

Sensitization of *Dictyostelium* chemotaxis by phosphoinositide-3-kinase-mediated self-organizing signalling patches

Marten Postma¹, Jeroen Roelofs^{1,2}, Joachim Goedhart^{3,4}, Harriët M. Looovers¹, Antonie J. W. G. Visser⁵ and Peter J. M. Van Haastert^{1,*}

¹Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

²Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115-5730, USA

³Laboratory of Molecular Biology, Department of Plant Sciences, Wageningen University, 6703 HA Wageningen, The Netherlands

⁴Section of Molecular Cytology, Swammerdam Institute for Life Sciences, University of Amsterdam, Kruislaan 316, 1098 SM Amsterdam, The Netherlands

⁵MicroSpectroscopy Centre, Laboratory of Biochemistry, Wageningen University, 6703 HA Wageningen, The Netherlands

*Author for correspondence (e-mail: haastert@chem.rug.nl)

Accepted 3 February 2004

Journal of Cell Science 117, 2925-2035 Published by The Company of Biologists 2004

doi:10.1242/jcs.01143

Summary

The leading edge of *Dictyostelium* cells in chemoattractant gradients can be visualized using green fluorescent protein (GFP) tagged to the pleckstrin-homology (PH) domain of cytosolic regulator of adenyl cyclase (CRAC), which presumably binds phosphatidylinositol-(3,4,5)-triphosphate [PtdIns(3,4,5)P₃]. Uniform cyclic AMP (cAMP) concentrations induce persistent translocation of PH_{CRAC}-GFP from the cytosol to multiple patches, which are similar to the single patch of PH_{CRAC}-GFP at the leading edge in a cAMP gradient. We show that cAMP determines the probability of patch formation (half-maximal effect at 0.5 nM cAMP) but not the size, lifetime or intensity of patches, indicating that patches are self-organizing structures. A pseudopod is extended from the area of the cell with a PH_{CRAC}-GFP patch at about 10 seconds after

patch formation. Cells treated with the F-actin inhibitor latrunculin A are round without pseudopodia; uniform cAMP still induces localized patches of PH_{CRAC}-GFP. Inhibition of phosphoinositide-3-kinase (PI3K) activity with LY294002 inhibits PH_{CRAC}-GFP patches and inhibits chemotaxis towards nanomolar cAMP but has no effect at higher cAMP concentrations. Thus, very low cAMP concentrations induce self-organizing PH_{CRAC}-GFP patches that serve as a spatial cue for pseudopod formation, which enhances the sensitivity and amplitude of chemotactic movement.

Key words: Chemotaxis, PtdIns(3,4,5)P₃, Self-organization, Patches, Pseudopod

Introduction

Many cells exhibit chemotactic behaviour – the ability to move in the direction of a chemical gradient. Eukaryotic cells measure the difference in chemoattractant concentration between the ends of the cell and extend a pseudopod up the gradient of chemoattractant (Devreotes and Zigmond, 1988). This process is composed of several steps during which a shallow gradient is processed, yielding one pseudopod at the leading edge. The cascade starts with sensing the chemoattractant concentration around the cell by means of surface receptors. These receptors activate G-proteins, resulting in the formation of second messengers. It appears that receptors are approximately homogeneously distributed on the cell surface during chemotaxis (Servant et al., 1999; Xiao et al., 1997), whereas G-protein activation might be stronger at the side of the highest cAMP concentration (Janetopoulos et al., 2001; Jin et al., 2000; Ueda et al., 2001). At the inner phase of the plasma membrane, several second messengers are formed, including cAMP, cGMP, inositol-(1,4,5)-triphosphate and phosphatidylinositol-(3,4,5)-triphosphate [PtdIns(3,4,5)P₃] (Bosgraaf and Van Haastert, 2002; Chung et al., 2001; Huang et

al., 2003; Iijima et al., 2002). These compounds might be produced more strongly at the leading edge of the cell than at the back of the cell. Owing to the rapid diffusion of soluble compounds as cAMP or inositol-(1,4,5)-triphosphate, these second messengers are not likely to establish strong intracellular gradients. However, the membrane phospholipid PtdIns(3,4,5)P₃ diffuses about 100 times more slowly, which allows this molecule to establish and maintain high local concentrations (Postma and Van Haastert, 2001).

The phospholipid PtdIns(3,4,5)P₃ forms binding sites for PH-domain-containing proteins, such as the cytosolic regulator of adenyl cyclase (CRAC) and protein kinase B (PKB) in *Dictyostelium* (Huang et al., 2003; Iijima et al., 2002; Meili et al., 1999; Parent et al., 1998). In gradients of chemoattractant, these PH-domain-containing proteins translocate to the leading edge of the cell. Phosphoinositide-3-kinase (PI3K) translocates to the front of the cell while the degrading 3-phosphatase PTEN accumulates in the back (Funamoto et al., 2002; Iijima and Devreotes, 2002). In leukocytes, a similar translocation was observed of PH-domain-containing proteins to the leading edge (Bourne and Weiner, 2002; Haugh et al., 2000; Servant

et al., 2000). These observations led to the proposal that the local activities of PI3K and PTEN provide the accumulation of PtdIns(3,4,5) P_3 in the leading edge, and that PtdIns(3,4,5) P_3 forms binding sites for specific PH-domain-containing proteins that control the local activity of the cytoskeleton (Bourne and Weiner, 2002; Huang et al., 2003; Iijima et al., 2002). Despite the elegant mechanism of PtdIns(3,4,5) P_3 -gradient formation by reciprocal translocated PI3K and PTEN, the exact function of PI3K in chemotaxis has been surprisingly difficult to define. Chemoattractant-mediated PtdIns(3,4,5) P_3 synthesis is mainly mediated by PI3K1 and PI3K2 in *Dictyostelium*, and by PI3K γ in neutrophils (Hirsch et al., 2000; Huang et al., 2003; Zhou et al., 1995). The chemotactic defects of *Dictyostelium pi3k1-pi3k2*⁻ null cells or mammalian PI3K γ -null cells are only partial; these modest defects are also observed in wild-type cells treated with reasonable concentrations of the PI3K inhibitors LY294002 or wortmannin (Funamoto et al., 2002; Iijima et al., 2002; Wang et al., 2002). Furthermore, it has been demonstrated that the N-terminal segment of *Dictyostelium* PI3K is essential and sufficient to mediate chemoattractant-induced translocation of PI3K to the front in wild-type cells and also in *pi3k1-pi3k2*⁻ cells that have no significant PI3K activity (Funamoto et al., 2002). This suggests that the primary intracellular gradient that is induced by the chemotactic signal acts upstream of PI3K.

Extending previous work by others on translocation of fusions between the CRAC PH domain and green fluorescent protein (PH_{Crac}-GFP) (Funamoto et al., 2002; Funamoto et al., 2001; Haugh et al., 2000; Iijima and Devreotes, 2002; Meili et al., 1999; Parent et al., 1998; Servant et al., 2000), we have begun to analyse the spatial and temporal properties of cAMP-induced PH_{Crac}-GFP translocation to specific areas of the membrane in conjunction with the formation of pseudopodia (Postma et al., 2003). This study was performed in a perfusion chamber to provide fully controlled up- and downshifts of the cAMP concentration without cAMP gradients. We observed that PH_{Crac}-GFP translocation to the membrane of *Dictyostelium* cells exhibits two response phases: transient translocation to the entire membrane followed by continuous translocation to smaller areas of the cell. We show here that very low cAMP concentrations induce PH_{Crac}-GFP patches but that cAMP does not affect the size and intensity of the patches, suggesting that patches are self-organizing structures triggered by cAMP. The concept of self-organization originates from technical engineering; in cell biology, a structure or protein complex is self-organizing if its properties are determined only by the components of that structure, whereas components outside that structure might trigger its appearance but do not effect its properties (Misteli, 2001). We also show that uniform cAMP still induces multiple PH_{Crac}-GFP patches in cells treated with latrunculin A, which inhibits F-actin, leading to round symmetric cells without pseudopodia. In control cells, large pseudopodia are extended within a few seconds from area of the cell containing PH_{Crac}-GFP patches. These results suggest that very low cAMP concentrations trigger self-organizing PH_{Crac}-GFP patches, which act as spatial cues for pseudopodium formation. The PI3K inhibitor LY294002 prevents translocation of PH_{Crac}-GFP to the membrane and strongly inhibits chemotaxis to nanomolar cAMP but has no effect at higher cAMP concentrations. We conclude that receptor-stimulated PI3K activity triggers the formation of

PH_{Crac}-GFP patches, which enhance chemotactic movement by increasing both the cAMP sensitivity and amplitude of cAMP-induced pseudopod extensions.

