Locally controlled inhibitory mechanisms are involved in eukaryotic GPCR-mediated chemosensing

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G protein–coupled receptor (GPCR) signaling mediates a balance of excitatory and inhibitory activities that regulate Dictyostelium chemosensing to cAMP. The molecular nature and kinetics of these inhibitors are unknown. We report that transient cAMP stimulations induce PIP3 responses without a refractory period, suggesting that GPCR-mediated inhibition accumulates and decays slowly. Moreover, exposure to cAMP gradients leads to asymmetric distribution of the inhibitory components. The gradients induce a stable accumulation of the PIP3 reporter PHCrac-GFP in the front of cells near the cAMP source. Rapid withdrawal of the gradient led to the reassociation of G protein subunits, and the return of the PIP3 phosphatase PTEN and PHCrac-GFP to their pre-stimulus distribution. Reapplication of cAMP stimulation produces a clear PHCrac-GFP translocation to the back but not to the front, indicating that a stronger inhibition is maintained in the front of a polarized cell. Our study demonstrates a novel spatiotemporal feature of currently unknown inhibitory mechanisms acting locally on the PI3K activation pathway.

Introduction

Eukaryotic cells can detect and move up concentration gradients of chemoattractants, a process known as chemotaxis (Zigmond, 1978; Chung et al., 2001a; Iijima et al., 2002; Van Haastert and Devreotes, 2004). This behavior plays an important role in a number of processes, including metastasis, angiogenesis, immune responses, and inflammation (Murphy, 1994; Parent and Devreotes, 1999; Condeelis et al., 2005). Furthermore, chemotaxis is essential for cell aggregation in the life cycle of the social amoeba, Dictyostelium discoideum (Gerisch, 1982; Devreotes and Zigmond, 1988; Devreotes, 1994; Van Haastert and Devreotes, 2004). Chemotaxis is a coordinated phenomenon of three fundamental cell processes: gradient sensing, cell polarization, and cell motility. Chemotactic cells, such as neutrophils and D. discoideum, display polarized morphology, involving asymmetric distributions of many signaling molecules (Parent and Devreotes, 1999; Comer and Parent, 2002; Iijima et al., 2002; Devreotes and Janetopoulos, 2003), and heightened responsiveness to the attractant at their leading edge (Parent et al., 1998; Jin et al., 2000; Xu et al., 2003). These crawling cells extend their leading edges by assembling a force-generating network of actin filaments beneath the plasma membrane (Chung et al., 2001a; Pollard and Borisy, 2003). Elsewhere in the cell, actin collaborates with myosin to retract the rear of the advancing cell and to prevent errant pseudopod extension (Chung et al., 2001a). Consequently, the actin-depolymerizing agent Latrunculin can be used to eliminate polarization and motility of D. discoideum cells, and thus facilitate quantitative spatiotemporal analyses of the mechanisms underlying gradient sensing (Parent et al., 1998; Jin et al., 2000; Xu et al., 2005).

Gradient sensing is mediated by G protein–coupled receptors (GPCRs) and associated signaling components that detect the spatiotemporal changes of chemoattractants and translate shallow gradients of chemoattractants into steep intracellular gradients of signaling components (Parent and Devreotes, 1999; Chung et al., 2001b; Funamoto et al., 2002; Iijima et al., 2002). Binding of cAMP to the GPCR cAR1 induces the dissociation of heterotrimeric G proteins into Gα2 and Gβγ subunits (Jin et al., 2000; Janetopoulos et al., 2001; Xu et al., 2005). Free Gβγ activates Ras, thereby leading to the activation of PI3K, which converts P(4,5)P2 (PIP2) to P(3,4,5)P3 (PIP3) in the plasma membrane (Li et al., 2000; Funamoto et al., 2001; Stephens et al., 2002; Sasaki et al., 2004; Wessels et al., 2004). The phosphatase PTEN acts as an antagonist of PI3K, dephosphorylating

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Abbreviations used in this paper: CRAC, cytosolic regulator of adenylyl cyclase; GPCR, G protein–coupled receptor; PH, pleckstrin homology; WT, wild type.

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PI3K to regenerate PIP3 (Funamoto et al., 2002; Iijima and Devreotes, 2002; Li et al., 2005). PIP3 mediates cellular processes by recruiting proteins with pleckstrin homology (PH) domains, such as cytosolic regulator of adenyl cyclase (CRAC) and Akt/PKB, to the plasma membrane (Parent et al., 1998; Meili et al., 1999). Both CRAC and Akt/PKB play roles in the regulation of actin polymerization during chemotaxis (Meili et al., 1999; Comer et al., 2005). Recent progress in fluorescence microscopy has permitted measurements of the spatiotemporal changes of many signaling events in living cells with high spatiotemporal resolution required to test models of gradient sensing (Ueda et al., 2001; Sasaki et al., 2004; Xu et al., 2005).

