁺ signalling induced by exogenous PtdIns(3,4,5)P₃ was inhibited by a PtdIns-3-OH kinase (PI(3)K) inhibitor, LY294002 (50 mM), suggesting that part of its action was mediated indirectly through the increased PI(3)K activity caused by exogenous PtdIns(3,4,5)P₃ (Weiner *et al.*, 2002). The dependence of the polarizing effect of exogenous PtdIns(3,4,5)P₃ on PI(3)K activity has been shown previously (Niggli, 2000). Although PI(3)K activation might in some way be 'non-specific', it is triggered only by an elevation in PtdIns(3,4,5)P₃ inside the cell, whether by an AM ester, a carrier or by microinjection, thus eliminating a transmembrane signalling route.

The data in this paper thus indicate that PtdIns(3,4,5)P₃-induced morphological polarization in neutrophils results from a combination of an influx of Ca²⁺ and, ultimately, membrane tethering of PtdIns(3,4,5)P₃ by the cytoskeleton in the uropod. We propose that cytoskeletally immobilized PtdIns(3,4,5)P₃ suppresses pseudopod formation, and that by forming an immobile uropod it defines the polarity of the neutrophil. The uropodal PtdIns(3,4,5)P₃ at the rear of the cell might also have a function in anchoring cytoskeletal elements to the membrane to provide leverage for the contraction of the uropod during chemotaxis (Hallett, 1997; Eddy *et al.*, 2000).

METHODS

Neutrophils were isolated from heparinized blood of healthy volunteers, as described previously (Pettit & Hallett, 1998), and were loaded with fluorescent phospholipid by the addition of preformed shuttle-carrier-lipid complexes, formed by incubating equal amounts of carrier (Shuttle PIP carrier-1; Molecular Probes) with lipid in chloroform for 30 min. Lipid-carrier complexes

were added to cell suspensions or delivered by soft lipid-assisted microinjection (SLAM) (Laffafian & Hallett, 1998) to give a final concentration of 66 µM for PtdInsP₂ molecules and 56 µM for PtdIns(3,4,5)P₃. In an attempt to quantify the amount of phospholipid delivered to individual cells, fluorescent shuttle-carrier was used. However, microinjected fluorescent shuttle-carrier accumulated in the nucleus, and extracellularly added shuttle remained associated with the membrane, making this approach unsuitable for quantifying the delivery of BODIPY–PtdIns(3,4,5)P₃ and other lipids to the cells. However, as the intensity of BODIPY–PtdIns(3,4,5)P₃ in the membrane was comparable to that of PH–Akt–GFP (Weiner *et al.*, 2002), it is unlikely that the amount of exogenous PtdInsP₃ incorporated into the cell was much higher than that achieved physiologically. In addition, at least 80% of the BODIPY–PtdIns(3,4,5)P₃ was delivered to the inner face of the plasma membrane, as more than this amount was metabolized by intracellular phosphatases.

Simultaneous cytosolic free-Ca²⁺ imaging and fluorescent imaging of BODIPY–PtdIns-phosphates or fura2 was achieved by using excitation wavelengths (480 nm for BODIPY; 340 nm and 380 nm for fura2) that were selected using a rapid monochromator (Delta RAM; PTI) that was connected to a Nikon Eclipse inverted microscope. The cells were maintained at 37 °C using a temperature-controlled microscope-stage heater. The images at each excitation wavelength were collected using an intensified charged-coupled device (CCD) camera (IC100; PTI) and the ratio image was calculated using Image Master software (PTI). All images were captured using an oil-immersion 100× objective lens. Phase-contrast images were taken under far-red illumination simultaneously with acquisition of fluorescent images using an appropriate dichroic mirror and a second red-sensitive CCD camera. The mobility of BODIPY lipids was assessed by photobleaching. A confocal plane was chosen that approximately bisected the cell to provide an image of the cell equator at low laser power. The voltage on the photomultiplier tube was set to maximum and line averaging was used. The portion of the cell to be photobleached was subjected to high-power laser-scanning for 10–30 s. Confocal images were then acquired as the fluorescence in the bleached area recovered. The ratios of the intensities of the bleached to non-bleached regions of the cell (*I*/*I*₀) were measured at time intervals (3–30 s) during the following two minutes. The rate of recovery after photobleaching (*k*) and the fraction of mobile molecules (*Mf*) was calculated as $I/I_0 = Mf(1 - e^{-kt})$, where $k = 1/\ln_2 t_{0.5}$ and *t* is the time after bleaching. Zymosan particles were presented as described previously (Dewitt & Hallett, 2002).