Materials and Methods

Strain and culture conditions.

A cell line expressing the PH domain of CRAC fused to enhanced (eGFP) (Parent et al., 1998; Xiao et al., 1997) was made by electroporation of wild-type AX3 cells with plasmid WF38 (a generous gift from P. Devreotes). Cells were grown in HG5 medium with 10–40 $\mu\text{g ml}^{-1}$ neomycin in dishes to 80% confluence and starved in the dish in 10 mM Na/K phosphate buffer, pH 6.5 (PB) for 5–8 hours. Chemotaxis was assayed with the small population assay (Konijn, 1970), using hydrophobic agar containing different concentrations of LY294002 (Sigma).

Fluorescence microscopy

Starved cells were detached from the dish and seeded onto the bottom coverslip (type #1, 24 \times 60 mm) of a home-made flow chamber [a generous gift from E. Potma (Potma et al., 2001)], resulting in a cell density of about 2×10^5 cells cm^{-2} . After adherence for about 10 minutes, the flow chamber was assembled on a Zeiss LSM510 (Carl Zeiss, Germany) confocal fluorescence microscope with a Plan-Neofluor 40 \times magnification 1.30 numerical aperture oil-immersed objective. The flow chamber has entrance and exit tubes that were filled with PB to the same level. By adding fluid to the entrance tube, the chamber automatically and rapidly fills with the new liquid. In previous experiments, it has been shown that exchange of solutions results in a laminar flow without occurrence of gradients, with a delay time of approximately 1 second (Potma et al., 2001); all kinetic data presented have been corrected for this delay time. Before the start of every experiment, the chamber was rinsed with PB; the time between subsequent experiments was at least 3 minutes to let cells recover from the previous experiment.

Data analysis

A series of confocal images of 115 \times 115 μm (512 \times 512 pixels) were recorded and saved as 12-bit greyscale bitmaps; images were taken every 2 seconds. About 20 cells in each image were processed in Matlab 5.3 as described (Postma et al., 2003). Briefly, a cytosolic region of interest was selected for a specific cell and the mean fluorescence intensity I_C was determined for each image in the series. The fluorescence intensity after stimulation was normalized to the fluorescence intensity of the cell before stimulation with cAMP, yielding \hat{I}_C .

To determine the fluorescence signal at the boundary of the cell, a contour of the cell was calculated with the help of the computer, which was divided into 1000 contour points. The fluorescence intensity of each contour point (I_M) was then calculated and normalized to the fluorescence intensity of the cytosol before stimulation, yielding \hat{I}_M . Two methods were used to obtain the values of I_M (Postma et al., 2003). A quick, nearly automatic, method was used to generate the time-space concentration plots; this method introduces some noise. A more time-consuming method involves the precise adjustment of the contours with the boundary of the cell by hand; this more-accurate method was used to obtain statistical data on patches. The data shown are the means and standard deviations, unless mentioned otherwise.

Protrusions and pseudopodia

The outline of the cell was compared for two subsequent images. The new area of the cell is defined as a protrusion if it is a small extension (<1 μm); multiple thin protrusions are often formed from a small area

of the cell. The new area of the cell is defined as a pseudopod if it is a larger extension ($>1 \mu\text{m}$); generally, one broader pseudopod is formed from a small area of the cell. Parts of the cell body that do not have ruffles, filopodia or pseudopodia usually have a convex appearance.

Results

Translocation of PH_{Crac} -GFP to the entire plasma membrane or to patches at different cAMP concentrations

Cells expressing PH_{Crac} -GFP were stimulated in a perfusion chamber with $1 \mu\text{M}$ or 1nM cAMP. A series of confocal fluorescence images recorded before and during 40 seconds after this uniform stimulation is presented in Fig. 1. Before stimulation, PH_{Crac} -GFP is localized mainly to the cytosol. As shown previously, $1 \mu\text{M}$ cAMP induces a strong increase of

fluorescence intensity at nearly the entire boundary of the cell at 8 seconds after stimulation, which has disappeared at 20 seconds. A second response appears after 30-40 seconds, but now the enhanced fluorescence at the boundary is localized to a few separate regions, called patches (Postma et al., 2003). Cells stimulated with 1nM cAMP do not show a biphasic response but only patches of PH_{Crac} -GFP are induced. These patches remain present as long as cells are stimulated with cAMP, which was 4 minutes in some experiments (Postma et al., 2003) (Fig. 2). We have examined the responses of *Dictyostelium* cells to different cAMP concentrations and focused on the spatial localization of PH_{Crac} -GFP along the cell boundary. To limit the large amount of computer-assisted analysis, we have chosen relatively short stimulations from which all the essential kinetic parameters of the PH_{Crac} -GFP translocation response could be obtained.

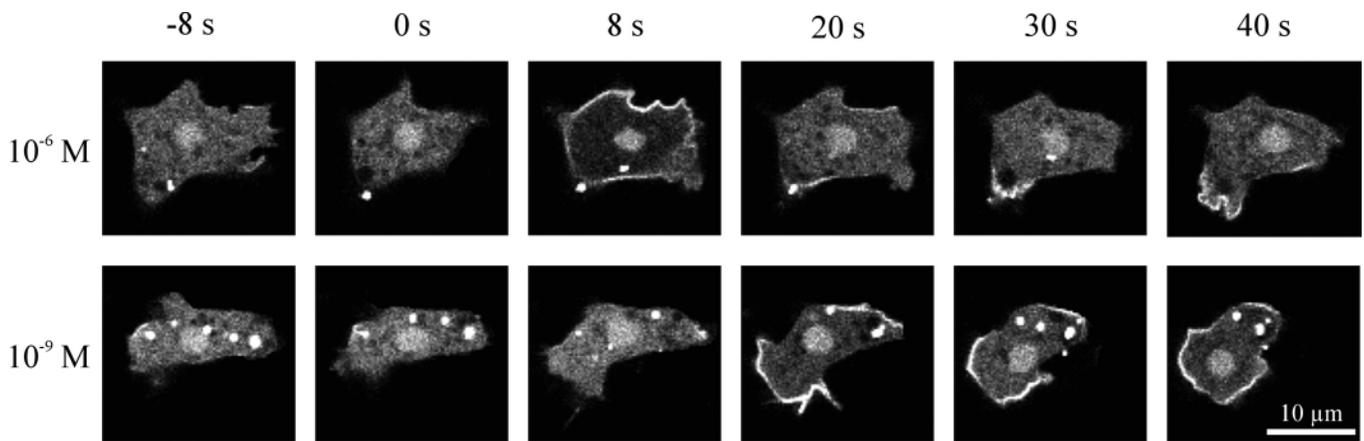


Fig. 1. cAMP-mediated translocation of PH_{Crac} -GFP. *Dictyostelium* cells were stimulated at $t=0$ seconds in a perfusion chamber with a homogeneous cAMP concentration of $1 \mu\text{M}$ or 1nM cAMP. Confocal images were taken at the times indicated. PH_{Crac} -GFP is in the cytosol before cAMP stimulation, and is translocated to the entire boundary (8 seconds) and to patches (30 seconds and later) after stimulation with $1 \mu\text{M}$ cAMP, whereas stimulation with 1nM cAMP induces only patches of PH_{Crac} -GFP (20 seconds and later).

