Protein Kinase A regulates the Ras, Rap1, and TORC2 pathways in response to the chemoattractant cAMP in Dictyostelium

Margarethakay Scavello*, Alexandra R. Petlick*, Ramya Ramesh, Valery F. Thompson, Pouya Lotfi, and Pascale G. Charest‡.

Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, 85721-0088, USA.

*Equal contribution
‡Author for correspondence (pcharest@email.arizona.edu)

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SUMMARY STATEMENT

Protein Kinase A is necessary for gradient sensing and chemotaxis to cAMP in Dictyostelium, in part, through the proper spatiotemporal regulation of key upstream chemoattractant signal transduction pathways.

ABSTRACT

Efficient directed migration requires tight regulation of chemoattractant signal transduction pathways in both space and time, but the mechanisms involved in such regulation are not well understood. Here, we investigated the role of Protein Kinase A (PKA) in controlling signaling of the chemoattractant cAMP in Dictyostelium. We found that cells lacking PKA display severe chemotaxis defects, including impaired directional sensing. Although PKA is an important regulator of developmental gene expression, including the cAMP receptor cAR1, our studies using exogenously expressed cAR1 in cells lacking PKA, cells lacking adenylyl cyclase A (ACA), and cells treated with the PKA-selective pharmacological inhibitor H89, suggest that PKA controls chemoattractant signal transduction, in part, through the regulation of RasG, Rap1, and TORC2. As these pathways control the ACA-mediated production of intracellular cAMP, they lie upstream of PKA in this chemoattractant signaling network. Consequently, we propose that the PKA-mediated regulation of the upstream RasG, Rap1, and TORC2 signaling pathways is part of a negative feedback mechanism controlling chemoattractant signal transduction during Dictyostelium chemotaxis.
INTRODUCTION

Chemotaxis, the ability of cells to detect and migrate up chemical (chemoattractant) gradients, is a conserved cellular behavior that is not only essential to normal human physiology but is also implicated in the onset and progression of diseases (Bravo-Cordero et al., 2012; Kolaczkowska and Kubes, 2013; Richardson and Lehmann, 2010; Sadik and Luster, 2012; Theveneau and Mayor, 2012; Zernecke and Weber, 2010). The detection of chemoattractants by cells triggers an intracellular network of signal transduction pathways that coordinately control their ability to interpret the gradient, polarize, and move (Artemenko et al., 2014). Moreover, these processes are often coupled to the paracrine release of chemoattractants that relay the original signal to promote group cell migration (Afonso et al., 2012; Garcia and Parent, 2008; Wyckoff et al., 2004). In order for cells to efficiently perform chemotaxis, chemoattractant signal transduction pathways are tightly regulated in both space and time, and adapt to persistent stimulation (Charest and Firtel, 2006; Hoeller et al., 2014; Wang, 2009). Adaptation is believed to be necessary for cells to sense and respond to increasing chemoattractant concentrations as they migrate up the gradient (Chang and Levchenko, 2013). However, very little is understood about the mediators of adaptation and the regulatory mechanisms involved in the spatiotemporal control of chemotactic signaling.

In the established chemotaxis experimental model *Dictyostelium discoideum*, Ras family GTPases, including RasC, RasG, and Rap1, are critical mediators of the chemotactic response to cAMP (Charest and Firtel, 2007). Rap1 controls cell-substrate adhesion and cell polarity by promoting remodeling of both actin and myosin, in part, through phosphoinositide 3-kinase (PI3K), Rac, the serine/threonine kinase Phg2, as well as through the regulation of the Target of Rapamycin Complex 2 (TORC2) (Jeon et al., 2007a; Khanna et al., 2016; Kortholt et al., 2006; Kortholt et al., 2010; Mun and Jeon, 2012; Plak et al., 2013). RasG activates PI3K, thereby controlling the site of F-actin polymerization and the direction of migration (Bolourani et al., 2006; Sasaki et al., 2004; Zhang et al., 2008). Tight spatiotemporal regulation of RasG is critical for gradient sensing during chemotaxis (Takeda et al., 2012; Zhang et al., 2008). Finally, RasC
directly promotes TORC2 activation, thereby modulating F-actin dynamics and controlling cAMP production by adenylyl cyclase A (ACA) (Bolourani et al., 2006; Cai et al., 2010; Charest et al., 2010; Khanna et al, 2016). In addition, we have found that a negative feedback mechanism promotes adaptation of chemoattractant-stimulated RasC activity (Charest et al., 2010). Downstream from RasC, TORC2-activated Akt/protein kinase B (PKB) and PKB-related PKBR1 phosphorylate the scaffold Sca1, which assembles a RasC activating complex containing the RasC specific guanine exchange factor (GEF) Aimless/gefA. PKB/PKBR1 phosphorylation of Sca1 inhibits the localization of the Sca1 complex to the plasma membrane, thereby preventing further RasC activation. Interestingly, PKB/PKBR1 phosphorylation of Sca1 is elevated in cells lacking the Protein Kinase A catalytic subunit (PKA-C; pkaC null cells) (Charest et al., 2010). Since the RasC-TORC2-PKB/PKBR1 pathway controls the ACA-mediated intracellular production of cAMP, this pathway promotes PKA activation in response to extracellular cAMP chemoattractant stimulation (Charest et al., 2010; Lee et al., 2005; Lim et al., 2001). Thus, these previous observations indicate PKA may regulate the RasC-TORC2-PKB/PKBR1 signaling pathway during chemotaxis through a negative feedback loop.

cAMP in *Dictyostelium* plays two different and completely separate roles: that of an extracellular chemoattractant and that of an intracellular signaling molecule (Reymond et al., 1995). Since cAMP is membrane-impermeable, the extracellular cAMP acting as chemoattractant for *Dictyostelium* does not directly activate PKA, it only acts through stimulation of specific seven-transmembrane cAMP chemoattractant receptors (cAR; Insall et al., 1994). In response to the chemoattractant stimulation, intracellular cAMP is produced by ACA, and part of this cAMP is used to activate PKA and part is used for relaying the chemoattractant signal to neighboring *Dictyostelium* cells by being actively exported outside the cells through ABC transporters (Garcia et al., 2008; Miranda et al., 2015).