There are several key features of gradient sensing. First, cells have the ability to spontaneously terminate responses under a sustained cAMP stimulation in a process called “adaptation” (Parent et al., 1998; Xu et al., 2005). Second, if cAMP is removed from adapted cells, the cells will enter a de-adaptation phase—a refractory period lasting several minutes during which the cells progressively regain their ability to respond to another cAMP stimulation (Dinu et al., 1980a,b). Third, cells have the capability of translating shallow cAMP gradients across the cell diameter into highly polarized intracellular responses, a process called “amplification” (Parent and Devreotes, 1999; Servant et al., 2000; Chung et al., 2001a). To explain these features, it has been proposed that an increase in receptor occupancy activates two antagonistic signaling processes: a rapid “excitation” that triggers cell responses, such as the membrane accumulation of PIP3, and a slower “inhibition” that turns off those responses (Parent and Devreotes, 1999). Although many of the molecular mechanisms of the excitatory process have been identified, those of the inhibitory process have remained elusive.

The dynamic relationship between excitation and inhibition that leads to activation, adaptation, and amplification has been studied by direct visualization and quantitative analysis of the spatiotemporal changes in receptor occupancy, G protein dissociation, PIP3 and PTEN distribution, and PIP3 level along the membrane (Xu et al., 2005; Meier-Schellersheim et al., 2006). Over the years, models have been proposed to explain how the excitatory and the inhibitory processes interact in cells responding to chemoattractants to achieve adaptation or amplification (Meinhardt, 1999; Parent and Devreotes, 1999; Postma and Van Haastert, 2001; Devreotes and Janetopoulos, 2003; Arriumeolou and Meyer, 2005; Charest and Firtel, 2006; Levine et al., 2006; Meier-Schellersheim et al., 2006). Although inhibitors are essential components of all gradient sensing models, the spatial-temporal presence of inhibitors has not been examined experimentally.

In this study, we designed sequential stimulation protocols to detect temporal and spatial aspects of the inhibition process in single living cells. We found that repeated transient activations of cAR1 receptor trigger repetitive PHC1ac-GFP membrane translocations without detectable refractory periods, demonstrating that a short pulse of cAR1 activation elevates excitation but little long-lasting inhibition. This result provides evidence that cAR1 activation induces an immediate excitation and a delayed recruitment of long-term inhibition leading to PIP3 accumulation. More significantly, we have revealed spatial distribution of the inhibition process induced by a cAMP gradient. Exposing a cell to a sustained cAMP gradient leads to a stable PHC1ac-GFP accumulation in the front of the cell. We found that a sudden withdrawal of the cAMP gradient from this biochemically polarized cell leads to a rapid return of G protein activation, PTEN, and PIP3 distributions to basal levels around the cell membrane. However, there was a short time period during which reactivation of receptors and G proteins around the membrane induced a clear PIP3 response in the back but not the front of the cell. This inverted PIP3 response indicates that a cAMP gradient induces a stronger inhibition of PI3K in the front of a cell.

Results

Brief cAMP stimuli activate excitation but not inhibition of PHC1ac-GFP membrane translocation

Previous studies suggest that activation of cAR1 triggers a fast increase of the excitation level and a slower elevation of the inhibition, allowing a cell to respond transiently and then adapt (Iijima et al., 2002; Devreotes and Janetopoulos, 2003; Janetopoulos et al., 2004; Xu et al., 2005). When a sustained cAMP stimulus is removed, cells that had adapted enter a refractory period during which the cells progressively regain their ability to respond to cAMP, suggesting that the inhibition returns to its prestimulus level more slowly than does excitation (Devreotes and Steck, 1979; Dinu et al., 1980a,b). To test whether there is a temporal difference between the cAR1-induced excitatory and inhibitory processes controlling PIP3 production, we measured the kinetics of PIP3 levels around the membrane of cells that were stimulated by multiple transient cAMP stimuli (Fig. 1). We simultaneously visualized and quantitatively measured transiently applied cAMP stimulations and PIP3 production, reported by the membrane translocation of PHC1ac-GFP, a PIP3 reporter (Parent et al., 1998). Chemotaxis-competent PHC1ac-GFP-expressing cells (“PH cells”) were treated with Latrunculin B, which eliminates morphological polarity and motility by disrupting the actin cytoskeleton. A micropipette filled with cAMP (1 μM) was placed within 30 μm of PH cells and used in conjunction with a microinjector to deliver a series of brief cAMP stimulations. Alexa 594 was included in the micropipette as a measure of the applied cAMP concentration (Xu et al., 2005). Each cAMP stimulation induced a transient response of PHC1ac-GFP membrane translocation (Fig. 1). Temporal changes in cAMP concentration around the cell were determined as the average intensity change of the dye in the R1 and R2 regions (Fig. 1 B). The kinetics of PHC1ac-GFP membrane translocation were measured as intensity changes in cytosolic PHC1ac-GFP pool (Fig. 1 B), which is inversely related to the amount of membrane-associated PHC1ac-GFP (Xu et al., 2005). Quantitative analyses showed that PHC1ac-GFP translocation reached its maximal level in 4 s after the cAMP concentration reached its peak, which reflects the temporal delay that is expected for PIP3 production upon the activation of cAR1 (Fig. 1 C). In our experimental setup, the shortest interval between two transient cAMP stimuli was ~24 s, a minimal time required for the cAMP concentration to return to its basal level between stimuli (Fig. 1 D). Sequential transient cAMP stimuli with as short as 24-s intervals generated repetitive transient responses of PHC1ac-GFP.
translocation, and transient responses displayed kinetics without a refractory period (Fig. 1 D). Our data indicate that a transient receptor activation quickly activates excitatory pathways leading to an increase in PIP3 and upon cAMP removal, these pathways quickly return to prestimulated levels and can be activated again by another cAMP stimulation. Transient cAR1 activations do not signal long enough to substantially elevate the slower inhibition process from its basal level. Therefore, this result supports the idea that cAR1-mediated excitation and inhibition process increases and decreases by following a fast and a slow temporal mode, respectively.

