A spatially restricted increase in receptor mobility is involved in directional sensing during Dictyostelium discoideum chemotaxis

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Summary
The directed cell migration towards a chemotactic source, chemotaxis, involves three complex and interrelated processes: directional sensing, cell polarization and motility. Directional sensing allows migrating eukaryotic cells to chemotax in extremely shallow gradients (<2% across the cell body) of the chemoattractant. Although directional sensing has been observed as spatially restricted responses along the plasma membrane, our understanding of the ‘compass’ of the cell that controls the gradient-induced translocation of proteins during chemotactic movements is still largely lacking. Until now, the dynamical behaviour and mobility of the chemoattractant-receptor molecule has been neglected in models describing the directional sensing mechanisms. Here, we show by single-molecule microscopy an agonist-induced increase in the mobile fraction of cAMP-receptor at the leading edge of chemotaxing Dictyostelium discoideum cells. The onset of receptor mobility was correlated to the uncoupling and activation of the Gα2-protein. A finite-element simulation showed that the increase in mobile fraction of the activated receptor enabled the amplified generation of activated Gβγ-dimers at the leading edge of the cell, faithfully representing a primary linear amplification step in directional sensing. We propose here that modulation of the receptor mobility is directly involved in directional sensing and provides a new mechanistic basis for the primary amplification step in current theoretical models that describe directional sensing.

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Key words: Single-molecule microscopy, Chemotaxis, Dictyostelium discoideum, G-protein-coupled receptor

Introduction
Most eukaryotic cells chemotax by converting shallow differences of extracellular chemoattractant gradient into highly localized intracellular responses (Affolter and Weijer, 2005; Parent et al., 1998). Directional sensing is the ability of the cell to determine the direction of extracellular cues and can be thought of as a ‘compass’ (Franca-Koh et al., 2006). The local accumulation of PtdIns(3,4,5)P3 at the leading edge of chemotaxing cells as a result of the reciprocal localization of the phosphatidylinositol 3-kinase (PI3K) and the PI3 phosphatase (PTEN) has been proposed to act as the internal compass (Weiner, 2002). However, recent evidence in the amoebae Dictyostelium discoideum shows that a mutant lacking all PI3K and the PTEN phosphatase is still able to sense direction (Hoeller and Kay, 2007). Another study in which the function of the six PI3Ks in D. discoideum was characterized suggests that other signalling pathways are possibly sufficient in directional sensing in response to a high signal, but that PI3K signalling is crucial for detecting weak signals (Takeda et al., 2007). Indeed, recently, another pathway was found in D. discoideum, involving the phospholipase A2, that acts in parallel to the PI3K pathway (Chen et al., 2007). As the signalling network is branched into these parallel pathways after G-protein-coupled receptor (GPCR)-mediated activation of a G protein, and the GPCR and G-protein are the only crucial components of the pathway, one might expect the compass of the cell to be at this level of the signalling pathway.

Until now, this idea has been rejected because the cAR1-receptor and the Gα2/Gβγ-proteins, remain uniformly distributed along the cell surface, as demonstrated using fluorescent cAR1 and Gβ subunit in D. discoideum (Jin et al., 2000; Xiao et al., 1997). FRET studies between the G-protein subunits revealed a higher GPCR-mediated activation of the G-proteins at the leading edge (Xu et al., 2005). However, this was interpreted as directly reflecting the receptor occupancy. Owing to technical difficulties, the GPCR activation of the G-protein has never been directly visualized, although the dynamics of activation is of the highest importance when interpreting the FRET data correctly. It has been found using single-molecule microscopy of fluorescence-labelled cAMP, that the dissociation rate of cAMP at the leading edge of the cell was twice that at the trailing edge (Ueda et al., 2001). It is therefore tempting to speculate that directional sensing is directedly related to the acceleration of the activation step of the G-protein-linked signalling pathways at the leading edge. This theory is addressed here by exploring whether the mobility of the receptor exhibits an asymmetry, which in turn could account for the primary decision in the spatially restricted response of the downstream signalling pathway during cell migration.
Results

In order to analyze the mobility of the receptor in detail, it was necessary to monitor individual fluorescent receptors. Therefore, the cAMP receptor cAR1 of D. discoideum was fused to eYFP and stably expressed in receptor-deficient car1\(^{-}\) cells to an expression level that resembles that of the endogenous receptor in wild-type cells (see Fig. 1A, and Materials and Methods). The fusion protein proved to be effectively synthesized and targeted to the plasma membrane (Fig. 1B). The molecular weight and stability of the fusion protein was validated by immunoblot of whole-cell extracts of transformed car1\(^{-}\) cells using a purified GFP antibody. As shown in Fig. 1A, the receptors appeared as a single band at the predicted size of ~70 kDa. cAR1-eYFP was functionally indistinguishable from wild-type cAR1, as the fusion protein complemented the deficiency of the cAR1 protein and completely rescued the developmental program (Devreotes, 1994) of car1\(^{-}\) cells, including the aggregation process and the formation of a fruiting body (Fig. 1C, bottom right). By contrast, car1\(^{-}\) cells could not undergo development beyond the one-cell state (Fig. 1C, top left).