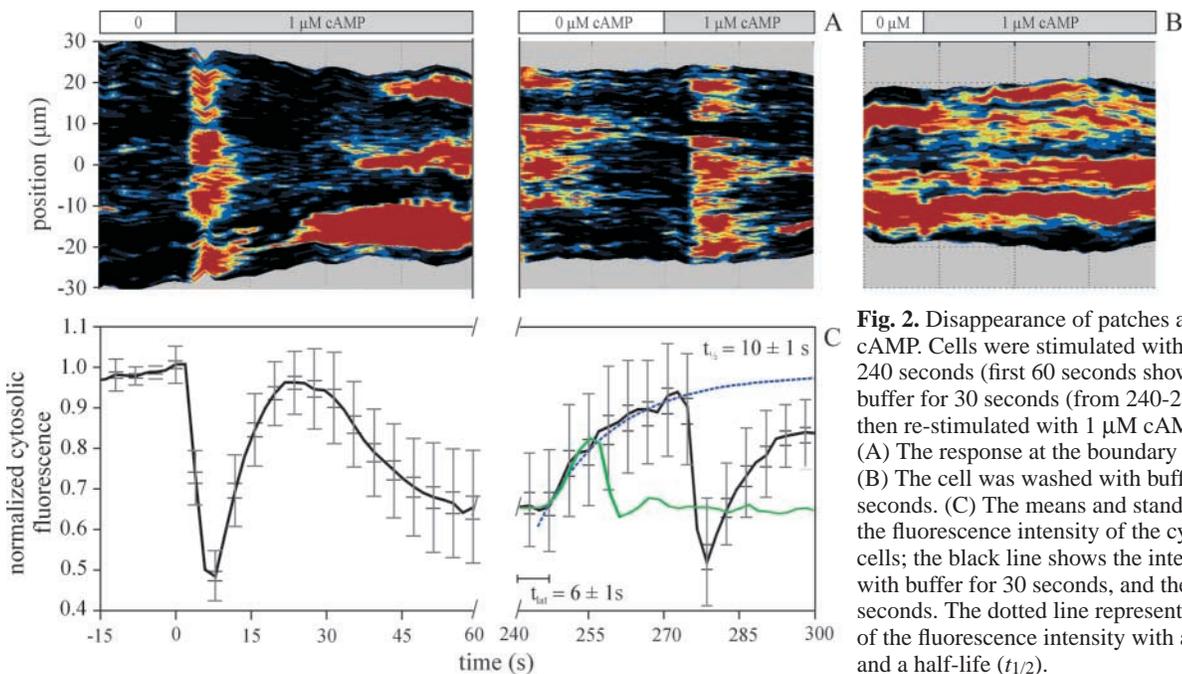


Fig. 2. Disappearance of patches after removal of cAMP. Cells were stimulated with $1 \mu\text{M}$ cAMP for 240 seconds (first 60 seconds shown), washed with buffer for 30 seconds (from 240-270 seconds), and then re-stimulated with $1 \mu\text{M}$ cAMP for 30 seconds. (A) The response at the boundary of a typical cell. (B) The cell was washed with buffer for only 10 seconds. (C) The means and standard deviations of the fluorescence intensity of the cytosol from 20 cells; the black line shows the intensity for the wash with buffer for 30 seconds, and the green line for 10 seconds. The dotted line represents a first-order decay of the fluorescence intensity with a latency time (t_{lat}) and a half-life ($t_{1/2}$).

cAMP-concentration dependency of PH_{Crac}-GFP association with the boundary

Dictyostelium cells were stimulated uniformly for 30 seconds with different cAMP concentrations (Fig. 3). This figure also presents a second experiment, discussed below, in which the stimulation with different cAMP concentrations was followed by an upshift of the cAMP concentration to 1 μ M. For analysing PH_{Crac}-GFP at the plasma membrane, the boundary of the cell is divided into 1000 points for which the fluorescence intensity is determined for each time after stimulation (Postma et al., 2003). The fluorescence intensity in these space-time plots are colour coded and are obviously unique for each cell (Fig. 3A-E). Data on the cytosolic fluorescence intensity of PH_{Crac}-GFP are presented as the average of about 20 cells (Fig. 3F-J), which allows accurate statistical analysis of the data.

Before cAMP stimulation, the cells were rinsed with buffer. During this period, the fluorescence at the boundary is similar to the fluorescence in the cytosol, apart from regions of the boundary that are in the vicinity of strongly fluorescent vesicles or the nucleus (Fig. 3D, around -15 seconds). The boundary plot of a cell stimulated with a high cAMP concentration (Fig. 3A) clearly reveals the two responses: uniform translocation around 8 seconds after stimulation and patches after 30 seconds. Cells stimulated with lower cAMP concentrations exhibit a first translocation response that started somewhat later and was less uniform than the response induced by higher cAMP concentrations. In addition, the subsequent dissociation from the membrane was slower and less complete at lower cAMP concentrations. Finally, the patches in the second phase of the response were formed sooner at lower cAMP concentrations. As a consequence of these general characteristics, the two phases become increasingly mixed at lower cAMP concentrations. At 10 nM cAMP the first response starts with a relatively uniform translocation but converts to patches without the intervening transient low levels of translocation (Fig. 3C). At 1 nM cAMP, nearly all cells show a patchy translocation from the beginning (Fig. 3D). At the lowest concentration tested, 0.1 nM cAMP, only about 15% of the cells responded, showing one or two clear patches (Fig. 3E).

To compare the translocation to the boundary of many cells, we have determined the fraction of translocation (FoT), which is defined as the fraction of boundary points that have a fluorescence intensity that is significantly higher than the fluorescence intensity in the cytosol (Postma et al., 2003). During the first phase of the response around 8-18 seconds, the FoT values are above 90%, except for the responses induced by 1 nM cAMP, which shows a FoT value of $57 \pm 15\%$. The FoT value for cells stimulated with 0.1 nM cAMP could not be calculated with sufficient accuracy, because only a few cells exhibited a patch-like response.

cAMP-concentration dependency of PH_{Crac}-GFP depletion in the cytosol

The relative fluorescence intensity in the cytosol is presented in Fig. 3F-J. Stimulation with cAMP between 10 nM and 1 μ M cAMP induces the same amount of depletion of PH_{Crac}-GFP from the cytosol (0.47 ± 0.10); depletion by 1 nM cAMP is smaller (0.33 ± 0.12). Stimulation with 0.1 nM cAMP induces

a patch response in only 15% of the cells and, in these cells, the fluorescence intensity of the cytosol decreases by about 0.32. Interestingly, the 85% of the cells that do not exhibit a patch response still show a decrease of the fluorescence intensity in the cytosol; this decrease of 0.095 ± 0.074 ($n=18$) is statistically very significant ($P < 0.001$).

We observed that depletion of fluorescence intensity of the cytosol starts increasingly later at lower cAMP concentrations, showing latency times that change from 2.2 seconds at 1 μ M cAMP and 3.6 seconds at 10 nM cAMP to 6.6 seconds at 1 nM cAMP. Once depletion has started, it rapidly reaches a maximum; the time required from offset to half-maximal depletion does not strongly depend on the cAMP concentration: it varies between 1.6 seconds at 1 μ M cAMP and 2.9 seconds at 1 nM cAMP. After depletion is maximal (at around 8-18 seconds after stimulation), the fluorescence intensity in the cytosol increases to reach a maximal level around 25 seconds after stimulation. The time of maximal recovery (24 seconds) appears to be largely independent of the cAMP concentration. By contrast, the extent of recovery is much larger at higher cAMP concentrations. The recovery (presented as the percentage of the maximal depletion in the cell) is nearly complete (91%) at 1 μ M cAMP, is 67% at 100 nM cAMP, 43% at 10 nM and only 16% at 1 nM. This partial recovery of cytosolic fluorescence at 10 nM and 1 nM cAMP closely parallels the observed remaining fluorescence at the boundary of the cell.