Exposure to cAMP gradient diminishes responsiveness at the front of the cell

The spatial distribution of inhibition in a cell exposed to a cAMP gradient has never been tested. Several models propose that cAR1-induced inhibitors diffuse quickly in a cell, and thus are assumed to be evenly distributed in the inner membrane (Parent and Devreotes, 1999; Iijima et al., 2002; Janetopoulos et al., 2004). However, our dynamic analyses and computer simulations suggest that inhibition mechanisms act locally and predict that inhibition affecting PI3K activity is strongest in the front of a cell at steady state in a cAMP gradient (Xu et al., 2005; Meier-Schellersheim et al., 2006). Because the molecular nature of the inhibitors in chemosensing of *D. discoideum* is still unknown, we developed an approach to indirectly measure the relative extent of inhibition in the front and back of a biochemically polarized cell. We reasoned that after a removal of the cAMP gradient, the signaling network would rapidly return from the polarized to the resting steady state. During this transition, the time required to regain responsiveness to cAMP (the refractory period) may differ between the front and back depending on local

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**Figure 1.** Transient cAMP stimuli induce repetitive transient PIP3 responses visualized by the PHcrac-GFP membrane translocation. (A) Transient cAMP stimulations (red channel) trigger membrane translocations of PHcrac-GFP (green channel) in a living cell. Images were captured at 4-s intervals, and the frames at selected time points are shown here. (B) Temporal changes in cAMP concentration around the cell were measured as the average of fluorescence intensity of Alexa 594 in the regions of R1 and R2. PHcrac-GFP translocation was quantified as the intensity decreases of GFP in the cytoplasm (cyto) of the cell. (C) Dynamic changes in cAMP concentration around the cell (red) and in levels of PHcrac-GFP in cytosol (green) are shown for the selected time period shown in A. (D) Temporal changes in the intensity of Alexa 594 were measured in R1 (the front region) and R2 (the back region), which were almost identical in each short pulse under our experimental conditions. Temporal changes in cAMP concentration around the cell are shown as the average fluorescence intensity in R1 and R2 in the time course. The results for two cells are shown.
concentrations of inhibitors induced by the prior gradient. In our experiments (Fig. 2), PH cells were first exposed to a cAMP gradient until they achieved a stably polarized state, in which PHCrac-GFP accumulated in the front. After quickly withdrawing the gradient at time 0, we observed that PHCrac-GFP rapidly returned to the cytosol. Before the cells could fully return to the resting state, which may take \( \frac{1}{2} \) hr (Devreotes and Steck, 1979; Devreotes, 1994), they were challenged with a uniformly applied cAMP stimulus. Interestingly, this induced an “inverted” response in which PHCrac-GFP transiently translocated to the back of the cells, demonstrating that the original front sides of the cells were less responsive to cAMP than were the back sides. Moreover, cells that had been exposed to gradients of various cAMP concentrations for the initial stimulus also exhibited inverted PHCrac-GFP responses upon a uniform cAMP stimulation (Fig. 2, B–G; and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200611096/DC1).

After the removal of a cAMP stimulus, G proteins quickly reassociate and can be reactivated
The observed inverted PIP3 response is likely caused by a slow return of the intracellular components to their “resting” states because the average time period for cAMP binding to the receptor is in the range of seconds (Ueda et al., 2001). Several components in the pathways may be responsible for the differential refractory behaviors of the front and back of the cells. For example, receptors may remain asymmetrically desensitized or G proteins may not be completely reassOCIated upon the second cAMP stimulation. We addressed this issue by directly measuring the kinetics of G protein reassociation and reactivation in living cells using FRET analysis (Fig. 3). Cells expressing Go2-CFP and YFP-Gβ (“G cells”) were suddenly exposed to 10 \( \mu \)M cAMP (Fig. 3, A and B), a saturating dose for cAR1, or 1 \( \mu \)M cAMP (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200611096/DC1). Addition of cAMP induced a rapid FRET loss, which reached a steady state in \(< 20 \) s, indicating G protein dissociation (Fig. 3 B; Fig. S2) (Xu et al., 2005). After the removal of cAMP, FRET returned to the prestimulus level in \( \frac{1}{2} \) hr, indicating that the G proteins were completely reassOCIated. A second sudden exposure to the same concentration of cAMP triggered an instant FRET loss that displayed kinetics very similar to those in response to the first stimulation (Fig. 3 B; Fig. S2), demonstrating that cAR1 receptor and G proteins rapidly returned to their prestimulation states and could be fully reactivated within 60 s after the removal of cAMP.