Whereas the role of PKA in *Dictyostelium* development and morphogenesis is well characterized (Loomis, 1998), its role in chemotaxis is not understood. PKA is
required for the starvation-induced aggregation of Dictyostelium cells (Mann and Firtel, 1991; Mann et al., 1997), a process driven by chemotaxis, and was found to be involved in controlling the directional extension of pseudopods during migration (Stepanovic et al., 2005; Zhang et al., 2003). In mammalian cells, PKA was shown to play a central role in actin-based cell migration through the differential regulation of Rac, Rho and Rap1 GTPases, as well as VASP, PI3K, PAK, and LIM kinase, at the leading edge of migrating cells (Chen et al., 2005; Howe, 2004; Howe et al., 2005; Jones and Sharief, 2005; Lim et al., 2008; Nadella et al., 2009; Paulucci-Holthauzen et al., 2009; Takahashi et al., 2013; Toriyama et al., 2012; Zimmerman et al., 2013).

The present study was undertaken to investigate the role of PKA in controlling chemotactic signaling pathways and directed cell migration in Dictyostelium. We performed a detailed characterization of the effect that the lack of PKA activity in Dictyostelium has on chemotaxis to cAMP and on the regulation of known cAMP-induced responses in Dictyostelium. We found that, in cells lacking PKA function, many early cAMP-induced chemotactic responses, including RasG, Rap1, PI3K, TORC2, and PKB/PKBR1 activation, are upregulated and fail to be spatially restricted upon exposure to cAMP gradients. Therefore, our results suggest that PKA is necessary for proper spatiotemporal regulation of early chemoattractant signal transduction pathways, which is critical to Dictyostelium chemotaxis. Whereas this regulatory mechanism may include the PKA-mediated transcriptional control of additional players, our study suggests that direct control of the signaling pathways by PKA could explain the observed effects.

RESULTS

pkaC null cells are unable to perform chemotaxis

To investigate the role of PKA in Dictyostelium chemotaxis to cAMP, we started with characterizing the chemotaxis phenotypes of Dictyostelium cells lacking the PKA catalytic subunit (PKA-C; pkaC null) and compared these phenotypes to those of wild-
type cells. *pkaC* null cells are viable and, although they were shown to lack expression of ACA (Mann et al., 1997), *pkaC* and *acaA* null cells were found to express key aggregative genes when provided with exogenous cAMP pulses (Mann et al., 1992; Mann et al., 1997; Pitt et al., 1993). Hence, in such conditions, the use of *pkaC* null cells should be informative as to the role of PKA in chemotaxis. Using cells responsive to the chemoattractant cAMP (pulsed with exogenous cAMP for 5.5 h), we found that *pkaC* null cells exhibit severe chemotaxis defects (Fig. 1). Whereas developed wild-type cells placed in an exponential cAMP gradient polarize and migrate efficiently, *pkaC* null cells do not polarize and they extend pseudopods in random directions, often opposite to the gradient (Fig. 1A; Movies 1, 2). As a consequence, *pkaC* null cells display poor persistence of movement (0.16 ± 0.07 versus 0.79 ± 0.09 for wild-type cells) and completely fail to migrate towards the chemoattractant source, in this case a micropipette filled with 150 μM cAMP (chemotactic index of 0.03 ± 0.07 versus 0.76 ± 0.12 for wild-type cells) (Fig. 1B,C). However, the *pkaC* null cells are motile and display an averaged displacement speed of 4.9 ± 1.4 μm/min compared to 5.8 ± 0.9 μm/min for wild-type cells (Fig. 1C). To verify that this phenotype is not unique to this *pkaC* null strain, we tested another strain, in which PKA-C was independently disrupted by another group (HBW1; Primpke et al., 2000). However, we found that these cells are a little heterogeneous and unstable, as the phenotypes changed with passages in cell culture. Nevertheless, we found that young HBW1 cells, compared to their control cells (HBW2), have severe chemotaxis defects and have cAMP-induced responses similar to the other *pkaC* null cells (Fig. S1).

Together, our analysis suggests that, although *pkaC* null cells are unable to perform chemotaxis, they do extend protrusions and move. These observations then suggest that PKA is not required for cell motility in *Dictyostelium*, but is essential for the establishment of cell polarity and chemotaxis towards cAMP.
pkaC null cells fail to properly localize and temporally regulate F-actin and myosin

To explore the underlying causes for the strong chemotaxis defects of pkaC null cells, we first assessed the spatiotemporal dynamics of F-actin in cells using the fluorescent reporter Lifeact-GFP (Riedl et al., 2008). As previously described (Chung and Firtel, 1999), we observe that migrating wild-type cells display cortical F-actin and F-actin-rich pseudopods at the leading edge (Fig. 2A, Movie 3). Cells lacking PKA also display cortical F-actin, but, consistent with the observed pkaC null chemotaxis phenotype, these cells exhibit many, small, randomly localized F-actin-rich protrusions (Fig. 2A, Movie 4). Lifeact-GFP labeling of F-actin allowed us to observe that these protrusions, although they sometimes produce a pseudopod that correlates with random movement of the cell, often form an endocytic cup or are just retracted. In addition, in cAMP pulsed, resting cells not exposed to the cAMP chemoattractant, we observed that, in contrast to wild-type cells displaying low basal levels of F-actin, most pkaC null cells display elevated basal levels of F-actin enriched in numerous spontaneous protrusions that often look like membrane ruffles rather than pseudopods (Fig. 2B,D, Movies 5, 6).