The second phase of depletion of cytosolic fluorescence starts at about 25 seconds after stimulation with 1 μ M cAMP. This offset time is approximately the same for different cAMP concentrations between 10 nM and 1 μ M. As described above, no two distinct phases can be discerned at 0.1 and 1 nM cAMP, as the first phase is already a patch-like response that continues as long as cAMP remains present.

cAMP-dose dependency of translocation

The depletion of PH_{Crac}-GFP from the cytosol is induced by very low cAMP concentrations (Fig. 4); half-maximal depletion occurs at about 0.5 nM and the response saturates between 1 nM and 3 nM (closed circles). The subsequent recovery of fluorescence intensity in the cytosol at about 24 seconds after stimulation requires much higher cAMP concentrations than its depletion (open circles); half-maximal recovery is induced at about 10 nM cAMP. The dose dependency for cAMP induced patches (expressed as the proportion of cells that show one or more patches, or as the mean number of patches per cell) is essentially identical to the dose response for the depletion of the cytosol. The number of patches per cell is variable from 0 to 4 patches with an average of 2.3 ± 1.1 patches per cell at 1 μ M cAMP. At lower cAMP concentrations the average number of patches per cell decreases strongly, which is mainly due to the increasing proportion of cells without patches; cells that do show patches in response to 0.1 nM or 1 nM cAMP still have 1.5 ± 0.7 patches per cell.

The effects of 0.1 nM and 1 nM cAMP on the fluorescence in the cytosol are relatively small, mainly because fewer patches are formed in a smaller proportion of the cells than at higher cAMP concentrations. We have analysed the properties of the patches induced by different cAMP concentrations. The

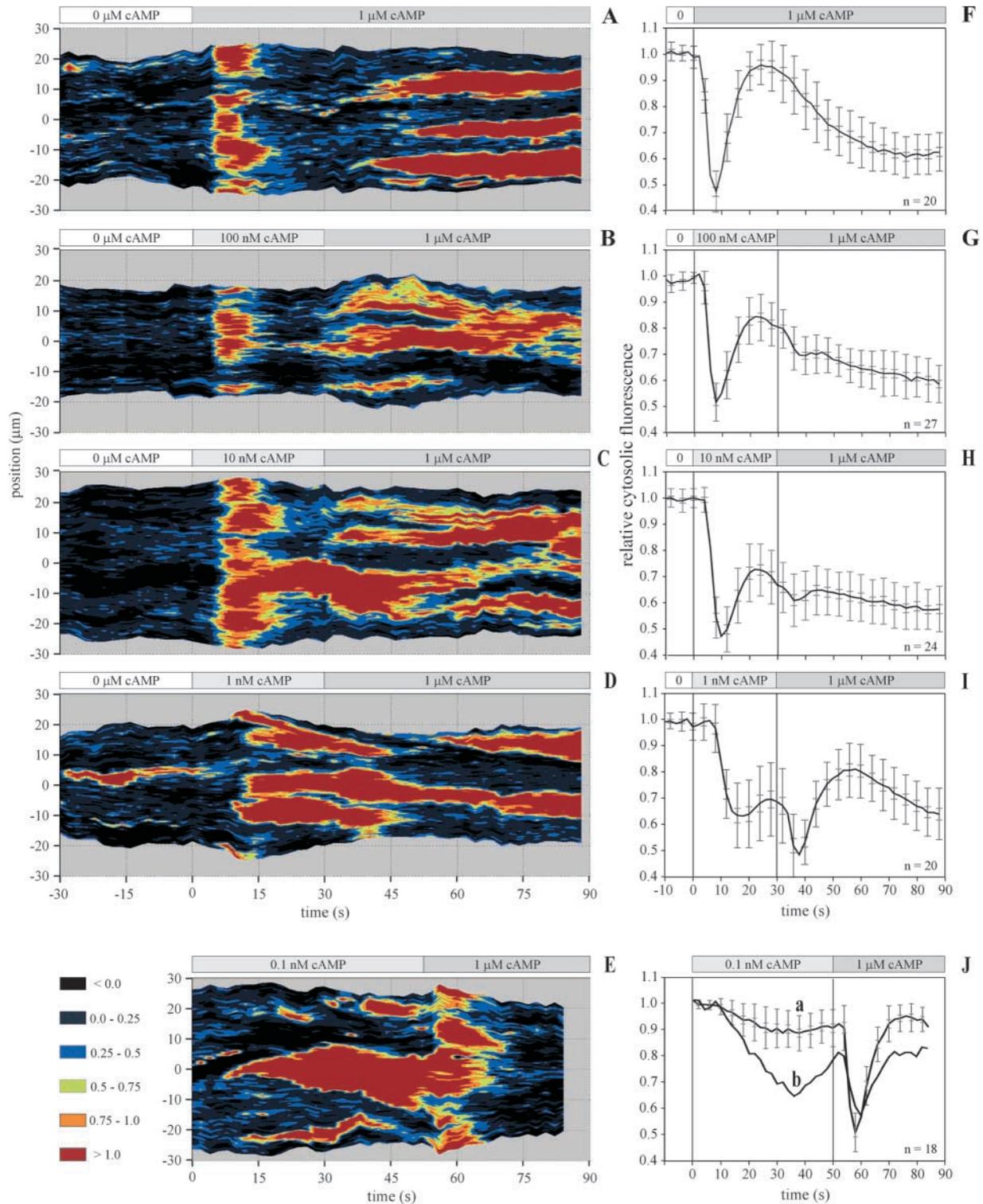


Fig. 3. cAMP dependency of PH_{Crac}-GFP localization. Cells were incubated in buffer for 30 seconds, followed at $t=0$ seconds by perfusion with the indicated cAMP concentrations. At $t=30$ seconds, all cells were perfused with 1 μM cAMP. (A-E) The response at the boundary of a typical cell; the colours represent the differences between fluorescence intensity at the boundary and the cytosol. (F-J) The response in the cytosol, shown as the fluorescence intensity after stimulation relative to the fluorescence intensity before stimulation; the figure shows the means and standard deviations (large bars) and standard errors of the means (small bars) of about 20 cells. The experiment with 0.1 nM cAMP was slightly different: cells were stimulated for 45 seconds followed by the upshift. Because only about 15% of the cells responded to 0.1 nM cAMP with a patch, the fluorescence intensity in the cytosol (J) is presented for 18 cells without a patch (a) and two cells with a patch (b). (E) The response at the boundary in a cell with a patch. (D) The patch before cAMP stimulation is a bright cytosolic vesicle located next to the boundary.

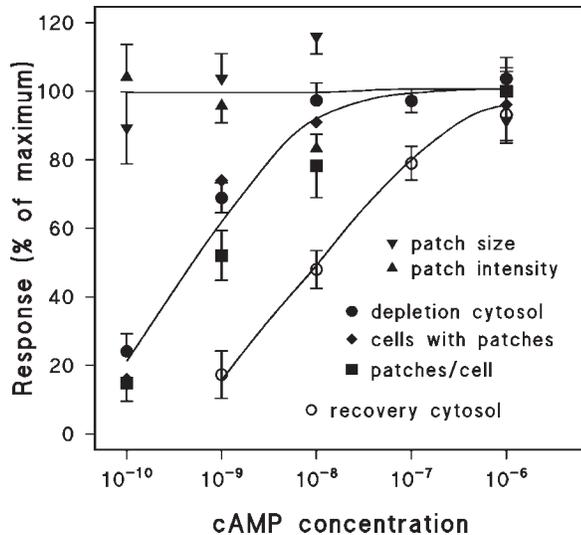


Fig. 4. Dose response curves of PH_{Crac}-GFP translocation and patch formation. Cells were incubated in buffer for 30 seconds, followed at $t=0$ seconds by perfusion with the indicated cAMP concentrations. At $t=30$ seconds, all cells were perfused with 1 μ M cAMP. The experiment with 0.1 nM cAMP was slightly different: cells were stimulated for 45 seconds followed by the upshift. Different aspects of these responses are presented as a percentage of the maximal response: the first depletion of the fluorescence intensity in the cytosol (maximum 53%); recovery of the fluorescence intensity in the cytosol (maximum 100%); the proportion of cells with one or more patches (maximum 100%); the number of patches per cell (maximum 2.2); the fluorescence intensity of the patches (maximum 2.4); and the size of the patches (maximum 9.85 μ m). The results shown are the means and standard error of the means of about 20 cells (except the size and fluorescence intensity of the patches at 0.1 nM cAMP, which were for five patches).