We then simultaneously monitored temporal changes in the receptor/G protein activation and PHCrac-GFP translocation in the front and back of the cells previously exposed to a cAMP gradient (Fig. 3, C–F). We measured FRET changes in one G cell...
Basal levels are indicated as dashed line. The G cell (Fig. 3 E), while a distinctive accumulation of PHCrac-fluorescence increase (FRET loss), in both the front and back of the cell, was measured as a CFP intensity change, in response to the uniform cAMP field. A normalized FRET change (expressed as CFP/YFP ratio), indicates relative level of G protein activation at the cell membrane. Kinetics of cAMP-mediated changes in the FRET ratio in the entire membrane of single G cells are shown as means ± SD (n = 7). The thick gray bars show temporal changes in CFP intensity changes. The thin black dashed line shows the basal level of FRET, which is 1. (C) The spatiotemporal relationship of G protein activation and the inverted PHCrac-GFP response. cAMP-mediated G protein activation was measured in the front and back regions of a G cell, and cAMP-induced PIP3 changes were monitored by PHCrac-GFP dynamics in the front and back of a nearby (within 20 μm) PH cell. A CAMP gradient (1 μM cAMP released from a micropipette) was suddenly withdrawn from the G and PH cells at 0 s. A uniform CAMP field (100 nM), applied at 134.7 s, induces a clear PHCrac-GFP translocation to the back of the PH cell. An animated version is in Video 2 (available at http://www.jcb.org/cgi/content/full/jcb.200611096/DC1). Regions of interest for simultaneous measurement of G protein activation in one G cell and the inverted PIP3 response in a PH cell are shown. (D) Selected images show the inverted PHCrac-GFP translocation in the experiment shown in C. The arrows point to direction of PHCrac-GFP membrane translocation: the original at 0 s and the inverted at 171 s. (E) The temporal changes in the G protein dissociation in the front (black) and back (gray) of the G cell, reflected as CFP intensity changes, in response to the uniform CAMP field. The basal level of G protein activation is 1, shown as the thin line. The thick gray bar indicates the temporal changes in CAMP concentration. (F) Temporal changes in PHCrac-GFP in the front and back of the PH cell in response to the uniform CAMP stimulation applied at 134.7 s. The basal levels of PHCrac-GFP in the front and back are 1, indicated as the thin dashed line. The thick gray bar indicates the temporal changes in CAMP concentration. (G) After a withdrawal of a gradient, a new uniformly applied cAMP field induced similar levels of G protein dissociation in the front and back of G cells, but triggered a clear PHCrac-GFP translocation only to the back regions of PH cells. Normalized maximal FRET changes and PHCrac-GFP increases in the front and back of G and PH cells are shown as means ± SD (n = 5). Basal levels are 1, indicated as dashed line.

Spatiotemporal dynamics of PHCrac-GFP membrane translocation in PTEN null cells in response to cAMP stimuli

cAR1 activates an excitatory signaling branch that induces PTEN to translocate from the membrane to the cytosol and elevate an inhibitory mechanism that allows cytosolic PTEN to return to the membrane (Funamoto et al., 2002; Iijima and Devreotes, 2002). To determine spatiotemporal changes in PIP3 in the cells lacking PTEN, we measured kinetics of PHCrac-GFP membrane translocation in pten−/− cells and compared the kinetics to those in wild-type (WT) cells (Xu et al., 2005; Meier-Schellersheim et al., 2006), in response to uniformly applied cAMP and a CAMP gradient (Fig. 4). WT and pten−/− cells expressing PHCrac-GFP were stimulated uniformly with cAMP (1 μM) at 0 s (Fig. 4 A). The CAMP-triggered PHCrac-GFP membrane translocation is fast and transient in WT cells. In contrast, the response in pten−/− cells was clearly slower, peaking in ~12 s and returning to prestimulus levels in more than 40 s (Fig. 4 A).
When the cells were suddenly exposed to a gradient (Fig. 4 B), membrane translocations of PH \textsubscript{Cac}-GFP occurred initially in both front and back regions in both WT and \textit{pten} \textsuperscript{−} cells. However, the kinetics of PH \textsubscript{Cac}-GFP translocation in the front was clearly abnormal in \textit{pten} \textsuperscript{−} cells. There was no clear decrease in PH\textsubscript{Cac}-GFP amount at the front for more than 150 s, which differs from the biphasic response in WT cells (Fig. 4 B). We also examined kinetics of the PIP\textsubscript{3} in response to the removal of cAMP stimuli in \textit{pten} \textsuperscript{−} cells (Fig. 4 C). After cells were exposed to a gradient, PH\textsubscript{Cac}-GFP accumulates in the front regions of WT or \textit{pten} \textsuperscript{−} cells. Upon a removal of the gradient at 0 s, PH\textsubscript{Cac}-GFP gradually returned to the cytosol. The returning process was clearly slower in \textit{pten} \textsuperscript{−} cells than in the WT cells, whose \( t_{1/2} \) were \( \approx 22 \) s and 14 s, respectively (Fig. 4 C).