cAMP stimulation of wild-type Dictyostelium cells produces a biphasic F-actin polymerization response, with a sharp first peak at 5 s, which is linked to actin cytoskeleton reorganization (cringe response), and a lower but wider peak at 30-60 s post-stimulation associated with pseudopod protrusion (Fig. 2B,C,D, Movies 5, 6) (Hall et al., 1989). By contrast, pkaC null cells display considerably higher basal F-actin levels (near significant difference with p = 0.07) and, as a likely consequence, a blunted F-actin polymerization response, with a markedly smaller first peak followed by limited depolymerization and prolonged elevated levels of F-actin (Fig. 2B,C,D, Movies 5, 6). Together, these results suggest that F-actin levels are upregulated in resting pkaC null cells and that F-actin is both temporally and spatially mis-regulated in migrating cells lacking PKA-C.
We similarly investigated the spatiotemporal profile of myosin II in cells exposed to cAMP using GFP-fused myosin heavy chain A (GFP-MyoII) (Chung and Firtel, 1999). As reported previously, GFP-MyoII is excluded from the leading edge and localizes to the sides and posterior of migrating wild-type cells (Fig. 2A) (Chung and Firtel, 1999). Resting, unstimulated wild-type cells display basal levels of GFP-MyoII at the cell cortex, which is rapidly delocalized to the cytosol upon uniform cAMP stimulation (Fig. 2B) (Jeon et al., 2007a). This delocalization is then followed by an increase in cortical GFP-MyoII, peaking at ~30-40 s, and a return to basal levels by 60 s post-stimulus (Fig. 2B,E). In pkaC null cells, we observed some heterogeneity in the spatiotemporal dynamics of GFP-MyoII. On average, though, pkaC null cells that were either unstimulated or exposed to a cAMP gradient display relative cortical levels of GFP-MyoII similar to those of wild-type cells, with GFP-MyoII being excluded from extended protrusions and enriched in retracting protrusions (Fig. 2A,B). Consistent with the observed random extension of membrane protrusions in migrating pkaC null cells, the cortical localization of GFP-MyoII in these cells is not restricted to the side of cells facing the lowest cAMP concentrations but is observed on any side of the cells, changing frequently and displaying no directional bias (Fig. 2A). In addition, we observed that, upon uniform cAMP stimulation, GFP-MyoII at the cell cortex of pkaC null cells translocates to the cytosol with delayed kinetics compared to those in wild-type cells and then only slowly comes back to basal cortical levels (Fig. 2B,E). These results suggest that, in addition to F-actin, the timing and localization of myosin II assembly in cells lacking PKA-C are mis-regulated during chemotaxis.

Cells lacking PKA function have elevated PI3K, TORC2 and PKB/PKBR1 activity

We previously found that the PKB/PKBR1-mediated phosphorylation of Sca1 is elevated in pkaC null cells (Charest et al., 2010). To then investigate the mechanism by which PKA controls cytoskeletal remodeling and chemotaxis, we assessed the kinase activity of PKB and PKBR1 in cells lacking PKA-C. We observed that the basal levels of PKB and PKBR1 kinase activity are elevated and the cAMP-induced stimulation of PKB and PKBR1 activity is prolonged in pkaC null cells compared to that in wild-type cells.
We also observed that in cells lacking ACA, PKB/PKBR1 display similarly elevated basal activity levels and prolonged cAMP-induced activation, although these effects are less pronounced than what we observe in pkaC null cells (Fig. 3A). This finding is not surprising as, although ACA is responsible for most of the chemoattractant-induced cAMP production in Dictyostelium, some cAMP is produced in acaA null cells and, thus, these cells are expected to have remaining PKA activity (Bagorda et al., 2009; Kim et al., 1998; Söderbom et al., 1999). Consistent with the observed elevated PKB/PKBR1 activity levels in pkaC null cells, these cells also display higher basal phosphorylation levels and extended cAMP-induced phosphorylation of cellular PKB/PKBR1 substrates compared to that found in wild-type cells, reminiscent of the Sca1 phosphorylation profile in these cells (Charest et al., 2010) (Fig. 3B). Thus, these results suggest that PKB and PKBR1 activities are upregulated in cells lacking PKA function.

Two upstream pathways co-regulate the activity of PKB/PKBR1 during cAMP chemotaxis in Dictyostelium, the PI3K-dependent pathway that leads to the PDK-mediated phosphorylation of PKB/PKBR1’s activation loop, and the TORC2 pathway leading to phosphorylation of PKB/PKBR1’s hydrophobic motif (Kamimura and Devreotes, 2010; Liao et al., 2010). We asked whether the elevated activity of PKB/PKBR1 in pkaC null cells results from upregulated PI3K and/or TORC2 activity. To assess PI3K activity, we expressed a fluorescent PI(3,4,5)P3 reporter consisting of the pleckstrin homology domain of the cytosolic regulator of adenylyl cyclase fused to GFP (PHcrac-GFP) (Dormann et al., 2002; Parent et al., 1998). Interestingly, we observed that whereas PHcrac-GFP is mostly localized to the cytosol of resting wild-type cells with occasional enrichment to the plasma membrane of extended protrusions, many unstimulated pkaC null cells display membrane-localized PHcrac-GFP at structures corresponding to protrusions, membrane ruffles, and endocytic cups (Fig. 3C). During chemotaxis to cAMP, whereas PHcrac-GFP localizes to the front of chemotaxing wild-type cells (Dormann et al., 2002; Parent et al., 1998), in pkaC null cells exposed to the cAMP gradient, the PI(3,4,5)P3 reporter localizes randomly around the cell cortex,
corresponding to the numerous randomly extended protrusions (Fig. 3D), reminiscent of the localization of the F-actin reporter Lifeact-GFP described above.

As previously reported, uniform cAMP stimulation induces the temporary translocation of PHcrac-GFP from the cytosol to the plasma membrane, peaking at ~8 s, reflecting PI3K activation and transient PI(3,4,5)P₃ accumulation at this site (Fig. 3E, Movie 7). After peaking at ~8 s post-cAMP stimulation, the levels of PHcrac-GFP at the membrane of wild-type cells decrease below pre-stimulus levels, consistent with previous observations (Tang et al., 2014). In contrast, pkaC null cells display a reduced PHcrac-GFP translocation response, when normalized to their respective basal level, with a slightly delayed and broader peak that reaches a maximum at ~10-12 s, before returning to pre-stimulus levels (Fig. 3E, Movie 8). Therefore, these observations suggest that PI(3,4,5)P₃ production is mis-regulated both temporally and spatially in cells lacking PKA.

As a measure of TORC2 activity, we assessed the TORC2-mediated phosphorylation of PKB and PKBR1 at their hydrophobic motifs (HM) before and upon cAMP stimulation as previously described (Kamimura et al., 2009). In contrast to wild-type cells in which the TORC2 phosphorylation of PKB/PKBR1-HM is very transient, peaking at 5-10 s and returning to basal levels by 40 s post-stimulus, pkaC null cells display considerably elevated and extended PKB/PKBR1-HM phosphorylation (Fig. 3F). Similarly, we observed that the TORC2-mediated PKB/PKBR1-HM phosphorylation is elevated in cells lacking ACA as well as in cells treated with the PKA-selective pharmacological inhibitor H89 (Zeng et al., 2001) (Figs 3F, S2). Hence, these results suggest that loss of PKA function leads to an increased activation of TORC2. Together, our observations indicate that the elevated PKB/PKBR1 activity levels in cells lacking PKA function likely result from the combination of upregulated PI3K and TORC2 pathways in these cells.
Lack of PKA function has different effects on RasC, RasG, and Rap1