Single-molecule microscopy (SMM), a combination of regular wide-field microscopy with laser excitation and ultra-sensitive CCD camera detection, was used to obtain high spatial (~40nm) and wide-field microscopy with laser excitation and ultra-sensitive CCD camera detection, was used to obtain high spatial (~40nm) and temporal (~44 milliseconds) resolution information on the mobility of cAR1 receptors. In order to reach a density of fluorescent receptors at which individual molecules could be observed (~1/µm\(^2\)), cells were photobleached prior to imaging (Fig. 1D). As photobleaching occurs at random, we assume that the unbleached population of receptors is a fully representative subpopulation of all receptors. As predicted for individual molecules, fluorescence signals were characterized by diffraction-limited spots on the camera that exhibited single-step photobleaching typical for single molecules (Fig. 1E). This finding was independent of position, stage of cellular development, mutations or stimulation protocol. Images were taken at a rate of 23 frames/second. Automated analysis yielded values for the integrated fluorescence signal and the lateral position of the receptors (accuracy ~40nm) (Schmidt et al., 1996). From the receptor positions in consecutive images, trajectories of individual cAR1-eYFP were reconstructed (Fig. 1F).

Trajectories of cAR1-eYFP similar to that shown in Fig. 1F from the top membrane of control cells were further analyzed to study the receptor mobility. For the analysis, the cumulative probability (P) of the squared displacements (r\(^2\)) was determined (Schutz et al., 1997) for a time delay between images set to 44 milliseconds (Fig. 2). Two-thousand-and-sixty trajectories were analyzed, and squared displacements up to 0.2 µm\(^2\) were found. Fig. 2A shows the data for a control cell that followed the developmental cycle for 2-3 hours (see Materials and Methods), at which point Go2 proteins were expressed (Kumagai et al., 1989) but no endogenous cAMP was detected (Kesbeke et al., 1986). It should be noted that Gβγ proteins were constitutively present in the plasma membrane of D. discoideum. Subsequently, cells were brought to an early aggregation stage by starvation during which they are gradient-sensing competent (Fig. 2B). This procedure is referred to in the following as natural assay. The leading and trailing edge of each cell was defined with respect to the centre of the aggregate. The cumulative probability distributions of the squared displacements of receptors located at the anterior and posterior of chemotaxing cells, respectively, showed a slight difference (Fig. 2B). In order to quantify the significance of that difference, we applied a statistical two-sample Kolmogorov-Smirnov test (KS-test).

![Fig. 1. Generation of cell lines expressing cAMP-receptor/eYFP fusion proteins at endogenous levels. (A) Detection of cAR1-eYFP fusion protein by western blot using an anti-GFP antibody. Free YFP showed the expected band at ~30 kDa (lane 3). As cAR1 has a size of 40 kDa, the correct size for the fusion protein is 70 kDa, which was observed (lane 2). Transformed car1\(^{-}\) cells with the cAR1-eYFP fusion protein exhibited a protein-band at the correct size (lane 2), whereas car1\(^{-}\) cells did not (lane 1). Free eYFP was not detected in cAR1-eYFP/car1\(^{-}\) cells. (B) cAR1-eYFP was localized at the plasma membrane of car1\(^{-}\) cells, as detected by confocal microscopy. (C) The first image shows the aggregation-deficient phenotype of car1\(^{-}\) mutant 24 hours after starvation. These cells were not able to initiate the developmental cycle. The following images display the different developmental stages of car1\(^{-}\) cells transformed with the cAR1-eYFP construct. The developmental defect of car1\(^{-}\) cells was rescued by the cAR1-eYFP transformation. (D) The left picture shows a fluorescence image of the top membrane of a typical unstimulated car1\(^{-}\) cell transformed with cAR1-eYFP. After a brief photobleaching pulse (2.5-5.0 seconds) individual receptors were detected (peaks of fluorescence in the right image). (E) Fluorescence signal of an individual cAR1-eYFP molecule as a function of time, showing a single-step photobleaching event characteristic for individual molecules. (F) Two examples of trajectories of individual cAR1-eYFP molecules diffusing in the top plasma membrane.](image_url)
cumulative probability distribution of squared displacements, $P(r^2)$, of the mobile fraction of the receptors was not different between anterior and posterior regions of the cell were defined as the regions closest or farthest away from the position of the needle, respectively.