**PTEN quickly redistributes to the membrane after cAMP removal**

PTEN is involved in regulating spatiotemporal dynamics of PIP\textsubscript{3} levels around the membrane of a cell in response to cAMP stimulation. Therefore, the dynamic distribution of PTEN affects the local PIP\textsubscript{3} levels. When a cell reaches the “polarized” steady state in a stable cAMP gradient, PTEN is enriched at the back side of a cell (Funamoto et al., 2002; Iijima and Devreotes, 2002; Li et al., 2005). After withdrawal of the cAMP gradient, PTEN starts to redistribute itself from the polarized to the resting steady state. During this transition, a transient accumulation of PTEN in the front could potentially explain the inverted PIP\textsubscript{3} response. To address this possibility, we measured the spatiotemporal dynamics of PTEN under these conditions (Fig. 5). After a rapid withdrawal of the gradient at time 0 (Fig. 5, A and B), PTEN redistributed from the back and became uniformly distributed around the membrane in \( \approx 80 \) s without over-accumulating in the front (Fig. 5). Furthermore, reapplying a uniform stimulus (Fig. 5, A–D) or gradient (Fig. 5, E–H) of cAMP induces PTEN translocation with kinetics (Fig. 5) similar to those observed in the cells that had not previously been stimulated (Meier-Schellersheim et al., 2006), indicating that the cAR1-controlled regulatory components of PTEN returned to their “resting” states and PTEN molecules in both the front and back were responsive to a second cAMP stimulation when the inverted PH\textsubscript{Cac}-GFP response occurred. Therefore, the excitatory and the inhibitory mechanisms that control PTEN membrane distribution are not the likely explanation for this inverted response.

**Inhibitory pathways controlled by G\( \alpha \)\textsubscript{1} or G\( \alpha \)\textsubscript{9} subunits are not essential for cAR1-mediated PH\textsubscript{Cac}-GFP responses**

Other mechanisms may also be involved in inhibition in the signaling network of cAMP gradient sensing. For example, Go9 and G\( \alpha \)\textsubscript{1}-mediated PLC pathways in \textit{D. discoideum} have been shown to function as negative regulators in the cAR1-mediated signaling (Bominaar and Van Haastert, 1993; Brzostowski et al., 2004). To test whether either pathway is essential for the gradient sensing, we examined PH\textsubscript{Cac}-GFP responses in Go9 and G\( \alpha \)\textsubscript{1} null cells (Fig. 6). We measured PH\textsubscript{Cac}-GFP membrane translocation by monitoring intensity changes of GFP fluorescence.

![Figure 4. Dynamics of PH\textsubscript{Cac}-GFP membrane translocation in PTEN null cells upon a uniform and a gradient of cAMP stimulations. (A) WT and \textit{pten} \textsuperscript{−} cells expressing PH\textsubscript{Cac}-GFP were uniformly stimulated with cAMP (1 \( \mu \)M). Kinetics of PH\textsubscript{Cac}-GFP membrane translocation are shown as the normalized intensity changes in cytosolic PH\textsubscript{Cac}-GFP, where the intensity at time 0 is defined as 1 and the minimal intensity is defined as 0. (B) WT (top panel, as a control) and \textit{pten} \textsuperscript{−} cells expressing PH\textsubscript{Cac}-GFP were suddenly exposed to a cAMP gradient. Temporal changes in relative levels of PH\textsubscript{Cac}-GFP in the front and back of the cells. Means \( \pm SD \) (\( n = 8 \)) are shown. (C) Kinetics of membrane-bound PH\textsubscript{Cac}-GFP in the front of WT, as a control, and \textit{pten} \textsuperscript{−} cells in response to a withdrawal of an applied cAMP stimulation. Means \( \pm SD \) (\( n = 10 \)) are shown.]
in the cell membrane (Xu et al., 2005). In response to a uniform stimulation, the spatiotemporal kinetics of PH\text{Crac-GFP} membrane translocation in either $g_\alpha^9$ or $g_\alpha^1$ were similar to those in the WT cells (Fig. 6, A and C). When the $g_\alpha^9$ or $g_\alpha^1$ cells were suddenly exposed to stable cAMP gradients, PH\text{Crac-GFP} translocation, as in WT cells, consisted of two phases, an initial transient translocation around the cell membrane followed by a second phase producing a highly polarized distribution (Fig. 6, B and D). Because our observed dynamics in both mutant cells are similar to those displayed in WT cells (Xu et al., 2005; Meier-Schellersheim et al. 2006), we suggest that $G_\alpha^1$ or $G_\alpha^9$ controlled signaling are not essential inhibitory mechanisms for cAR1-mediated gradient sensing.

Local inhibition mechanism of PI3K revealed by a cAMP-gradient-induced inverted PH\text{Crac-GFP} translocation

We speculated that the inverted PH\text{Crac-GFP} translocation may be induced by a reapplied cAMP gradient. PH\text{Crac-GFP} translocation, as in WT cells, consisted of two phases, an initial transient translocation around the cell membrane followed by a second phase producing a highly polarized distribution (Fig. 6, B and D). Because our observed dynamics in both mutant cells are similar to those displayed in WT cells (Xu et al., 2005; Meier-Schellersheim et al. 2006), we suggest that $G_\alpha^1$ or $G_\alpha^9$ controlled signaling are not essential inhibitory mechanisms for cAR1-mediated gradient sensing.