In Dictyostelium, the Ras protein RasG is the main mediator of PI3K activation in response to cAMP stimulation (Bolourani et al., 2006; Sasaki et al., 2004), RasC is a major activator of TORC2 (Cai et al., 2010; Charest et al., 2010), and Rap1 has been shown to regulate both PI3K and TORC2 activities (Khanna et al., 2016; Kortholt et al., 2010), in addition to other effectors. To then investigate potential mechanisms underlying the elevated activity levels of TORC2 and PI3K in cells lacking PKA function, we examined the activity of RasC, RasG, and Rap1 in such conditions. First, we assessed the activity of Flag-tagged RasC in pkaC null cells and in wild-type cells treated with H89. Interestingly, we observed that cAMP-induced RasC activation is considerably decreased in cells lacking PKA activity (Fig. 4A). We also found that Sca1 fails to translocate from the cytosol to the plasma membrane in pkaC null cells stimulated with cAMP and that Sca1 translocation is considerably reduced in acaA null cells compared to that in wild-type cells (Fig. 4B,C). These observations are consistent with our previous findings that PKB/PKBR1 phosphorylation of Sca1 is elevated in pkaC null cells and that this phosphorylation inhibits the translocation of the Sca1 complex to the plasma membrane and RasC activation (Charest et al., 2010). These results show that RasC activity is down-regulated in cells lacking PKA function and, therefore, suggest that PKA is necessary for optimal RasC activation. Consequently, this PKA-mediated positive regulation of RasC cannot explain the elevated TORC2 activity observed in pkaC null cells.

In contrast to the decrease in RasC activation observed in cells that lack PKA activity, we found that both RasG and Rap1 display elevated basal activity levels and elevated and extended cAMP-induced activation in pkaC null cells compared to that in wild-type cells (Fig. 5A). To image RasG activity dynamics, we used the Ras activity reporter consisting of the Ras binding domain (RBD) of human Raf1 fused to GFP [Raf1(RBD)-GFP], which binds RasG and not RasC (Kae et al., 2004; Kortholt et al., 2013; Sasaki et al., 2004). Although Raf1(RBD)-GFP also binds the active forms of Dictyostelium RasB and RasD (Zhang et al., 2008), RasG is believed to be the
predominant Ras protein expressed in 5h-developped, cAMP-responsive cells (Rot et al., 2009), and thus Raf1(RBD)-GFP is believed to be a good reporter of active RasG. Whereas Raf1(RBD)-GFP is mostly found in the cytosol of resting wild-type cells, many resting pkaC null cells display cortex-localized Raf1(RBD)-GFP similar to the observed localization of both the F-actin and PI(3,4,5)P3 reporters in these cells, including membrane protrusions, membrane ruffles, and endocytic cups (Fig. 5B). Also reminiscent of the observed F-actin and PI(3,4,5)P3 dynamics in pkaC null cells, Raf1(RBD)-GFP is highly enriched in the randomly extended membrane protrusions of pkaC null cells exposed to a cAMP gradient (Fig. 5C). Consistent with the profile of the PI(3,4,5)P3 response, which lies downstream from RasG, the Ras activity reporter exhibits a reduced translocation to the cell cortex in pkaC null cells upon uniform cAMP stimulation compared to that in wild-type cells, when normalized to their respective basal levels (Fig. 5D, Movies 9, 10).

To image Rap1 activity dynamics, we used the Rap1 activity reporter consisting of the RBD of human RalGDS [RalGDS(RBD)-GFP] (Jeon et al., 2007b). In resting cells, although some pkaC null cells are found with slightly more RalGDS(RBD)-GFP localized to the membrane, for the most part, RalGDS(RBD)-GFP displays localization patterns more similar to those in wild-type cells (Fig. 5E). However, RalGDS(RBD)-GFP is clearly enriched in the randomly extended membrane protrusions of pkaC null cells exposed to a cAMP gradient (Fig. 5F). In addition, the Rap1 activity reporter displays increased translocation to and prolonged localization at the cell cortex in pkaC null cells in response to uniform cAMP stimulation compared to that in wild-type cells (Fig. 5G, Movies 11, 12). Therefore, together, our observations suggest that, in contrast to the PKA-dependent positive regulation of RasC, PKA has a negative regulatory effect on RasG and Rap1 activation.
**pkaC null cells have severe gradient sensing defects**

Spatiotemporal regulation of RasG activity is required for directional sensing of cAMP gradients in *Dictyostelium* (Zhang et al., 2008). Our findings that *pkaC* null cells are motile but fail to migrate in a cAMP gradient and that RasG activity is upregulated in the absence of PKA suggest PKA may be necessary for gradient sensing. To test if PKA plays a role in cAMP gradient sensing, we assessed the Ras response, using Raf1(RBD)-GFP, in cells treated with the F-actin inhibitor Latrunculin B (LatB). LatB treatment generates cells that are unable to polymerize F-actin and are, thus, paralyzed, symmetrical, and spherical (Parent et al., 1998). LatB-treated cells are then used to interrogate cAMP gradient sensing capabilities in the absence of feedback from the actin cytoskeleton (Zhang et al. 2008). As previously reported (Zhang et al., 2008), LatB-treated wild-type cells respond to the cAMP gradient and accumulate Raf1(RBD)-GFP in the form of a crescent at the cell cortex on the side closest to the chemoattractant source, a cAMP-filled micropipette (Fig. 6A). Upon repositioning of the micropipette to the opposite side of the cell, Raf1(RBD)-GFP is quickly delocalized from its previous site and accumulates at the cortex on the side closest to the new position of the micropipette within 10-15 s, reflecting the rapid deactivation and activation of Ras at each site, respectively (Fig. 6A,B, Movie 13). In wild-type cells, Raf1(RBD)-GFP accumulation is highly restricted to the side closest to the cAMP chemoattractant source and never accumulates at the side opposite to the micropipette or the lateral sides, illustrating the highly spatially regulated Ras response.