Immediately before applying cAMP, polarized cells were randomly oriented with respect to the position of the needle, which led to an average receptor mobility irrespective of its position on the cell (Fig. 2C) that was equal to that of the control (Fig. 2A). Hence, as expected for randomly oriented cells, receptor mobility in the ensemble average. After the cells sensed a gradient of cAMP (30-60 seconds after cAMP application) a difference in mobility was clearly observed (Fig. 2D): receptors at the leading edge had a higher mobility compared with those at the posterior, with the latter being indistinguishable from the control (Fig. 2A). In order to confirm those results obtained by the needle assay, cells were exposed to a global cAMP level of 1 mM for less than 5 minutes. Receptor mobility on the whole cell was found to be indistinguishable from that found at the front of gradient-sensing cells in the needle assay (see supplementary material Fig. S1E).

For a better understanding what this higher mobility entailed, a quantitative description of the data was obtained by global analysis of the squared displacement distributions (Fig. 2). The cumulative probability distributions of squared displacements, $P(r^2)$, as shown in Fig. 2, were fitted to a two-population model, reflecting a mobile receptor fraction and an immobile receptor fraction (Schutz et al., 1997):

$$P(r^2) = 1 - \alpha \exp \left( -r^2/\text{MSD} \right) - (1-\alpha) \exp \left( -r^2/4\sigma^2 \right)$$

Equation 1 leads to a characteristic mean squared displacement, MSD, and a fraction of mobile receptors, $\alpha$. In our experiments, the lateral accuracy was found to be $\sigma = 40 \text{ nm}$. Assuming that mobile receptors were characterized by one characteristic MSD, all data (Fig. 2) were fitted simultaneously, yielding a fraction $\alpha$ (Fig. 3) for each data set and the corresponding characteristic MSD. The receptors were characterized by MSD=0.034$\mu$m$^2$/s, which, using the delay between two observations of $t_{lag}=44$ms, translates into a diffusion constant of $D=(\text{MSD}/4\sigma^2) t_{lag}=0.17\pm 0.02\mu$m$^2$/second. In the control, 38% of the receptors were mobile (Fig. 2A), whereas 44% and 39% were mobile in the natural assay at the anterior and the posterior of the cell, respectively (Fig. 2B). Herewith, receptor mobility at the anterior of the cell by a factor of 54%/31% (i.e. 1.7) when compared with receptors at the posterior. Furthermore, the shift in mobile fraction observed in the natural assay (Fig. 2B) was reduced compared with the shift in the needle assay (Fig. 2D). This difference is readily explained by taking into account that, in the natural assay, cAMP is produced in waves within which the local gradient exists for only part (50%) of the cycle. Consequently, the difference in mobile fraction observed in the needle assay (54% versus 31%) is reduced by half for the natural assay which fully accounts for our findings (Fig. 2B).

The change in mobile fraction was not due to a change in membrane viscosity, as the mobile fraction of an inert membrane.
marker (individual concanavalin A conjugated with Alexa647 molecules were followed) was position independent even for polarized cells (see supplementary material Fig. S1). The distributions of receptors in the posterior for both the natural and the needle assay were, within experimental error, indistinguishable from the distribution in control cells. This indicates that only receptors at the anterior exhibit a specific, spatially restricted response to the chemotactic receptor. The latter finding is taken here as a first evidence that the chemoattractant receptor shows a spatially restricted response upon cAMP-induced activation in chemotaxis.