size of the patches in cells stimulated with 1 μ M cAMP (9.0 \pm 3.2 μ m at $t=58$ seconds) is identical to the size of the patches induced by 1 nM cAMP (8.8 \pm 1.6 μ m at $t=30$ seconds). We also noticed that the fluorescence intensity of the patches is essentially independent of the stimulus concentration. The small variation observed at different cAMP concentration is not statistically significant. Interestingly, the few patches that appear after stimulation with 0.1 nM cAMP also have this size and fluorescence intensity (Fig. 4), which is consistent with the large decrease of fluorescent intensity of the cytosol in the few cells with a patch compared with the cells without a patch (Fig. 3J). We conclude that the cAMP concentration determines the probability of patch formation but, when patches appear, all patches essentially have the same size and fluorescence intensity.

Upshift of the cAMP concentration after stimulation with lower cAMP concentrations

Cells that were stimulated for 30 seconds with 0.1–100 nM cAMP were further stimulated with an upshift to 1 μ M cAMP. The experiment was performed especially to investigate which response 1 μ M cAMP will induce in cells that were prestimulated with very low concentrations of cAMP. Are the patches induced by low concentrations stable, or does 1 μ M

cAMP induce a uniform translocation as in unstimulated cells?

Cells stimulated with 100 nM cAMP for 30 seconds show the beginning of patch formation, and an upshift to 1 μ M cAMP just induces a stabilization of these patches (Fig. 3B). Cells stimulated with 10 nM cAMP for 30 seconds often have one or two patches that evolved from the uniform response (Fig. 3C); a 100-fold increase in the cAMP concentration does not induce a uniform response but supports the patches. The conclusion that the upshift does not induce a new uniform response is also supported by the analysis of the fluorescence intensity in the cytosol (Fig. 3G,H), showing only a small shoulder around 10 seconds after the upshift.

Cells stimulated with 1 nM cAMP for 30 seconds show patches. The response to the 1000-fold upshift of the cAMP concentration appears to be a mixture of stabilization and extinction of the patches, and some translocation to new areas between the patches. The fluorescence intensity in the cytosol reveals a transient depletion at around 8 seconds after the upshift (Fig. 3I) and the fluorescence intensity at the boundary suggest that this translocation has occurred to the existing patches (Fig. 3D). However, two observations suggest that the response to the upshift shows some properties of the ‘uniform’ response. First, the FoT value increases from 57 \pm 15% before the upshift to 78 \pm 10% at 8 seconds after the upshift, suggesting that PH_{Crac}-GFP translocated to some areas of the boundary not previously occupied.

In about 15% of the cells stimulated with 0.1 nM cAMP, a translocation response was observed, which was usually the formation of one or two patches. In these cells, the upshift induces a nearly uniform response (Fig. 5E) and the existing patches disappear at about 15 seconds after the upshift, together with the uniform response.

Disappearance of patches after removal of cAMP

Cells were stimulated with 1 μ M cAMP for 4 minutes, followed by a shift of the medium to buffer without cAMP. Fig. 2 reveals the progressive disappearance of the patches. At the same time, the fluorescence intensity in the cytosol reappears. This recovery of the fluorescence intensity in the cytosol shows a latency time of 6 seconds before dissociation from boundary becomes apparent and then proceeds with first-order kinetics with a half-life of 10 seconds. These results imply that patches depend on the continuous presence of cAMP.

To investigate the possible existence of a type of adaptation or memory at the site of the membranes where a patch was present, cells were re-stimulated with 1 μ M cAMP after a wash with buffer for 30 seconds; at this time, the patches have just disappeared. This new stimulus induces a new translocation to nearly the entire boundary. This translocation is transient, although PH_{Crac}-GFP stays a little longer at the boundary than in cells that were stimulated with cAMP for the first time. When cells are washed for only 10 seconds, about 40% of the fluorescence at the boundary has returned to the cytosol, with the remaining fluorescence being present in patches. Re-stimulation of these cells with 1 μ M cAMP induces the reassociation of PH_{Crac}-GFP with the boundary at the patches. We conclude that a wash with buffer for only 30 seconds is sufficient to clear the translocation response and, apparently, cells are fully responsive to a new stimulus. Translocation

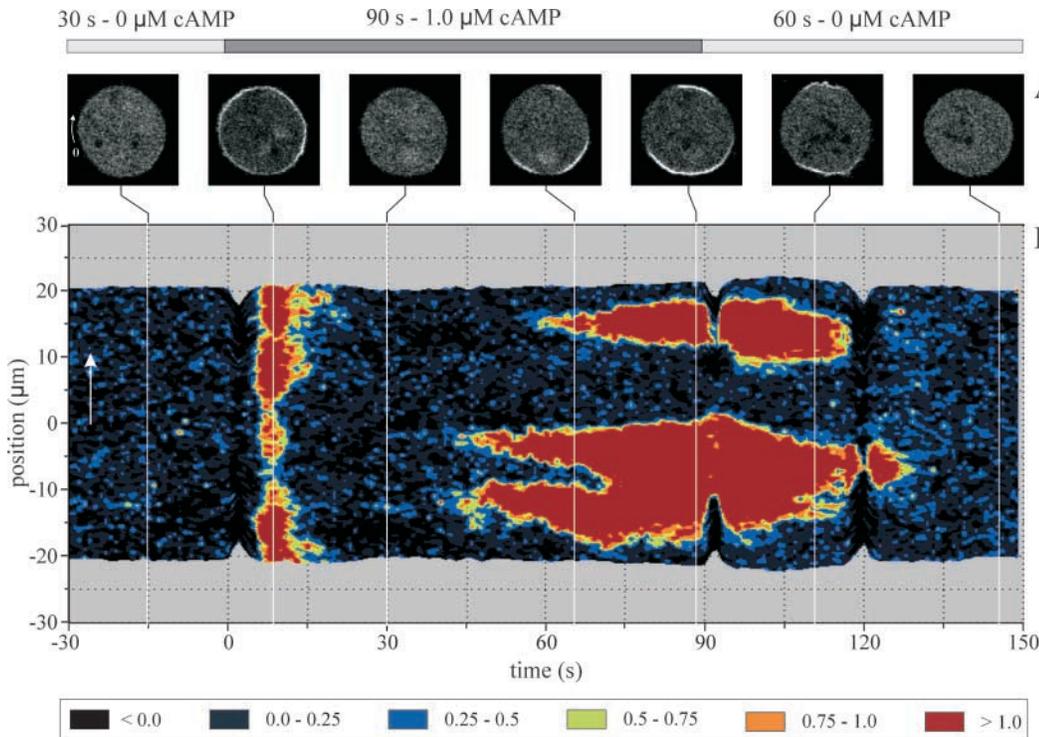


Fig. 5. Patch formation in latrunculin-A-treated cells. Cells in the perfusion chamber were incubated with $1\ \mu\text{M}$ latrunculin A for 15 minutes, subsequently stimulated at $t=0$ seconds with $1\ \mu\text{M}$ cAMP and washed with buffer at $t=90$ seconds. (A) The fluorescence intensity and the shape of a representative cell before and after stimulation with cAMP. (B) The fluorescence intensity at the boundary for the same cell. Before mounting the perfusion chamber, cells were overlaid with a thin layer of agarose to prevent elution of these spherical cells. The small indentations at 0 seconds, 90 seconds and 120 seconds are perfusion artefacts; at the onset of perfusion, the cover slip bends for a few seconds and cells move in the plane of focus.

occurs to (residual) patches as long as they are still present but no memory is observed beyond the life-time of these patches.