In contrast to the rapid and restricted accumulation of Raf1(RBD)-GFP to the cell cortex of LatB-treated wild-type cells upon cAMP gradient sensing, accumulation of the Ras reporter to the cortex of LatB-treated *pkaC* null cells does not as strongly correlate with the position of the chemoattractant source (Fig. 6, Movies 14, 15). In *pkaC* null cells, accumulation of Raf1(RBD)-GFP on the side of the cell closest to the chemoattractant becomes enriched, but with very slow kinetics (~ 1 min) compared to that for wild-type cells (within 15 sec) and localization of the reporter is not restricted there. Raf1(RBD)-
GFP is often observed localized to the lateral sides and even sometimes on the opposite side from that facing the micropipette, which is never observed for wild-type cells. In *pkaC* null cells, the Ras reporter appears to be moving around the cell cortex as if the Ras signal is propagating, reminiscent of the previously reported self-organized waves of PI(3,4,5)P3 that are generated spontaneously on the membrane in the absence of the chemoattractant (Arai et al., 2010). These observations suggest that, although *pkaC* null cells respond to the cAMP gradient, PKA is necessary for cells to properly restrict Ras activation to the side closest to the chemoattractant source, which likely underlies the inability of cells lacking PKA to accurately determine the direction of the gradient during chemotaxis to cAMP.

**cAR1 expression in pkaC null cells**

Our results using the *pkaC* null cells, as well as *acaA* null cells and the H89 inhibitor suggest a role for PKA in regulating the Ras and Rap1 pathways in response to the cAMP chemoattractant in *Dictyostelium*. However, it is also possible that part of the observed effects of the lack of PKA are indirect and due to its role in controlling gene expression. We determined that, at least, Ras, Rap1, Pia (an essential TORC2 component), PKB, and PKBR1 are well expressed in *pkaC* null cells (Figs 7A, S3). Interestingly, the observed regulated decrease in PKB expression in 5.5 h pulsed cells indicate that some developmental gene expression is properly conserved in *pkaC* null cells (Stajdohar et al., 2015). On the other hand, we failed to detect the expression of the main cAMP chemotactic receptor cAR1 by immunoblot. However, the fact that *pkaC* null cells respond well to cAMP stimulation, whereas cells lacking cAR1 (*carA* null cells) mostly don’t, suggests that some cAR1 is likely present in *pkaC* null cells (Fig. 7D,E) (Liao et al., 2013). In order to test whether the shortage of cAR1 expression in *pkaC* null cells is responsible for the *pkaC* null phenotypes, we assessed the ability of exogenously expressed cAR1-GFP to rescue the chemotaxis, RasG, Rap1, and TORC2 activity defects of *pkaC* null cells. We found that, at similar cAR1-GFP expression levels in *carA* null and *pkaC* null cells, cAR1-GFP rescued *carA* null cells chemotaxis and only very partially rescued the *pkaC* null cells chemotactic defects (Fig. 7C, Movies 16, 17). Indeed,
some of the cAR1-GFP/pkaC null cells closest to the chemoattractant source did find the way to the tip of the micropipette, albeit inefficiently. Nonetheless, this result suggests that part of the pkaC null cells chemotaxis phenotype is due to lack of cAR1 expression.

We next assessed the cAMP-induced activation of RasG, Rap1, and TORC2 in carA null cells compared to those in pkaC null cells, and tested the effect of expressing exogenous cAR1-GFP at similar levels in both strains, as controlled by immunoblot and fluorescence measurements. As previously reported (Kae et al., 2004), the basal level of Ras activity, as well as that of Rap1 activity, is elevated in carA null cells and, by contrast to the pkaC null cells, the carA null cells fail to respond to a cAMP stimulus by further increasing the activity of RasG and Rap1 (Fig. 7D). In addition, whereas the cAMP-induced TORC2-mediated phosphorylation of PKB and PKBR1 is elevated and prolonged in pkaC null cells, cAMP stimulation produces minimal TORC2 activation in carA null cells, likely mediated by the cAMP receptor 3 expressed at low levels in aggregation-competent cells (Insall et al., 1994) (Fig. 7E). As expected, cAR1-GFP expression in carA null cells rescued the cAMP-induced activation of Ras, Rap1, and TORC2 in this strain (Fig. 7D,E). Interestingly, pkaC null cells expressing cAR1-GFP, at levels similar to those rescuing the carA null cell phenotypes, displayed the same elevated and extended cAMP-induced RasG, Rap1, and PKB/PKBR1 phosphorylation responses as in pkaC null cells (Fig. 7D,E). Together, these observations suggest that: some cAR1 is expressed in pkaC null cells; little cAR1 is sufficient to fully activate the RasG, Rap1, and TORC2 pathways, whereas high levels of cAR1 is necessary for chemotaxis; and, finally, the lack of cAR1 is not responsible for the observed upregulated signaling pathways in pkaC null cells.

DISCUSSION

It is widely appreciated that specific kinetics and localization of chemotactic signaling pathways, as well as their adaptation to the chemoattractant signal, is required for the directional response of cells to chemoattractant gradients. However, how
chemoattractant signaling is regulated and how signaling pathway dynamics and polarity is achieved is not understood. Our study suggests that PKA is essential to chemotaxis and plays an important role in the control of both the localized activation and adaptation of key cAMP-induced chemotactic signaling pathways in Dictyostelium, involving RasG, Rap1, PI3K, TORC2, and PKB/PKBR1, as well as the spatiotemporal regulation of the cytoskeleton (Fig. 8). The observed effect of lack of PKA function on chemotaxis is likely a combination of gene regulation and of a signaling role. Indeed, whereas it is hard to separate these two cellular functions of PKA, our experiments using acaA null cells, which produce a lot less cAMP and, thereby, have little PKA activity (Meima and Schaap, 1999), as well as our results with the PKA-selective pharmacological inhibitor H89, strongly suggest that PKA plays a signaling role in controlling the chemotactic pathways in Dictyostelium. Although we found that pkaC null cells lack detectable cAR1 expression, as assessed by immunoblotting, they are responsive to cAMP stimulation, which suggests that some cAR1 is present. Interestingly, vegetative cells, which express little cAR1, also produce a full cAMP-induced TORC2 activation response (Liao et al., 2013). This previous observation and our present study suggest that only small amounts of cAR1 are necessary to activate, at least some, cAMP-induced signaling pathways, whereas more cAR1 is necessary for efficient chemotaxis to cAMP. In fact, the partial rescue of pkaC null cell chemotaxis by the exogenous expression of cAR1 suggests that lack of cAR1 is partly responsible for the chemotaxis phenotype of these cells. However, we found that lack of cAR1 expression in pkaC null cells is not responsible for the upregulated cAMP signaling pathways in these cells.