The issue of whether the difference in mobile fraction between the anterior and the posterior of gradient-sensing polarized cells is a secondary effect caused by downstream signalling components. The latter must be absent in Gα2 cells in which the downstream signalling is impaired. The lack of change in membrane viscosity upon stimulation (supplementary material Fig. S1). Therefore, cells expressing cm1234-eYFP were measured very close to the aggregation centre, a situation reflecting that of the needle assay. For cm1234-eYFP, the fraction of mobile receptors at the anterior exceeded that at the posterior by a factor of 1.7 (72% versus 43%, see supplementary material Fig. S1). The change in receptor mobile fraction upon stimulation when compared with the mobile fraction in control cells (supplementary material Fig. S1). The mobile receptor fraction increased from 38% to 58% (see supplementary material Fig. S1). The change in receptor mobile fraction had already occurred in control cells, which confirmed that ligand-induced actin polymerization also did not play any role in those observations, given that Gα2 cells are deficient in a chemoattractant-induced response (Kumagai et al., 1989). Although the results reported above were compelling evidence for the hypothesis that Gα2, via cytoskeleton anchoring, controls the mobility of cAR1, one might argue that the change in mobile fraction was a secondary effect caused by downstream signalling components. The latter must be absent in Gα2 cells in which the downstream signalling is impaired. The lack of change in membrane viscosity upon stimulation (supplementary material Fig. S1), however, suggests that this is not the case. In addition, the mobility of cAR1-eYFP in a pI3K−/− background displayed the same change in mobile fraction upon stimulation when compared with the wt-cells (supplementary material Fig. S1). Furthermore, wild-type cells treated with the PI3K inhibitor LY294002 (1 μM) showed a pI3K−/−, pten−/− mutant in which all PI3K and PTEN genes were knocked out (Hoeller and Kay, 2007) exhibit a phenotype in terms of chemoattractant sensing similar to that of the pI3K−/−, pten−/− mutant. Together, this suggests that the cause of the shift in mobile fraction observed of cAR1 is located upstream of PI3K.

**Discussion**

In conclusion, our data suggest that the immobile fraction of receptors that become mobile upon stimulation reflects inactive receptors bound via Gα2 to cytoskeletal elements. In addition to this cAMP-responsive fraction, a second pool of immobile receptors that did not change their mobility upon stimulation was significantly different for the two cell lines. No difference in cAR1-eYFP mobility between control and starved cAR1-eYFP/gα2− cells was observed independently of the presence of a cAMP gradient (Fig. 2E,F). The mobile receptor fraction in gα2− cells (51%, Fig. 3) was, for all conditions, comparable with that at the anterior of gradient sensing cAR1 cells, and independent of receptor localization. These findings together suggest that the population of immobile receptors in cAR1-eYFP/cAR1 cells that become mobile upon cAMP addition would represent receptors that became uncoupled from their associated Gα2 protein upon cAMP stimulation. As binding of the receptor to the GDP-bound inactive Gα2-GDP-βγ is expected to result in only a minor decrease in receptor diffusion constant, given the logarithmic dependence of D on receptor size (Saffman and Delbruck, 1975), pure binding can not account for the dramatic slowdown observed. One explanation follows from the postulated idea that G proteins interact with the cytoskeleton meshwork via protein complexes on microtubule plus ends (Rogers et al., 2004; Hampeolz and Knoblich, 2004). The microtubule plus-end complex seems to be required for the capture of microtubule tips at cortical sites by mediating interactions of microtubule tips with cortical actin, as well as with membrane proteins; this process plays a major role in nuclear migration, spindle formation and directed cell movement (Hestermann et al., 2002; Siegrist and Doe, 2007). As the mechanisms underlying chemotaxis are highly conserved and various proteins of this complex have been identified in *D. discoideum* (Hestermann et al., 2002), we propose a similar linkage to explain our results in *D. discoideum*. A stable anchoring of the receptor via Gα2 to the cytoskeleton would lead to a larger fraction of immobile receptors. To test this hypothesis, we applied Latrunculin B to control cells, which confirmed our prediction. The mobile fraction increased from 38% to 58% (see supplementary material Fig. S1). The change in receptor mobile fraction in control cells, which confirmed that ligand-induced actin polymerization also did not play any role in those observations, given that Gα2 cells are deficient in a chemoattractant-induced response (Kumagai et al., 1989).
observed for all cells. This pool of cAMP-irresponsive receptors might be differently coupled to cellular structures that are static during the measurement (10-100 ms). Similarly, the mobile population found in control cells might indicate that there is a basal level of receptor activity. A more likely explanation though is that not all of cAR1 is precoupled to the G protein, a notion that has been suggested in several studies of mammalian GPCRs (Hein et al., 2005; Azpiazu and Gautam, 2004; Chisari et al., 2007). With reference to the results obtained on the cm1234-eYFP mutant, it should be noted that phosphorylation of the receptor is independent of G-protein signalling, as G-protein re-association still occurred on phosphorylated receptors (Janetopoulos et al., 2001).