Formation of patches in the absence of actin filaments and pseudopodia

Previously, we have shown that cAMP-induced $\text{PH}_{\text{Crac}}\text{-GFP}$ patches are often observed at the periphery of pseudopodia (Postma et al., 2003). To investigate the requirement of F-actin and pseudopodia for patch formation, we used cells treated with latrunculin A, which induces depolymerization of actin filament and leads to the retraction of all pseudopodia and immobilization of the cell. In these cells, cAMP stimulation from a micropipette still induces $\text{PH}_{\text{Crac}}\text{-GFP}$ translocation towards the cAMP source (Parent et al., 1998). Cells are completely round at about 5 minutes after perfusion with $1\ \mu\text{M}$ latrunculin A. Uniform stimulation of these round cells with $1\ \mu\text{M}$ cAMP induces a $\text{PH}_{\text{Crac}}\text{-GFP}$ translocation response that is essentially identical to the response of an untreated control cell (Fig. 5). At about 8 seconds after stimulation, translocation of $\text{PH}_{\text{Crac}}\text{-GFP}$ is present at nearly the entire boundary of the cell, followed by dissociation of $\text{PH}_{\text{Crac}}\text{-GFP}$ from the membrane to the cytosol. Subsequently, the second translocation of $\text{PH}_{\text{Crac}}\text{-GFP}$ to patches at the boundary of the cell is observed. The patches in latrunculin-A-treated cells are essentially identical in size and fluorescence intensity to the patches in control cells (data not shown). These results demonstrate that patch formation is not dependent on the presence of F-actin and pseudopodia. Moreover, uniform cAMP stimulation of a nearly symmetric latrunculin-A-treated cell still induces localized $\text{PH}_{\text{Crac}}\text{-GFP}$ patches, strongly supporting the notion that patches are autonomous signalling entities.

Pseudopodia are extended from areas of the cell with $\text{PH}_{\text{Crac}}\text{-GFP}$ patches

Inspection of many cells stimulated with uniform cAMP reveal that patches are dynamic structures with a life-time of about 1 minute and that reappear at other places as long as cAMP remains present. We have further analysed the correlation between $\text{PH}_{\text{Crac}}\text{-GFP}$ patches and pseudopodia in about 30 cells stimulated with different cAMP concentrations. Cells stimulated with $1\ \mu\text{M}$ cAMP stop locomotion, retract pseudopodia ('cringe') and then resume to extend pseudopodia (Varnum and Soll, 1984). Previously we have shown that, in these cells, most patches of $\text{PH}_{\text{Crac}}\text{-GFP}$ start in convex areas of the cell body and grow in the subsequent 10 seconds to a full-size patch (Fig. 3A,E). Between 12 seconds and 16 seconds after the onset of patch formation, a pseudopod was extended from the area of the cell with a $\text{PH}_{\text{Crac}}\text{-GFP}$ patch. After about 40-60 seconds, the $\text{PH}_{\text{Crac}}\text{-GFP}$ patches disappear, followed by retraction of the pseudopod after 7 ± 2 seconds (Postma et al., 2003).

Cells stimulated with $1\ \text{nM}$ cAMP do not stop locomotion and existing pseudopodia continue to be extended or retracted (Varnum and Soll, 1984). The patches of $\text{PH}_{\text{Crac}}\text{-GFP}$ induced by $1\ \text{nM}$ cAMP do not grow as slowly after stimulation with $1\ \mu\text{M}$ cAMP but appear at once at full size about 16-18 seconds after stimulation (Fig. 3D). In the images taken at 20 seconds after stimulation, we can distinguish three situations: patches in convex areas of the cell body, patches in pseudopodia (each about 50% of the patches) and pseudopodia without patches (about 30% of the pseudopodia). We followed the lifecycle of the patches and pseudopodia in each category. All patches in convex areas of the cell body transformed to pseudopodia after 10.8 ± 1.8 seconds. When a patch was present in an existing pseudopod at 20 seconds after stimulation, the pseudopod was

12±3 seconds old. By contrast, pseudopodia without patches were 31±11 seconds old. We determined that the lifecycle of a pseudopod is 41±10 seconds, indicating that 1 nM cAMP can induce patches before and during the first 30-40% of the lifetime of pseudopodia, but not in older pseudopodia. When these young pseudopodia with patches are followed in time, the patches remain present during the entire life of the pseudopod. Thus, 1 nM cAMP cannot induce PH_{Crac}-GFP-binding sites in an older pseudopod but, once PH_{Crac}-GFP-binding sites are induced, the patch of PH_{Crac}-GFP remains associated with the pseudopod.

We noticed that pseudopodia with a patch of PH_{Crac}-GFP have a more active appearance than pseudopodia without a patch. Quantitative analysis reveals that the movement step is about twice as long for a pseudopod with a patch (6.7±0.9 µm; *n*=6) than for a pseudopod without a patch (3.2±1.1 µm; *n*=6); the lifetimes of pseudopodia with or without patches are not different. In summary, these results reveal that patches of PH_{Crac}-GFP are closely associated with pseudopodia, that patches are often formed before pseudopodia, and that pseudopodia with patches make larger translocation steps than pseudopodia without patches.

Requirement of PI3K for chemotaxis to low cAMP concentrations

The translocation of PH_{Crac}-GFP and another PH-domain-containing protein, PhdA-GFP, from the cytosol to the plasma membrane, and the formation of PH_{Crac}-GFP patches are strongly inhibited by the PI3K inhibitor LY294002 (Funamoto et al., 2001) (data not shown). We tested the effect of different concentrations of LY294002 on the chemotactic response of wild-type cells to different cAMP concentrations using the small population assay, which allows us to record such double-dose response curves with the same batch of cells (Fig. 6). Relatively low concentrations of LY294002 (40 µM) inhibit chemotaxis to nanomolar cAMP, with half-maximal inhibition by about 30 µM LY294002. This LY294002 concentration is similar to the concentration giving half-maximal inhibition of PhdA-GFP translocation in vivo, or GTPγS-stimulated PtdIns(3,4,5)P₃ production in vitro (both about 25 µM) (Funamoto et al., 2001; Huang et al., 2003). Although LY294002 completely inhibits chemotaxis towards 1 nM and 10 nM cAMP, it has little effect on chemotaxis to higher cAMP concentrations. Even 100 µM LY294002, which completely inhibits PI3K activity in vitro, does not affect chemotaxis towards 100 nM cAMP.

Discussion

Dictyostelium cells exhibit a biphasic translocation response of PH_{Crac}-GFP to the plasma membrane after stimulation in a perfusion chamber with uniform cAMP: a rapid, transient translocation of PH_{Crac}-GFP to nearly the entire plasma membrane, as has been observed previously (Parent et al., 1998), is followed by a second translocation of cytosolic PH_{Crac}-GFP to the membrane that persists as long as cAMP remains present. In this second response, PH_{Crac}-GFP is not located along the entire plasma membrane but appears as distinct patches of intense fluorescence (Postma et al., 2003).