Consistent with a role for cAMP in the spatiotemporal control of signaling pathways, acaA null cells are unable to suppress the formation of lateral pseudopods, leading to directionality defects during chemotaxis (Stepanovic et al., 2005). We propose that this intracellular role of ACA and cAMP in Dictyostelium is mediated, in part, by the PKA-dependent regulation of the RasG and Rap1 pathways in space and time during chemotaxis. In addition, as the PI3K, TORC2, and PKB/PKBR1 signaling pathways contribute to the cAMP-stimulated activation of ACA and cAMP production (Charest et al., 2010; Comer and Parent, 2006; Lee et al., 2005; Lim et al., 2001) and, consequently,
to the downstream activation of PKA, we further propose that PKA indirectly controls its own level of activity during chemotaxis in a negative feedback fashion. This hypothesis is consistent with previous observations that cAMP-stimulated ACA activity fails to adapt in ACA-expressing pkaC null cells (Mann et al., 1997) and that cells with elevated PKA activity display reduced intracellular levels of cAMP compared to that in wild-type cells (Abe and Yanagisawa, 1983; Anjard et al., 1992).

Our analysis of the pkaC null cells chemotaxis and cAMP gradient sensing phenotypes suggests that PKA is not necessary for basic cell motility in Dictyostelium, but that it is crucial to cAMP chemotaxis and plays a key role in restricting the extent as well as the site of chemotactic pathway activation and, thereby, pseudopod protrusion. Our finding that PKA is essential to cAMP chemotaxis is surprising because, whereas pkaC null cells are unable to aggregate by themselves with or without the ability to produce cAMP (Mann et al., 1997), they have been found to co-aggregate with wild-type cells (Mann and Firtel, 1991). We propose that, in such case, cell-cell contacts between pkaC null and wild-type cells may have enabled their co-aggregation. On the other hand, the chemotaxis phenotype of pkaC null cells is consistent with the considerable reduction in chemotaxis observed for cells with decreased PKA activity due to the expression of PKA regulatory subunit (PKA-R) mutants (Harwood et al., 1992). Intriguingly, cells lacking the PKA-R, in which PKA-C is constitutively active, also display reduced polarity, reduced directionality, the inability to repress the formation of lateral pseudopods, and, consequently, severely impaired chemotaxis to cAMP (Zhang et al., 2003). However, given the promiscuous nature of PKA whose specificity is normally tightly regulated through anchoring complexes (Greenwald and Saucerman, 2011; Pidoux and Taskén, 2010), careful consideration must be given to the phenotype of cells with constitutive PKA activity. Nonetheless, the similarity of pkaC and pkaR null cell phenotypes could support the idea of an auto-regulatory circuit, linked by feedback loops that function to tightly regulate PKA activity during chemotaxis, such as was previously proposed by Soll and colleagues (Stepanovic et al., 2005). Disruption of any one component of such circuit destroys the circuit, and is likely to yield a common phenotype that reflects the circuit’s role. In the case of cAMP-PKA signaling, we propose that the
wiring of this circuit allows the coordinated control of directional pseudopod extension and signal relay (Fig. 8).

The cAMP chemotaxis phenotype of pkaC null cells is also highly reminiscent of cells lacking the RasG GAP DdNF1 (nfaA null cells), which exhibit elevated levels of chemoattractant-induced RasG activity (Zhang et al., 2008), as well as of cells expressing a constitutively active Rap1 mutant (Rap1G12V; Jeon et al. 2007). nfaA null cells display increased phagocytosis, macropinocytosis, are motile but fail to properly sense cAMP gradients, and extend multiple lateral pseudopods leading to severe chemotaxis defects (Bloomfield et al., 2015; Zhang et al., 2008). Rap1G12V-expressing cells also produce lateral pseudopods during migration, and perform chemotaxis to cAMP with little directionality (Jeon et al. 2007). Consequently, we propose that the elevated and spatially mis-regulated RasG and Rap1 activities in pkaC null cells underlies the inability of these cells to properly sense gradients, to define a leading front, and to migrate in a cAMP gradient. Hence, we suggest that PKA regulates the cAMP gradient sensing abilities of cells mainly through controlling RasG and Rap1 activation. The PKA-mediated regulation of RasG and Rap1 can explain, at least in part, the effects of PKA on the downstream activation of PI3K, TORC2 and PKB/PKBR1, as well as F-actin polymerization and MyoII assembly (Fig. 8). Indeed, both RasG and Rap1 mediate PI3K activation, which, in turn, promotes F-actin polymerization, and Rap1 regulates TORC2, as well as MyoII assembly through a parallel pathway involving its effector Phg2 (Khanna et al., 2016; Kortholt et al., 2010; Swaney et al., 2010). Similar to pkaC null cells, cells with elevated RasG activity display elevated PI3K activity and F-actin dynamics (Zhang et al., 2008), and cells with elevated Rap1 activity exhibit elevated TORC2 activity and reduced MyoII assembly (Jeon et al., 2007a; Khanna et al., 2016). Of course, we cannot exclude the possibility that PKA directly regulates PI3K, PKB/PKBR1, F-actin, and MyoII, in addition to RasG, Rap1, and TORC2, and that PKA may have additional roles in chemotaxis to cAMP such as regulating Rac GTPases and various actin modulators (Howe, 2004).
In addition to the PKA-dependent regulation of Rap1, which likely affects downstream TORC2 activity, we propose that TORC2 is also regulated by PKA independently of Rap1. We recently showed that RasC is essential for TORC2 activation while Rap1 regulates the RasC-mediated TORC2 activity (Khanna et al., 2016). Since RasC activity in cells lacking PKA is strongly reduced, we propose that the highly elevated TORC2 activity in cells lacking PKA results from the PKA-dependent regulation of both Rap1 and TORC2. In mammalian cells, PKA has been found to associate with mTOR (Mavrakis et al., 2006) and to mediate inhibition of both mTORC1 and mTORC2 by inducing dissociation of the complexes (Xie et al., 2011). However, whether PKA directly phosphorylates mTOR or components of mTOR complexes is unknown. On the other hand, the observed PKA-dependent positive regulation of RasC is likely to be indirect and to result from the PKA-dependent inhibition of TORC2, as well as of RasG/Rap1/PI3K signaling, leading to reduced PKB and PKBR1 activity. This reduced activity of PKB and PKBR1, which normally inhibit the Seal complex-mediated RasC activation through a negative feedback loop (Charest et al., 2010), will consequently lead to greater RasC activation (Fig. 8).