Fig. 4A summarizes our findings and incorporates them into a model that relates a biological function for the increased mobile fraction of cAR1. Receptors normally reside in an inactive G\(\alpha_2\)/GDP precoupled state and are immobile. Upon receptor activation, G\(\alpha_2\)GDP is processed into G\(\alpha_2\)GTP and dissociates both from the receptor and from its G\(\beta\gamma\) partners. In turn, the receptor is decoupled from structural elements and becomes mobile. Accordingly, it is able to activate other G\(\alpha_2\)GDP hetero-trimers. In the last step, receptor reassociation with the G protein is paralleled by anchorage to structural elements and receptor immobilization.

Inherent to most models describing directional sensing (Charest and Firtel, 2006; Janetopoulos et al., 2004; Postma and Van Haastert, 2001) is an initial linear amplification step for which our findings possibly yield a molecular interpretation. Assuming that G-protein activation is solely a diffusion-limited process, the higher mobile fraction of receptors at the anterior will increase the rate of activation of G proteins in proportion to the diffusion constant. Taking the off-rate of cAMP from the receptor, \(k_{\text{off}}=0.39/\text{second}\) (Ueda et al., 2001), as a typical timescale, the associated distance of an activated receptor movement becomes \(\sqrt{4D/k_{\text{off}}}=1.40\ \mu\text{m}\) which is far enough to activate additional G proteins at an estimated concentration of \(~10^2/\mu\text{m}^2\). The amplification step proposed here, one receptor activating multiple G-proteins, will lead to a higher local G-protein excitation at the anterior, as a result of the difference in receptor occupancy. We suggest here that this initial linear amplification step might be crucial for crossing a threshold, as set by constitutive signal inhibition (e.g. by PTEN), and for subsequent signal propagation. Higher local G-protein excitation at the anterior has been monitored previously by fluorescence resonance energy

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**Fig. 4.** Model describing accelerated signalling at the leading edge. (A) In resting cells, we found two receptor populations: immobile and mobile receptors. A fraction of the immobile receptors is coupled to G\(\alpha_2\)GDP/G\(\beta\gamma\), which, in turn, is coupled to protein-protein networks and/or the cytoskeleton which inhibits diffusion (1). This fraction of immobile receptors becomes mobile by uncoupling of G\(\alpha_2\)GTP upon cAMP activation. Free G\(\alpha_2\)GTP and free G\(\beta\gamma\) subunits activate intracellular signalling (2). The mobile receptors have the ability to further activate other G\(\alpha_2\)GDP/G\(\beta\gamma\) complexes in a diffusion-limited process (3). In a final step, re-association of the receptor with G\(\alpha_2\)GDP/G\(\beta\gamma\) and corresponding loss of cAMP immobilizes the receptor again (4). (B) An ellipsoidal cell is exposed to a gradient of 0.4nM/\mu\text{m} cAMP. The concentration at the leading edge is 66nM and that at the trailing edge 58nM. The density of active cAR1 receptor (cAR1*) is plotted versus the position along the cell membrane. At the leading edge the density of active cAR1 is higher by a factor of 1.05 when compared with the density at the trailing edge (6.1 molecules/\mu\text{m}^2 versus 5.8 molecules/\mu\text{m}^2) following the cAMP gradient. The density of activated G\(\beta\gamma\) at the leading edge was 73.0 molecules/\mu\text{m}^2, whereas that at the trailing edge was 71.4 molecules/\mu\text{m}^2. Hence, diffusion leads to a linear amplification of the gradient by a factor of 5.
transfer between $\alpha$ and $\beta\gamma$, but has been interpreted only in terms of receptor occupancy (Xu et al., 2005). If we further assume that the probability of the loss of cAMP from the receptor is related to the process of G-protein activation, i.e. collision, the above mechanism also explains the increased cAMP off-rate at the anterior of the cell (Ueda et al., 2001).