Asymmetric delivery of PtdIns(3,4,5)P₃ to neutrophils was achieved by placing a micropipette (tip diameter approximately 1–2 µm) containing the lipid-carrier complex within 5–15 µm of the neutrophil and applying a low-pressure (40–50 mbar) pulse (Laffafian & Hallett, 1995). Ejected BODIPY-labelled lipid-carrier complex was seen as fluorescent clouds touching the edge of the nearby cells, which 'stained' the near side of the neutrophils. All fluorescent reagents were purchased from Molecular Probes. TLC of extracted lipids was performed on oxalate-impregnated silica gel as described in Traynor-Kaplan *et al.* (1989), except that precautions were taken to minimize bleaching of the fluor during extraction and separation (by omitting strong acids and using subdued lighting) so that the fluorescent signals could be detected and quantified on the TLC plates.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We are grateful to the Wellcome Trust (UK) for supporting this work.

REFERENCES

- Ching, T., Hsu, A., Johnson, A.J. & Chen, C. (2001) Phosphoinositide 3-kinase facilitates antigen-stimulated Ca²⁺ influx in RBL-2H3 mast cells via a PI(3,4,5)P₃-sensitive entry mechanism. *J. Biol. Chem.*, **276**, 14814–14820.
- Costello, P.S., Gallagher, M. & Cantrell, D.A. (2002) Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. *Nature Immunol.*, **3**, 1082–1089.
- Cox, D., Berg, J.S., Cammer, M., Chingwundoh, J.O., Dale, B.M., Cheney, R.E. & Greenberg, S. (2002) Myosin X is a downstream effector of PI(3)K during phagocytosis. *Nature Cell Biol.*, **4**, 469–477.
- Davies, E.V. & Hallett, M.B. (1996) Near membrane Ca²⁺ changes resulting from store release in neutrophils: detection by FFP-18. *Cell Calcium*, **19**, 355–362.
- Dewitt, S. & Hallett, M.B. (2002) Cytosolic free Ca²⁺ changes and calpain activation are required for β 2 integrin-accelerated phagocytosis by human neutrophils. *J. Cell Biol.*, **159**, 181–189.
- Eddy, R.J., Pierini, L.M., Matsumura, F. & Maxfield, F.R. (2000) Ca²⁺-dependent myosin II activation is required for uropod retraction during neutrophil migration. *J. Cell Sci.*, **113**, 1287–1298.
- Ellson, C.D., Anderson, K.E., Morgan, G., Chilvers, E.R., Lipp, P., Stephens, L.R. & Hawkins, P.T. (2001) Phosphatidylinositol 3-phosphate is generated in the phagosomal membranes. *Curr. Biol.*, **11**, 1631–1638.
- Hallett, M.B. (1997) Controlling the molecular motor of neutrophil chemotaxis. *Bioessays*, **19**, 615–621.
- Hirsch, E., Katanaev, V.L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F. & Wymann, M.P. (2000) Central role for G-protein-coupled phosphoinositide 3-kinase γ in inflammation. *Science*, **287**, 1049–1052.
- Lafffian, I. & Hallett, M.B. (1995) Does cytosolic free Ca²⁺ signal neutrophil chemotaxis? *J. Cell Sci.*, **108**, 3199–3205.
- Lafffian, I. & Hallett, M.B. (1998) Lipid-assisted microinjection: introducing material into the cytosol and membranes of small cells. *Biophys. J.*, **75**, 2558–2563.
- Li, Z., Jiang, H., Xie, X., Zhang, Z., Smrcka, A.V. & Wu, D. (2000) Roles of PLC- β 2 and β 3 and PI3K γ in chemoattractant-mediated signal transduction. *Science*, **287**, 1046–1049.