Figure 5. Spatiotemporal dynamics of PTEN in single cells upon a withdrawal of a cAMP gradient and then stimulated with a uniform dose (A–D) or a gradient (E–H) of cAMP. (A) A PTEN-GFP expressing cell was highly polarized in a stable cAMP gradient (red, 1 $\mu$M in the micropipette, red) at 0 s. The gradient was suddenly withdrawn from the cell at 0 s. A uniform cAMP stimulation (100 nM) was applied at 144 s. Regions of interest for the data reported in B and C are also shown. (B) Temporal changes in cAMP concentrations in the front (black) and back (gray) are shown in the time course. (C) Temporal changes in relative levels of membrane-bound PTEN in the front (black) and back (gray) are shown in the time course. (D) Kinetics of membrane-bound PTEN in the front and back are shown as means ± SD ($n = 10$) in response to a withdrawal and then to a uniformly applied stimulation. (E) A PTEN-GFP expressing cell was highly polarized in a stable cAMP gradient (red, 1 $\mu$M in the micropipette) at 0 s. The gradient was suddenly withdrawn from the cell start at 0 s, and reapplied at the 90 s. Regions of interest for the data reported in B and C are also shown. Animated version is in the supplemental materials (Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200611096/DC1). (F) Temporal changes in cAMP concentrations in the front (black) and back (gray) are shown in the time course. (G) Temporal changes in relative levels of membrane-bound PTEN in the front (black) and back (gray) are shown in the time course. (H) Kinetics of membrane-bound PTEN in the front and back are shown as means ± SD ($n = 13$) in response to a withdrawal and reapplied of the cAMP gradient.
polymerization leads to recruitment of additional PI3K from cytosol to the membrane, thereby increasing the amount of active PI3K (Sasaki et al., 2004). In Latrunculin-treated cells, PI3Ks were uniformly distributed around the membrane of the cells even when they were exposed to the cAMP gradient (Sasaki et al., 2004; unpublished data). Therefore, under our experimental condition, we monitored the spatiotemporal regulations of PI3K activity without complications from the second layer of actin-dependent PI3K recruitment. In addition to the signaling pathway leading to PI3K activation, the cAMP receptor also regulates another pathway mediating the redistribution of membrane-bound PTEN, which is important for the proper directional response of PIP3. In pten−cells, PHCrac-GFP was still able to accumulate in the front when the cells were exposed to a cAMP gradient (Janetopoulos et al., 2004; Sasaki et al., 2004). We found that the crescents of PHCrac-GFP in pten−cells were broader than those formed in WT cells (Fig. 7, E and F; at time 0), as previously described (Janetopoulos et al., 2004; Sasaki et al., 2004). Furthermore, the directions of the crescents, unlike those in WT cells, did not always perfectly point to the direction of the gradient (Fig. 7 F; at time 0). These results indicate, as expected, that a cAMP gradient-induced PTEN redistribution ensures the PIP3 response in the restricted front region, and this localized inhibition acts on the signaling pathway between free Gβγ to PI3K.

**Kinetics of the asymmetrical inhibition induced by a cAMP gradient**

We examined the temporal appearance and disappearance of the gradient-induced asymmetrical inhibition (Fig. 8; Fig. S4, B and C, available at http://www.jcb.org/cgi/content/full/jcb.200611096/DC1). We found that a brief gradient stimulation of ~50 s was not sufficient to induce an inverted PHCrac-GFP response (Fig. 8, A and B; Fig. S4 B). Thus, exposure to a stable gradient for ~2 min is needed to establish an asymmetrical inhibition. Furthermore, cells that were removed from a gradient for 6 min and rechallenged with either uniform cAMP stimulation or a cAMP gradient displayed a noninverted PHCrac-GFP translocation response as in naive cells (Fig. 8, C and D; Fig. S4 C), indicating that asymmetrical inhibition disappears within 6 min after the gradient is removed.