To test whether the measured values would lead to the proposed linear amplification, a finite element model of the cellular processes was implemented in VCell (see Materials and Methods). In the model, the receptor $\text{cAR1}$ was activated upon cAMP binding, resulting in an activated $\text{cAR1}$ (hereafter referred to as $\text{cAR1}^*$). $\text{cAR1}^*$ in turn was allowed to catalyze the dissociation of the $\alpha_2\beta\gamma$ heterotrimer into membrane-localized $\beta\gamma$ and cytosolic Go2 (see supplementary material Fig. S2). Using estimates for concentrations known (see supplementary material Table S1) and for rate constants (see supplementary material Table S2), the effect of diffusion on the ability of $\text{cAR1}^*$ to activate multiple G proteins was investigated. As predicted, receptors activated multiple G proteins in the case of mobile receptors. At equilibrium conditions, 240 active $\text{cAR1}^*$ receptors at the leading edge activated 2866 $\beta\gamma$-proteins, whereas at the trailing edge, 228 $\text{cAR1}^*$ molecules activated 2802 $\beta\gamma$-proteins (Fig. 4B). The gradient is thus translated from an anterior/posterior difference of ten activated $\text{cAR1}^*$ receptors to a difference of 64 activated $\beta\gamma$-proteins, a fivefold linear amplification of the difference signal. It should be noted that signal amplification prevailed even at a ten times lower cAMP gradient, for which an even larger linear signal amplification of 20 was found (not shown). Next to a significant amplification of the primary signal, the finite-element model clearly confirms that gradient sensing must be transduced via activated membrane-bound $\beta\gamma$.

The high mobility of cytosolic Go2 completely washes out any external gradient, a result that had been predicted by the so-called depletion model (Postma and Van Haastert, 2001).

In this study, we found a cAMP-induced increase in the amount of mobile $\text{cAR1}$ receptors at the leading edge of cells. We showed that the acquired mobility of $\text{cAR1}$ allows for the receptor to activate multiple G proteins in the membrane and the simple model introduced above shows a fundamental type of physical amplification process that occurs at the very beginning of a signalling pathway. The experimental results, however, yielded a difference in mobile receptors between anterior and posterior of a factor of $\sim$1.7. Hence, in order to explain our experimental data fully, a non-linear process will have to be incorporated into an improved model. The latter might include, for example, a $\text{cAR1}^*$-induced $\text{cAR1}$ activation, the potential role of which must be verified in future experiments. Another explanation could be that the initial amplification, as predicted by the linear model, is enforced by an unknown feedback mechanism that affects the G protein coupling to the receptor. A recent study in mammalian cells revealed a shuttling of the G protein subunits between the cytoplasm and the membrane. It is therefore very feasible that the dynamic process of G protein coupling to the G protein-coupled receptor could be affected by downstream signalling component. This is currently tested in $D. discoideum$. Certainly, in order to understand the whole process of spatially restricted responses and chemosensing, including known feedback mechanisms and downstream processes (Charest and Firtel, 2006), more complex models, such as the local-excitation global-inhibition [LEGI (Ma et al., 2004)] or the diffusion-translocation model (Postma and Van Haastert, 2001), will have to be incorporated. These models do, however, benefit from the primary linear amplification our model predicts: the primary amplification may act as the compass of the cell during directional sensing. Considering the highly conserved nature of G-protein coupled receptor signalling in eukaryotes, our model might have even broader applications for other G-protein-coupled receptor signalling pathways.

Materials and Methods

$c\text{AR1-eYFP fusion protein}$

To create C-terminal YFP-tagged $\text{cAR1}$, eYFP DNA was created by PCR. The N-terminal primer CGGTCAGATCTGGTAAGGACGGCAAGGGAGGAG contained an XbaI site at 5’ end, followed by the N-terminal residues of eYFP. The C-terminal primer GCTTCTAGACTTGATCAAGGGACGGCAAGGG contained an Xhol site at 3’ end. The PCR products were double digested with XhoI and XbaI and then cloned into the Xhol site of the $D. discoideum$ $\text{cAR1}$ expression plasmid (Parent et al., 1998). The DNA was purified and transformed into JB4 cells ($\text{cAR1}$ or $\text{Go2}$ cells by electroporation (Zigmond et al., 1981). Clones were grown up in a Petri dish in HL5-medium containing 10 µg/ml G418. Cells were cultured in six-well plates in axenic medium with addition of 100 µg/ml ampicillin and 100 µg/ml mixture of penicillin and streptomycin (1:1) at 22°C. The expression level of the $\text{cAR1-eYFP}$ in $\text{cAR1}$ cells was calculated in the following manner. The fluorescence of the cells at the membrane before measurement was on average five-times higher than the fluorescence expected for a single molecule (1000 cts/3 msconds versus 185 cts/3 msconds) based on the fluorescence of single YFP molecules in an artificial lipid membrane (Harms et al., 2001). Thus, there were on average five receptors within each diffraction-limited area [see $\sigma=2r=0.03$ µm, with $2r=1.22\sqrt{3}/\lambda$ (i.e. 220 nm), for a wavelength ($\lambda$) of 514 nm. For the whole cell, the surface of the membrane was $S=4\pi R^2-3\pi R^2$ µm², where $R$=5 µm is the typical radius of the cell. This leads to a total number of receptors $n=5:s=4\times10^6$, which is comparable with the expression level of endogenous receptors in wild-type cells (Johnson et al., 1991; Van Haastert, 1987).