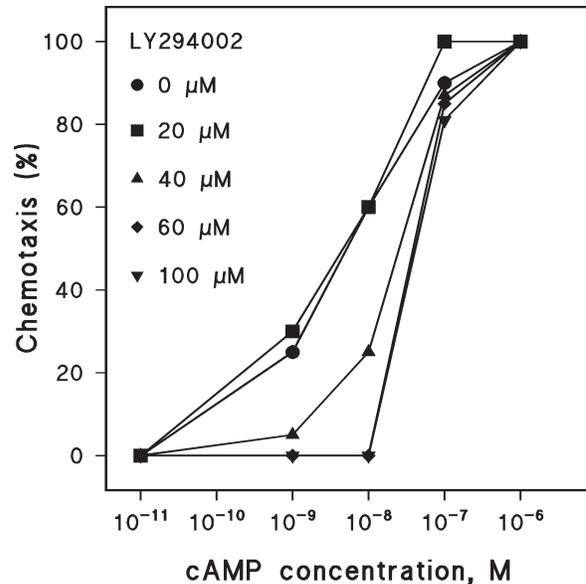


Fig. 6. Inhibition of chemotaxis by LY294002. Wild-type cells were starved for 5 hours on plates in phosphate buffer, resuspended in buffer and deposited as small droplets on hydrophobic agar containing different concentrations of LY294002. After about 15 minutes, droplets with different cAMP concentrations were deposited close to the amoebae and the chemotaxis response was observed during the following 30 minutes. The data are presented as a percentage of the populations (about 20 for each cAMP concentration) that showed a positive chemotactic response.

Properties of PH_{Crac}-GFP signalling patches

The translocation of PH_{Crac}-GFP to patches is a dynamic process, with patches disappearing and reappearing as long as cAMP is present. The average lifetime of a PH_{Crac}-GFP patch is about 1 minute. A patch has a radius of about 5 µm, yielding a patch surface area of about 10% of the surface of a cell (Postma et al., 2003). Very low cAMP concentrations induce translocation of PH_{Crac}-GFP to the membrane, with half-maximal effect at 0.5 nM cAMP. Because the cAMP receptor has an affinity of about 10-100 nM, this implies a strong amplification of the signal. The observation that even 100-times lower cAMP concentrations can induce PH_{Crac}-GFP translocation in cells with a deletion of the PtdIns(3,4,5)P₃ 3-phosphatase PTEN (Iijima and Devreotes, 2002), suggests that PtdIns(3,4,5)P₃ is a component of this amplification mechanism. There are probably also other components involved, because we observed cAMP-concentration-dependent latency times ranging from 2.2 seconds at 1 µM cAMP to 6.6 seconds at 1 nM cAMP, which were followed by equally rapid translocation of PH_{Crac}-GFP to the boundary, suggesting that an intermediate has to build up before translocation is triggered. The PH_{Crac}-GFP patches that are formed at low cAMP concentrations disappear upon an upshift of cAMP to 1 µM, indicating a cAMP-dependent production of inhibitor. The concentration dependency of inhibition shows half-maximal effect at about 10 nM, which is close to the affinity of the cAMP receptor. The data suggest that receptor occupancy induces excitation and inhibition responses, and that only the excitation response shows self-amplification.

Importantly, the dimensions, lifetime and fluorescence intensity of the patches are nearly identical in patches induced by 0.1 nM or 1000 nM cAMP, indicating that the cAMP concentration determines the probability of patch formation but has no effect on the temporal and spatial properties of the patches. These observations confirm the control of PH_{Crac}-GFP patch formation and PtdIns(3,4,5) P_3 synthesis by strong amplification mechanisms; once a receptor-stimulated threshold for PtdIns(3,4,5) P_3 synthesis is surpassed, a mature, full-size patch is formed.

Latrunculin A inhibits actin filaments, leading to a symmetrical, spherical cell without pseudopodia (Parent et al., 1998). We observed that latrunculin-A-treated cells exhibit the same biphasic PH_{Crac}-GFP translocation response to uniform cAMP as control cells (Fig. 5). Thus, a uniform cAMP stimulus in a perfusion chamber induces localized PtdIns(3,4,5) P_3 synthesis in a cell that is virtually symmetrical. Apparently, patches of PH_{Crac}-GFP are formed in the absence of actin filaments, pseudopodia or other visible morphological spatial cues related to actin polymerization. The patches of PH_{Crac}-GFP that are induced by uniform cAMP in latrunculin-A-treated cells have approximately the same size and fluorescence intensity as the PH_{Crac}-GFP patches of control cells, suggesting that pseudopodia do not strongly affect PtdIns(3,4,5) P_3 synthesis in *Dictyostelium*. In neutrophils, an important control loop has been demonstrated in which PtdIns(3,4,5) P_3 induces actin filaments in the leading pseudopod, which stimulates PtdIns(3,4,5) P_3 synthesis, thereby enhancing the chemotactic sensitivity of the pseudopod at the leading edge (Srinivasan et al., 2003; Xu et al., 2003). As a consequence, neutrophils are very polarized, even after stimulation with a uniform concentration of chemoattractant. We do not find evidence for this PtdIns(3,4,5) P_3 -pseudopod autocatalytic feedback loop in *Dictyostelium*, which could explain why *Dictyostelium* cells are less polarized than neutrophils.

Patches and pseudopodia

PH_{Crac}-GFP patches are often found in pseudopodia, both in uniform cAMP and in cAMP gradients (Parent et al., 1998; Postma et al., 2003). Several observations suggest that patches induce or enhance pseudopod formation instead of that patches are preferentially formed in pseudopodia. First, cAMP still induces patches in latrunculin-A-treated cells that are devoid of pseudopodia (Fig. 5), indicating that pseudopodia are not essential for patch formation. Second, patch formation generally precedes pseudopod formation by about 10 seconds, and pseudopodia will retract about 10 seconds after a patch disappears (Parent et al., 1998; Postma et al., 2003). Third, in cAMP-stimulated *pen*-null cells, the magnitude and time course of PtdIns(3,4,5) P_3 production are increased compared with wild-type cells (Huang et al., 2003). The absence of 3-phosphatase activity leads to broadening of the region with PH_{Crac}-GFP localization as well as the region from which F-actin-filled pseudopodia are extended (Iijima and Devreotes, 2002). These observations strongly suggest a causal link between PH_{Crac}-GFP patches and pseudopod formation.

We observed that stimulation of cells with 1-10 nM cAMP induces translocation of PH_{Crac}-GFP to small areas of the membrane that are either early pseudopodia or regions where

pseudopodia will be formed within the next 15 seconds. No translocation of PH_{Crac}-GFP was observed into old pseudopodia. Furthermore, we never observed that a new patch of PH_{Crac}-GFP reappears within 30 seconds at a position where a former patch-pseudopodium was retracted. These observations are consistent with the notion that, in chemoattractant gradients, a new pseudopodium is often made adjacent to a retracting pseudopodium and rarely at the same position (Varnum-Finney et al., 1987).

PI3K pathway sensitizes chemotaxis

The PI3K pathway appears to be a strong modulator of chemotaxis, especially affecting chemotaxis to low cAMP concentrations. Disruption of two PI3K-encoding genes in *Dictyostelium* or inhibition of the PI3K pathway with LY294002 leads to a significant reduction of the chemotactic activity (Funamoto et al., 2002), notably a 10-100-times reduction in cAMP sensitivity (Fig. 5). Furthermore, improving the PI3K pathway enhances the cAMP sensitivity about tenfold, as was observed in mutants with a deletion of PtdIns(3,4,5) P_3 5-phosphatases (Loovers et al., 2002) or deletion of *InsP6* kinase that produces the PtdIns(3,4,5) P_3 antagonist *InsP7* (Luo et al., 2003). Although *Dictyostelium* cells can extend pseudopodia in the absence of PI3K activity, the results reveal that a pseudopodium is preferentially formed at a region of the cell with a PH_{Crac}-GFP patch. Furthermore, PtdIns(3,4,5) P_3 /PH_{Crac}-GFP signalling patches are induced by very low cAMP concentrations and the movement steps of pseudopodia with a PH_{Crac}-GFP patch are about twice as large as those of pseudopodia without PH_{Crac}-GFP patches. Taking these observations together, we propose that localized PtdIns(3,4,5) P_3 synthesis at the leading edge of cells in a chemoattractant gradient increase the cAMP sensitivity of the cells to induce a pseudopodium in the direction of the gradient, and to enlarge the movement step of the emerging pseudopodium.