In mammalian cells, including neutrophils, PKA activity was shown to be enriched at the front of migrating cells and to promote actin cytoskeleton polarization (Jones and Sharief, 2005; Paulucci-Holthauzen et al., 2009). One of the suggested mechanisms through which PKA controls mammalian cell migration involves its direct phosphorylation of Rap1, thereby controlling Rap1’s membrane localization and activity cycle (Takahashi et al., 2013). Whether this is the mechanism through which PKA controls Rap1, and/or RasG, activity in Dictyostelium remains to be determined, but this is certainly a possibility. It is also conceivable that PKA targets specific RasG and Rap1 GEFs and/or GAPs, instead or in addition to directly targeting the GTPases. In any case, our findings highlight a previously unappreciated role of PKA in chemotaxis, through the spatial and temporal regulation of key chemotactic signaling pathways.
MATERIALS AND METHODS

Reagents

cAMP sodium salt monohydrate and anti-Flag M2 were from Sigma-Aldrich (St. Louis, MO, USA) and H2B was from Roche-Genentech (San Francisco, CA, USA). Phospho-p70 S6 kinase (Thr389; 1A5), phospho-Akt substrate (110B7), and phospho-(Ser/Thr) PKA substrate antibody were from Cell Signaling Technology (Danvers, MA, USA). Pan-Ras antibody (Ab-3; RAS10) was from Calbiochem/EMD Millipore (Billerica, MA, USA). H89 and DyLight™ secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The Living Colors® GFP monoclonal antibody was purchased from Clontech (now Takara Bio USA Inc.; Mountain View, CA, USA). HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). The Rap1 (directed against amino acids 169-182 of Dictyostelium Rap1) and Pia antibody (directed against amino acids 138-159 of Dictyostelium Pianissimo) were custom-made by ProSci Incorporated (Poway, CA, USA). PKB and PKBR1 antibodies, Lifeact-GFP, GFP-MyoII, and PHcrac-GFP constructs were gifts from Rick Firtel and were previously described (Bastounis et al., 2011; Jeon et al., 2007a; Meili et al., 1999; Sasaki et al., 2004). The cAR1 antibody was a generous gift from Peter Devreotes and described elsewhere (Hereld et al., 1994). Raf1(RBD)-GFP and RalGDS(RBD)-GFP constructs were kindly provided by Arjan Kortholt and were previously described (Kortholt et al., 2010; Kortholt et al., 2013). GFP-Sca1 was described in (Charest et al., 2010). Flag-tagged RasC was generated by fusing the Flag tag nucleotide sequence, GATTATAAAGATGATGATGATAAA, in frame at the N-terminus of the RasC sequence and cloned into the extra-chromosomal vector pDM304, obtained through the Dicty Stock Center, deposited by Douwe Veltman (Veltman et al., 2009). cAR1-GFP was generated by cloning cAR1 in the GFP-containing extra-chromosomal vector pDM323 (Veltman et al., 2009) and was a gift from Chris Janetopoulos.
Cell culture and strains used

*Dictyostelium* cells were grown attached to substrate in axenic HL5 medium (ForMedium, Hunstanton, Norfolk, UK) at 22°C and transformants were generated by electroporation. Transformed cells were selected in 10 or 20 µg/ml Geneticin, or 50 µg/ml Hygromycin B (both from Life Technologies, Grand Island, NY, USA) and expression confirmed by Western blot. Cells were developed by pulsing with 30 nM cAMP every 6 min for 5.5 h in 12 mM Na/K phosphate pH 6.1 at a confluency of 5x10^6 cells/ml for wild-type cells, and at 7x10^6 cells/ml for *pkaC* null cells, which are much smaller, in order to have equivalent densities. The wild-type cells used are AX2 and AX3, depending on the background of mutant used. *ScaA* null cells were described elsewhere (Charest et al., 2010). *pkaC* null and *acaA* null cells were obtained from the Dicty Stock Center where they were deposited by Rick Firtel and Bill Loomis, respectively; both strains were described elsewhere (Mann and Firtel, 1991; Mann et al., 1992; Stepanovic et al., 2005). The HBW1 and HBW2 cells were a kind gift from Robert Kay and described previously (Primpke et al., 2000). The *carA* null cells were generously provided by Alan Kimmel and previously described (Sun and Devreotes, 1991).

Biochemical assays

F-actin measurements using Phalloidin staining, PKB and PKBR1 kinase assays, Ras-GTP and Rap1-GTP pull-down assays were performed as previously described (Insall et al., 1996; Jeon et al., 2007a; Meili et al., 1999; Sasaki et al., 2004; Sasaki et al., 2007; Zhang et al., 2008). TORC2 activity was assessed by evaluating the TORC2-mediated phosphorylation of PKB and PKBR1 as described previously (Kamimura et al., 2009). Detection of phosphorylated PKB and PKA substrates by Western blot, using phospho-Akt substrate and phospho-(Ser/Thr) PKA substrate antibodies, was performed on whole cell lysates according to the antibodies’ manufacturer protocols. For H89 treatments, 5.5 h pulsed cells were treated either with 5% DMSO (control) or 50 µM of DMSO-dissolved H89 for 30 min prior to cAMP stimulation.
Chemotaxis and imaging

Assessment of chemotaxis, gradient sensing, global responses of fluorescent reporters to cAMP stimulation, and image acquisition were performed as previously described (Charest et al., 2010; Chung and Firtel, 1999; Sasaki et al., 2007; Zhang et al., 2008). Chemotaxis parameters were determined as described in (Cai et al., 2010). Images were acquired using a Marianas Spinning Disk Confocal Workstation (Intelligent Imaging Innovations, In., Denver, CO, USA) equipped with an Evolve™ 512 EMCCD camera (Photometrics, Tucson, AZ, USA), and image analysis was performed using the Slidebook software (Intelligent Imaging Innovations, In., Denver, CO, USA). Analysis of fluorescent reporter translocations between the cytosol and the cell cortex was performed by measuring changes in fluorescent intensities in the cytosol as described in (Takeda et al., 2012), normalized to each cell’s basal levels.
ACKNOWLEDGEMENTS

We thank Rick Firtel, Arjan Kortholt, Peter Devreotes, Alan Kimmel, and Chris Janetopoulos, as well as the Dicty Stock Center for providing reagents.

COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

M.S. and A.R.P. contributed equally to the work. P.G.C. conceptualized and designed the study, M.S., A.R.P., R.R., V.F.T., P.L., and P.G.C. performed and interpreted the experiments, M.S., A.R.P. and P.G.C. prepared the manuscript.