- Marshall, J.G., Booth, J.W., Stambolic, V., Mak, T., Balla, T., Schreiber, A.D., Meyer, T. & Grinstein, S. (2001) Restricted accumulation of PI3-kinase products in a plasmalemmal subdomain during FC γ receptor-mediated phagocytosis. *J. Cell Biol.*, **153**, 1369–1380.
- Niggli, V. (2000) A membrane permeant ester of PI(3,4,5)P₃ is an activator of human neutrophil migration. *FEBS Lett.*, **473**, 217–221.
- Ozaki, S., DeWald, D.B., Shope, J.C., Chen, J. & Prestwich, G.D. (2000) Intracellular delivery of phosphoinositides and inositol phosphates using polyamine carriers. *Proc. Natl Acad. Sci. USA*, **97**, 11286–11291.
- Pettit, E.J. & Hallett, M.B. (1998) Release of 'caged' cytosolic Ca²⁺ triggers rapid spreading of human neutrophils adherent via integrin engagement. *J. Cell Sci.*, **111**, 2209–2215.
- Rickert, P., Weiner, O.D., Wang, F., Bourne, H.R. & Servant, G. (2000) Leukocytes navigate by compass: roles of PI3K γ and its lipid products. *Trends Cell Biol.*, **10**, 466–473.
- Sasaki, T. et al. (2000) Function of PI3K γ in thymocyte development, T cell activation and neutrophil migration. *Science*, **287**, 1040–1046.
- Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W. & Bourne, H.R. (2000) Polarization of chemoattractant receptor signalling during neutrophil chemotaxis. *Science*, **287**, 1037–1040.
- Seveau, S., Keller, H., Maxfield, F.R., Piller, F. & Halbwachs-Mecarelli, L. (2000) neutrophil polarity and locomotion are associated with surface redistribution of leukosialin (CD43), an anti-adhesive membrane molecule. *Blood*, **95**, 2462–2470.
- Seveau, S., Eddt, R.J., Maxfield, F.R. & Pierini, L.M. (2001) Cytoskeletal-dependent membrane domain segregation during neutrophil polarization. *Mol. Biol. Cell*, **12**, 3550–3562.
- Shetlerline, P. & Rickard, J.E. (1989) in *The Neutrophil: Cellular Biochemistry and Physiology* (ed. Hallett, M.B.), 141–166. CRC, Boca Raton, Florida, USA.
- Shetlerline, P., Rickard, J.E., Boothroyd, B. & Richards, R.C. (1986) Phorbol ester induces rapid actin assembly in neutrophils independently of changes in [Ca²⁺]_i or pH_i. *J. Musc. Res. Cell Motil.*, **7**, 405–411.
- Stephens, L., Ellson, C. & Hawkins, P. (2002) Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr. Opin. Cell Biol.*, **14**, 203–213.
- Traynor-Kaplan, A.E., Thompson, B.L., Harris, A.L., Taylor, P., Omann, G.M. & Sklar, L.A. (1989) Transient increase in PI(3,4)P₂ and PIP₃ during activation of human neutrophils. *J. Biol. Chem.*, **264**, 15668–15673.
- Vossebeld, P.J.M., Homburg, C.H.E., Schweizer, R.C., Ibarrola, I., Koenderman, L., Roos, D. & Verhoeven, A.J. (1997) Tyrosine phosphorylation dependent activation of PI-3-kinase occurs upstream of Ca²⁺ signalling induced by Fc- γ R cross-linking in human neutrophils. *Biochem. J.*, **323**, 87–94.
- Weiner, O.D., Neilsen, P.O., Prestwich, G.D., Kirschner, M.W., Cantley, L.C. & Bourne, H.R. (2002) A PtdInsP3 and Rho GTP-ase-mediated positive feedback loop regulates neutrophil polarity. *Nature Cell Biol.*, **4**, 509–512.