Sensitization of chemotaxis by the PI3K pathway could be mediated by a PtdIns(3,4,5) P_3 -induced potentiation of a cAMP-activated pathway for pseudopodium formation. For instance, actin polymerization is probably mediated by Rac GTPases, which are activated by Rac guanine-nucleotide-exchange factors (RacGEFs). Two classes of RacGEFs have been described, with and without PH domains (Wilkins and Insall, 2001; Etienne-Manneville and Hall, 2002; Cote and Vuori, 2002). cAMP-mediated activation of RacGEFs without PH domains might occur at high cAMP concentrations, whereas activation of RacGEFs with a PH domain by PtdIns(3,4,5) P_3 occurs at very low cAMP concentrations.

Self-organizing properties of PH_{Crac}-GFP patches in chemotaxis

The concept of self-organization in cell biology is based on the capacity of a macromolecular complex to determine its own structure by means of the functional interaction of its components (Misteli, 2001; Nicolis and Prigogine, 1977; Xu et al., 2003). Self-organizing systems use metabolic energy to induce transitions between different states, and small perturbations trigger a chain of events leading to a new steady state. These small perturbations may be caused by outside

signals, which therefore induce the new steady state; however, outside signals have no effect on the architecture or functional features of the new steady state. Several observations suggest that PH_{Crac}-GFP patches are self-organizing structures. First, we noticed that, once PH_{Crac}-GFP starts to accumulate at some point on the boundary, either PH_{Crac}-GFP dissociates from the boundary or a full-size patch appears that remains present for at least 30 seconds; we did not observe patches that are small or had low fluorescence intensity. Second, as discussed above, the cAMP concentration determines the probability of patch formation but has no effect on the properties of the patches. Last, we observed that latrunculin-A-treated cells exhibit the same biphasic PH_{Crac}-GFP translocation response to uniform cAMP as control cells, indicating that patches of PH_{Crac}-GFP are formed in the absence of actin filaments, pseudopodia or other visible morphological spatial cues. These experiments suggest that PH_{Crac}-GFP patches and their underlying biochemistry of synthesis and degradation of PtdIns(3,4,5)P₃ have self-organizing properties. Systems that possess self-amplification exhibit a threshold-like behaviour (Levchenko and Iglesias, 2002). When the concentration of PtdIns(3,4,5)P₃ passes a threshold at some point on the plasma membrane, strong amplification will take place by local regulation of PtdIns(3,4,5)P₃ synthesis and degradation such that a full size patch is always formed, independent of the stimulus concentration that triggered patch formation.

Several models have been proposed to explain how cells could read chemotactic gradients. Most models consist of a local activator and a global inhibitor (Haugh and Lauffenburger, 1997; Iijima et al., 2002; Levchenko and Iglesias, 2002; Meinhardt and Gierer, 2000; Postma and Van Haastert, 2001). In a spatial gradient, the level of activation at the front of the cell exceeds the level of global inhibition providing a persistent response. In the back of the cell, activation is less than inhibition and so there is no response. In these models, activation and inhibition are operational in the whole cell and so these models have difficulties explaining the sharp boundary of the leading edge ('soft boundaries'), and especially the reversibility of the leading edge when the gradient changes direction ('freezing'). The concept of self-organizing signalling patches might be very useful in explaining chemotaxis. In self-organizing patches, the cAMP gradient induces some PtdIns(3,4,5)P₃ synthesis at the front above a threshold, while PtdIns(3,4,5)P₃ synthesis elsewhere is inhibited by global inhibition. This small amount of PtdIns(3,4,5)P₃ synthesis will then activate the self-organizing amplification mechanism by which a mature patch of PtdIns(3,4,5)P₃ is formed. The size, boundaries and lifetime of these patches are not determined by the cAMP stimulus and are thus also independent of the cAMP gradient. The main advantage of a switchable self-organizing element in chemotaxis is its ease by which it can be locally triggered, thereby eliminating the problems of soft boundaries and freezing that often occur when models are tuned to perform at shallow gradients of chemoattractant (Levchenko and Iglesias, 2002).

Recently, a different model was presented for neutrophil chemotaxis, consisting of two signalling pathways (Li et al., 2003; Xu et al., 2003). A 'frontness' pathway consists of an autocatalytic PtdIns(3,4,5)P₃-pseudopodium loop, leading to Rac-stimulated actin filaments and pseudopodia with enhanced

chemotactic sensitivity at the leading edge. A second 'backness' pathway depends on Rho-induced myosin filament formation in the back and at the sides of the cell, suppressing the formation of lateral pseudopodia. These two pathways are functionally incompatible, causing them to segregate to different domains, making the leading edge more sensitive to attractant than the back of the cell. The present observations in *Dictyostelium* showing self-organizing PtdIns(3,4,5)P₃ patches and pseudopodia are consistent with 'frontness' signalling, perhaps with the exception that F-actin does not appear to enhance PtdIns(3,4,5)P₃ production in *Dictyostelium* as shown by the latrunculin-A experiment. Our observations stress the importance of a switchable element triggered by extremely low cAMP concentrations, so we suspect a strong positive-feedback loop between the receptor and PtdIns(3,4,5)P₃ in *Dictyostelium*, rather than the PtdIns(3,4,5)P₃-actin loop of neutrophils. The 'backness' signal in *Dictyostelium* consists of actin-myosin filaments and suppression of lateral pseudopodia as in neutrophils, which is probably not mediated by Rho in *Dictyostelium* but by a unique cGMP-signalling pathway (Bosgraaf et al., 2002). It will be interesting to test the 'inverse pathways' model in *Dictyostelium* by investigating the interactions between the PtdIns(3,4,5)P₃-actin and cGMP-myosin pathways.

Absence of robust perfect adaptation and the consequence for chemotaxis

Cells stimulated for prolonged periods with uniform cAMP continue to show PH_{Crac}-GFP patches, indicating that the receptor-mediated PtdIns(3,4,5)P₃ response does not fully adapt to constant cAMP levels. This conclusion is consistent with the observation that activation of PH_{Crac}-GFP translocation occurs at 20-fold lower cAMP concentrations than its subsequent inhibition. The absence of perfect adaptation is also demonstrated by the experiment in which cAMP stimulation was interrupted by a brief period (30 seconds) with buffer, showing that patches of PH_{Crac}-GFP disappear upon addition of buffer but immediately reappear upon re-stimulation with cAMP. The observed partial inhibition of PH_{Crac}-GFP translocation is not sufficient to induce complete adaptation; together with cGMP-mediated myosin filaments, partial adaptation might be sufficient to inhibit pseudopod formation in the back of the cell.

The obvious advantage of partial adaptation is that the PI3K pathway continuously provides temporal and spatial information on the chemoattractant gradient. The cell may use this information to change the direction of movement by means of lateral pseudopodia when the direction of the chemoattractant gradient changes. Support for this hypothesis comes from experiments showing suppression of lateral pseudopodia upon inhibition of PI3K activity by LY294002 (Funamoto et al., 2001). In addition, upon changing the direction of the chemoattractant gradient, LY294002-treated cells make U-turns at the front, whereas untreated cells extend pseudopodia at the back of the cell in the direction of the new gradient (Chen et al., 2003).

In summary, uniform cAMP triggers the formation of multiple PtdIns(3,4,5)P₃ signalling patches that are self-organizing structures. These patches provide spatial cues for enhanced actin filament formation and pseudopodium

extension. In a chemoattractant gradient only one signalling patch is formed at the leading edge, which is the preferred site of pseudopodium formation. This pathway is especially important for chemotaxis in shallow gradients

We thank P. Devreotes for providing plasmid WF38, E. Potma for the flow chamber and Ruchira for helpful assistance with confocal microscopy. This research was supported by the Netherlands Organization for Scientific Research (NWO).

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