Developmental test in $\text{cAR1}$ expressing $\text{cAR1-eYFP}$

Transformants were plated on non-nutrient plates at a concentration of 10⁶ cells/ml. Development was monitored for the next 30 hours with a confocal microscope (Leica MZFLIII).

Immunoblotting

$c\text{AR1}$ and $\text{cAR1-eYFP}$ cells were solubilized with SDS-sample buffer and resolved by SDS-PAGE on 10% gels along with a set of protein MW standards. $\text{cAR1-eYFP}$ was detected by immunoblot with anti-GFP antibody. Free YFP was also run on the gel and immunoblotted.

Cell preparation and measurement

Control cells were transferred to phosphate buffer (0.534 g Na₂HPO₄, 0.952 g KH₂PO₄ in 1 litre of H₂O, set pH to 6.5) after one night in low-fluorescence medium (Liu et al., 2002) and measured after 1 hour. This procedure was required to lower the intrinsic auto-fluorescence as described by de Keijzer et al. (de Keijzer et al., 2007). Low-fluorescence medium contains few nutrients and therefore control cells are not vegetative, but their state is estimated to be comparable with 2-3 hours of starvation. These cells were not able to migrate towards the CAMP source in the needle assay. For the experiments with the natural assay, the cells were starved at a concentration of 10⁵ cells/ml in phosphate buffer for 20 hours at 16°C. For the chemotaxis needle assay, the cells were maintained overnight in low-fluorescence medium and starved for 6-8 hours in phosphate buffer at 22°C. Before measuring, the cells were tested to be aggregation competent. All measurements were performed in two-well, chambered coverglasses (1.5 Borosilicate Sterile, Lab Tek II).

The cells were placed in a distance of 75µm from the opening (r=0.25µm) of a needle. After applying cAMP, the cells, oriented in a front-back gradient way, were captured with the microscope and the measurement was performed in the middle to focus on the cell and perform single molecule measurements. At least 25 cells were measured for every condition.

Labelling membrane with marker

ConcanavalinA Alexa647 conjugate (Invitrogen) was used as a marker to label the plasma membrane. ConcanavalinA (ConA) selectively binds to $\alpha$-mannopyranosyl and $\alpha$-glucopyranosyl residues. Cells where incubated for 10-15 minutes in 1 ml of 80ng/ml ConA in PB. The excess marker was removed by washing the cells three times with PB before measuring. The low concentration in combination with the short incubation time prevented formation of ConA clustering, and allowed us to follow individual ConA bound to the plasma membrane.
Single-molecule microscopy

The experimental setup for single-molecule imaging has been described in detail previously (Schmidt et al., 1996). The samples were mounted onto an inverted microscope (Axiovert 100, Zeiss) equipped network. Centrosomal microtubule plus end 2005). Cytoskeletal dynamics during chemotaxis. Curr. Opin. Genet. Dev. 15, 621-630.


Figure S1

Receptor mobility controls

A. Cumulative probability distribution of squared displacements of concanavalin A (labelled by Alexa 647) in the anterior (gray circles) and posterior (black circles) top membrane of cAR1-eYFP/car1- cells. The distributions were equivalent in terms of a 2-sample Kolmogorov-Smirnoff test with an acceptance level set to 93.5%. Fluorescent labelled lipids
to assay membrane viscosity were not successful because of their fast uptake by *D. discoideum* cells.

**B.** Cumulative probability distributions of squared displacements of cm1234-eYFP from the top membrane of polarized *wt* cells from the anterior (*N*=398) and from the posterior (*N*=349), respectively. The data were taken in the natural assay. Fitting the distributions to the two-population model revealed 72±7% and 42±4% of the receptors were mobile, respectively.

**C.** Cumulative probability distribution of squared displacements of cAR1-eYFP in the top membrane of control (16 h in LoFlo and subsequently 2 h in PB) cAR1-eYFP/carl cells. In relation to the data presented in Figure 2A, these data were taken in a similar but independent experiment prior to the addition of the actin-depolymerization drug, Latrunculin B. The distributions of cAR1-eYFP in cells treated with 5 and 10 µM Latrunculin B were significantly different from cells which were not treated with Latrunculin B as tested by a 2-sample Kolmogorov-Smirnoff test with an acceptance level set to 93.5%, respectively. 58±6% and 59±6% of the receptors were found to be mobile in the 5 an 10 µM Latrunculin B-treated cells, respectively by fitting the distributions to the two-population model. In cells not treated with Latrunculin B 38 ±4% of the receptors were found to be mobile.