**Discussion**

The existence of inhibitory components in GPCR-mediated chemosensing has been proposed for more than thirty years, but the molecular mechanisms are still unknown and, thus, cannot yet be visualized directly. Here, we report insights into the temporal and spatial aspects of the inhibition based on measurements of the spatiotemporal dynamics of known components of the gradient sensing machinery aided by our computational modeling study.
We have constructed a quantitative model for cAR1-mediated signaling network (Meier-Schellersheim et al., 2006). The model, which includes receptor-mediated and locally controlled inhibitory mechanisms that regulate PI3K and PTEN (Fig. 9 B), simulates experimentally determined dynamics of receptor activation, G protein dissociation, PTEN membrane localization, and PIP3 accumulation (Meier-Schellersheim et al., 2006). For example, in response to a uniform cAMP stimulus, the model generates a transient PIP3 response that quickly returns to the resting stage (adaptation). When exposed to a cAMP gradient, a cell generates a steeper PIP3 gradient at a higher steady-state level in the front. However, this level does not continue to increase ($\Delta\text{PIP}_3/\Delta t = 0$) in spite of a lower level of PTEN. At the same time, in the back, a higher level of PIP3 is almost equal to that in the back (Sasaki et al., 2004). PIP3 is enriched in the back, which is expected from models containing only globe inhibition mechanisms (Parent and Devreotes, 1999; Iglesias and Levchenko, 2002; Iijima et al., 2002). Second, balances between the activities of PI3K and PTEN have been reached in both the front and back, and the balances are achieved by a stronger inhibition of PI3K activity in the front, which is stronger than that of PTEN in the front, and PIP3s are continuously produced. The PIP3 level remains steady in the front because it diffuses fast enough to be degraded by PTEN that is localized in the front of the cell. Images were captured at 2-s intervals, and the frames at selected time points are shown. Regions of interest used to assess concentration changes in cAMP and dynamics of PIP3 are shown. Video 5 shows a full set of images from one experiment (available at http://www.jcb.org/cgi/content/full/jcb.200611096/DC1). Another example is shown as Fig. S2 and Video 6. Regions of interest used to assess concentration changes in cAMP and PIP3 are shown. Front (DF) and back (DB) regions used to evaluate quantitative changes of Alexa 594 as a measure of cAMP concentration. PHF and PHB were selected membrane regions used for monitoring the responses of PHCrac-GFP translocation to the front and back of the cell, respectively. (B) Temporal changes in cAMP concentrations (top panel) and in PHCrac-GFP (bottom panel) in the front (black) and the back (gray) regions of the cell. (C) After a withdrawal of gradients, new gradients were applied to the PH cells that were in the transients from polarized stages to resting stages. The fronts of the cells were exposed to higher concentrations of cAMP, comparing DF to DB [cAMP concentration in the back]. Means ± SD are shown ($n = 8$). (D) PHCrac-GFP initially translocated only to the back of the cells, comparing PHB to PHF. Maximal PHCrac-GFP translocation responses are shown as means ± SD ($n = 8$). The basal levels are 1, indicated as the thin dashed line. [E and F] In planar cells, the inverted PHCrac-GFP translocations were induced by a reapplied cAMP stimulation. The white arrows indicate the direction of PHCrac-GFP accumulation. The red arrows indicate the directions of cAMP gradients. Images were captured at 3-s intervals, and the frames at selected time points are shown.
have been proposed in our model that includes local inhibition mechanisms (Xu et al., 2005; Meier-Schellersheim et al., 2006). Because different proposed mechanisms could lead to high chemotactic sensitivity in theory (Meinhardt, 1999; Postma and Van Haastert, 2001; Devreotes and Janetopoulos, 2003; Arrieumerlou and Meyer, 2005; Levine et al., 2006; Meier-Schellersheim et al., 2006), we designed experiments to determine which inhibitory mechanisms are likely used in GPCR-mediated chemosensing.

In this study, we revealed spatiotemporal features of an inhibitory process that acts locally on the activation pathway between Gβγ and PI3K.

There is a general agreement that inhibition increases and decreases slowly in response to the changes of cAMP receptor occupancy (Parent and Devreotes, 1999; Postma and Van Haastert, 2001; Devreotes and Janetopoulos, 2003; Arrieumerlou and Meyer, 2005; Levine et al., 2006; Meier-Schellersheim et al., 2006), we designed experiments to determine which inhibitory mechanisms are likely used in GPCR-mediated chemosensing. In this study, we revealed spatiotemporal features of an inhibitory process that acts locally on the activation pathway between Gβγ and PI3K.

Figure 8. Kinetics of the asymmetrically distributed inhibition. [A and B] Asymmetrically distributed inhibition is not induced by exposure to an initial cAMP gradient for just 50 s. PH cells were suddenly exposed to a cAMP gradient (1 μM) at 0 s for 50 s. After a withdrawal of the gradient for ~70 s, a uniform cAMP stimulation (100 nM) induced PHcra-GFP translocation in both the original front and the back regions [A]; and a reapplied cAMP gradient triggered a clear PHcra-GFP translocation in the original front (B). [C and D] The asymmetrical inhibition disappears 6 min after the removal of the cAMP gradient. PH cells had been exposed to a cAMP gradient (1 μM) for more than two min, and PHcra-GFP became stably polarized in the front of the cells. The cAMP gradient was removed from the cells at 0 s. After a removal of the gradient for 6 min, a uniform cAMP stimulation (100 nM) induced a PHcra-GFP translocation in both the original front and the back regions (C), and a reapplied cAMP gradient triggered PHcra-GFP translocations to both the original front and back regions.
period of time during which another cAMP stimulation triggers G protein dissociation and PTEN translocation in both the front and back but induces PIP₃ responses only in the back of the cell (Fig. 9). This suggests that inhibitors that are more abundant in the front block transmission of activating signals from G protein (Fig. 9). 