**D.** Cumulative probability distribution of squared displacements of cAR1-eYFP from the top membrane of the anterior (*N*=141) and posterior (*N*=207) of polarized *pi3k*- cells in a chemotaxis needle assay. The distributions of the anterior and posterior were identical to the anterior and posterior distributions of cAR1-eYFP in *car1* cells, respectively, whereas they were different from each other. The significance was tested in terms of a 2-sample Kolmogorov-Smirnoff test with an acceptance level set to 93.5%. 46±5% and 30±3% of the receptors were found to be mobile, respectively.

**E.** Cumulative probability distribution of squared displacements of cAR1-eYFP from the top membrane of cells globally stimulated with 1 µM cAMP for < 5 min (open gray circles, *N*=385) in comparison to those at the anterior (closed gray circles, *N*=225), and posterior (closed black circles, *N*=367) of polarized *car1* cells in a chemotaxis needle assay (see for the latter two also fig.2D). The global stimulation distribution of squared displacements was identical to the distribution at the anterior of chemotaxing cells in terms of a 2-sample Kolmogorov-Smirnoff test with an acceptance level set to 93.5%; it was different to the distribution at the posterior of chemotaxing cells. 54±5% of the receptors were found to be mobile on global stimulation.
Figure S2
Details of the model

Description of the components in the finite element model implemented in VCell. Extracellular cAMP binds to the cAR1 receptor with an on-rate of 9.3 μM$^{-1}$s$^{-1}$ leading to an activated receptor cAR1*. cAR1* inactivation is set by the off-rate of 0.39s$^{-1}$. The activated receptor catalyses the dissociation of the G-protein Gα2βγ hetero-trimer into a membrane bound Gβγ and a cytosolic Gα2 subunit. This reaction was assumed to be diffusion limited with a reaction rate of 0.66 μm$^2$/molecule/s. The diffusion-limited back-reaction of Gα2 re-coupling to Gβγ is characterized by a rate constant of 100 μM$^{-1}$s$^{-1}$. This reaction thereby deactivates G-protein signalling.
**Table S1:** Parameters of the finite-element model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>location</th>
<th>Init. conditions</th>
<th>Units</th>
<th>D(μm²*s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP</td>
<td>Fixed gradient of cAMP</td>
<td>extracellular</td>
<td>Varies</td>
<td>μM</td>
<td>fixed</td>
</tr>
<tr>
<td>cAR1</td>
<td>Inactive cAMP receptor</td>
<td>membrane</td>
<td>10.0</td>
<td>No./μm²</td>
<td>0.04</td>
</tr>
<tr>
<td>cAR1*</td>
<td>Active cAMP receptor</td>
<td>membrane</td>
<td>0.0</td>
<td>No./μm²</td>
<td>0.19</td>
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<tr>
<td>Gα₂βγ</td>
<td>Inactive G-protein heterotrimer</td>
<td>membrane</td>
<td>139.76</td>
<td>No./μm²</td>
<td>0.04</td>
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<tr>
<td>Gα₂</td>
<td>Gα subunit</td>
<td>cytosol</td>
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<td>μM</td>
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<tr>
<td>Gβγ</td>
<td>Membrane associated second messenger</td>
<td>membrane</td>
<td>0.0</td>
<td>No./μm²</td>
<td>0.5</td>
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</tbody>
</table>

**Table S2:** Rate constants for finite-element model

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate constants</th>
</tr>
</thead>
<tbody>
<tr>
<td>$cAMP + cAR1cAMP \rightleftharpoons_{K_f}^{K_r} cAR1$</td>
<td>$K_f$=9.3μM⁻¹*s⁻¹ $K_r$=0.39s⁻¹</td>
</tr>
<tr>
<td>$Gα₂βγ \rightarrow_{K_f}^{K_r} Gα + Gβγ$</td>
<td>$K_f$=0.7μm²<em>No.⁻¹</em>s⁻¹</td>
</tr>
<tr>
<td>$Gα₂ + Gβγ \rightarrow_{K_r}^{K_f} Gαβγ$</td>
<td>100μM⁻¹*s⁻¹</td>
</tr>
</tbody>
</table>