This inhibition is stronger in the front of the cell. The spatiotemporal features of the inhibition can shed light on unknown molecular mechanisms. Based on the fast-diffusive-inhibition models, small molecules, such as Ca²⁺ or cGMP, were suggested to be candidate inhibitors, which have not been verified by experiments. The “local excitation and global inhibition” model assumes the presence of a negative regulator, and suggests that it is likely to be PTEN. Based on our detailed spatiotemporal dynamics of PTEN and PIP₃, our computational model showed that PTEN alone cannot fully explain the experimentally determined dynamics (Meier-Schellersheim et al., 2006). We proposed, in addition to PTEN, other inhibitory mechanisms that may involve reversible modifications of components in the pathway from free Gβγ to Ras and then to PI3K. Previous studies in mammalian GPCR signaling indicated several inhibitory components. After GPCR activation, free Gβγ dimers interact with the receptor-associated kinase GRK2, blocking Gβγ signaling (Lodowski et al., 2003). GPCR activation can also induce a translocation of a RasGAP, which binds to PIP₃, to inner membrane deactivating Ras thereby inhibiting PI3K (Lockyer et al., 1999). In D. discoideum, it has been shown that a sustained cAR1 activation, which triggers a persistent G protein dissociation, induces a transient activation of RasG, which activates PI3K (Sasaki et al., 2004). The transient nature of RasG activation is consistent with the idea that the cAR1 activation also recruits inhibitors to the membrane to shut down signals from free Gβγ to Ras activation. Our computational model is able to simulate the observed spatiotemporal dynamics of known components in adaptation and in gradient sensing by including these putative inhibitor(s) (Meier-Schellersheim et al., 2006). Therefore, we propose that the inhibition process is performed by these negative regulators acting locally on the PI3K
signaling branch and those on PTEN branch, which act in concert to control the spatiotemporal dynamic of PIP3 around the cell membrane. Future studies are needed to identify inhibitors involved in the GPCR-mediated chemosensing network.

Materials and methods

Cell lines and live cell imaging

As previously described (Xu et al., 2005), D. discoideum cell lines expressing PHcrac-GFP (Xu et al., 2005), PTEN-GFP (liljina and Devreotes, 2002), PI3K1-GFP (Sasaki et al., 2004), and both Gα2CFP and YFP-Gβ (Xu et al., 2005); and pten gastrin null cells expressing PHcrac-GFP were developed to the chemotactic stage. Cells were plated on a 1-well chamber for the microrjector delivery of cAMP stimulation (Nalge Nunc International), allowed to adhere to the cover glass for 10 min, and then covered with additional DB buffer. Live cells were imaged using a Zeiss Laser Scanning Microscope, LSM 510 META, with a 40× NA 1.3 DIC Plan-NeoFluar objective. To monitor cAMP and PHcrac-GFP, PTEN-GFP, PI3K1-GFP cells were excited with two laser lines, 488 nm for GFP and 543 nm for Alexa 594, a water-soluble fluorescence dye. Images were simultaneously recorded in three channels. Channel one: fluorescent emissions from 505–530 nm for GFP (green); channel two: emissions from 580–650 nm for Alexa 594 (red).

Generation and measurement of applied cAMP stimulations

The temporal-spatial intensity changes of Alexa 594 and cells expressing PHcrac-GFP, PTEN-GFP, or PI3K1-GFP were directly imaged using a confocal microscope. Channel one: fluorescent emissions from 505–530 nm for GFP (green); channel two: emissions from 580–650 nm for Alexa 594 (red).

FRET measurement

Using a spectral confocal fluorescence microscope (LSM510 META), we measured intensity decrease of acceptor (YPF) and increase of donor (CFP) in response to stimuli. We monitored intensity changes of CFP (donor) and YFP (acceptor) following a stimulation using a time-lapse acquisition of Lambda Stacks. The cells were excited with a 454-nm laser line and the spectral emissions in each pixel of the fluorescence images were simultaneously recorded in 8 channels, each with a 10-nm width, from 464 to 544 nm. To separate multi-fluorescence signals, each of the fluorescence images was collected using Lambda Stack acquisition. The spectral emissions of fluorescence images were simultaneously recorded in a CHS-1 from 464 to 544 nm. The spectra of the cells expressing CFP, YFP or GFP only were collected using Lambda Stack acquisition. The spectral emissions of the cells expressing CFP, YFP or GFP only were obtained and used as the references for the Linear Unmixing Function. The digitally separated images of CFP and YFP of the G cells, and GFP of the PH cells were obtained. The intensities of each fluorophore in the regions of interest in the time-lapse experiments were measured, normalized, and expressed as a function of time in responses to CAMP stimulations, using the software of LSM510 META (Xu et al., 2005).

Imaging and data processing

Images were processed and analyzed by the LSM 510 META software, and converted to TIFF files by the Adobe Photoshop software. All frames of any given series were processed identically. Selected frames of the series were assembled as montages using Photoshop 7.0. Quantification of fluorescent intensities of Alexa 594, GFP, CFP, and YFP in the regions of interest was performed using the LSM 510 META software.

Online supplemental material

Fig. S1 shows inverted PIP responses. Fig. S2 shows FRET measurement of G protein dissociation and association and redissociation. Fig. S3 shows reapplied a cAMP gradient induced an inverted PHcrac-GFP membrane translocation. Fig. S4 A shows Gα9 null cells detect CAMP gradient normally.

Fig. S4 B and C show kinetics of the formation of the asymmetrical distributed inhibition. Fig. S5 shows PI3K activity, membrane-bound PTEN and the resulting dynamics of PIP3 in a cell when it is exposed to a CAMP gradient in a computer simulation and a schematic representation of the signaling network that describes spatiotemporal changes. Videos 1 and 2 show uniformly applied cAMP stimulation triggered inverted PHcrac-GFP translocation. Video 3 shows simultaneously measurement of G protein activation in the front and back of a cell and the inverted PHcrac-GFP response. Video 4 shows dynamics of PTEN in a cell upon a withdrawal of a CAMP gradient and then reapplied the gradient. Videos 5 and 6 show a CAMP gradient induced the inverted PHcrac-GFP membrane translocation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200611096/DC1.

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