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REFERENCES


**Fig. 1. The chemotaxis phenotype of pkaC null cells.**

(A) Morphology of wild-type (WT) and pkaC null cells (pkaC−) exposed to a cAMP gradient. The direction of the gradient is indicated by the arrow. Scale bars, 10 μm. (B) Traces of representative cells migrating in an exponential cAMP gradient created by a point source. The starting point of each cell was apposed to the axis’ origin. The arrow indicates the direction of the gradient. (C) Quantitative analysis of the chemotactic behavior of cells (13 WT and 8 pkaC null cells) from 3 independent experiments. Motility speed represents the total path length divided by time; Migration speed represents the total linear displacement (end point – starting point) divided by time; Persistence indicates path linearity and was calculated as the linear displacement divided by total path length; Chemotactic index represents the directionality of the cells’ movements relative to the gradient.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Speed (μm/min)</th>
<th>Persistence</th>
<th>Chemotactic Index</th>
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<tr>
<td></td>
<td>Motility</td>
<td>Migration</td>
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<tr>
<td>WT</td>
<td>5.8 ± 0.9</td>
<td>4.7 ± 1.2</td>
<td>0.79 ± 0.09</td>
</tr>
<tr>
<td>pkaC−</td>
<td>4.9 ± 1.4</td>
<td>0.8 ± 0.4</td>
<td>0.16 ± 0.07</td>
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Fig. 2. Spatiotemporal dynamics of F-actin and myosin in pkaC null cells.

Live imaging of F-actin (Lifeact-GFP) and Myosin II (GFP-labeled myosin light chain, GFP-MyoII) fluorescent reporters in cells exposed to an exponential gradient of cAMP (A) and in cells uniformly stimulated with 5 μM cAMP for the indicated times (B). Scale bars, 10 μm. (C) Relative fluorescence intensity of Lifeact-GFP in cells uniformly stimulated with cAMP for the indicated times. Data represent the mean fluorescence intensity +/- SEM of 71 WT and 48 pkaC null cells. (D) cAMP-induced F-actin polymerization measured by Phalloidin binding, expressed as fold over basal. Inset, basal
F-actin levels measured per protein mass, expressed as fold over WT basal levels. Data represents the mean +/- SEM of 5 different experiments. *, p < 0.05 in a two-tailed \( t \)-test.

(E) Relative cytosolic fluorescence intensity of GFP-MyoII in cells uniformly stimulated with cAMP for the indicated times. Data represent the mean fluorescence intensity +/- SEM of 91 WT and 50 \( \text{pkaC} \) null cells. Imaging data are representative of at least 3 independent experiments.
Fig. 3. Activity of chemotactic effectors PKB, PKBR1, PI3K and TORC2 in cells lacking PKA function.

(A) cAMP-induced PKB and PKBR1 kinase activity in WT, pkaC<sup>−</sup>, and in cells lacking adenylyl cyclase A (<i>acaA</i><sup>−</sup>). The kinase activity of immunopurified PKB and PKBR1 was assessed using H2B as a substrate. H2B phosphorylation was detected by autoradiography and PKB and PKBR1 were revealed by immunoblotting. (B) cAMP-induced phosphorylation of PKB and PKBR1 substrates was detected using an antiphospho-PKB substrate antibody (P-PKBS). (C) Localization of the PI(3,4,5)P<sub>3</sub> reporter PHcrac-GFP in developed, resting cells. (D) Live imaging of PHcrac-GFP in cells
exposed to an exponential gradient of cAMP. The direction of the gradient is indicated by the arrow. (E) Live imaging of PHcrac-GFP in cells uniformly stimulated with 5 μM cAMP for the indicated times, and relative cytosolic fluorescence intensity of PHcrac-GFP measured in cells uniformly stimulated with cAMP. Graph data represent the mean fluorescence intensity +/- SEM of 20 WT and 26 pkaC null cells. (F) TORC2-mediated phosphorylation of PKB and PKBR1 at their respective hydrophobic motif (HM) was assessed in WT, pkaC−, acaA−, and in WT cells treated with the PKA pharmacological inhibitor H89 or its vehicle (DMSO). CB, Coomassie blue staining. Scale bars, 10 μm. Data are representative of at least 3 independent experiments.
**Fig. 4. RasC pathway activation in cells lacking PKA function.**

(A) cAMP-induced activation of Flag-tagged RasC expressed in WT and *pkaC* null cells, and in WT cells treated with the PKA pharmacological inhibitor H89 or its vehicle (DMSO). Active RasC (RasC-GTP) was pulled-down with GST-Byr2(RBD). RasC-GTP and total RasC were revealed by Flag immunoblotting. (B) Live imaging of GFP-Scal1 in cells uniformly stimulated with 5 μM cAMP for the indicated times. Scale bars, 10 μm. (C) Relative cytosolic fluorescence intensity of GFP-Scal1 in cells uniformly stimulated with cAMP. Data represent the mean fluorescence intensity +/- SEM of 150 WT, 33 *pkaC* null cells, and 147 *acaA* null cells. Data represent or are representative of at least 3 independent experiments.
Fig. 5. Spatiotemporal dynamics of RasG and Rap1 activities in pkaC null cells.

(A) cAMP-induced RasG and Rap1 activation. Active RasG (Ras-GTP) and Rap1 (Rap1-GTP) were pulled-down with GST-Raf1(RBD) and GST-RalGDS(RBD), respectively, and revealed by anti-Pan-Ras and Rap1 immunoblotting. (B and E) Localization of Raf1(RBD)-GFP (B) and RalGDS(RBD)-GFP (E) in resting cells. (C and F) Live imaging of Raf1(RBD)-GFP (C) and RalGDS(RBD)-GFP (F) in cells exposed to an exponential gradient of cAMP. (D and G) Live imaging of Raf1(RBD)-GFP (D) and
RalGDS(RBD)-GFP (G) in cells uniformly stimulated with 5 μM cAMP for the indicated times. Relative reporter cytosolic fluorescent intensity is shown on the right. Scale bars, 10 μm. Quantified data represent the mean fluorescence intensity +/- SEM of 20 RafI(RBD)-GFP/WT, 9 RafI(RBD)-GFP/pkaC null, 109 RalGDS(RBD)-GFP/WT, and 135 RalGDS(RBD)-GFP/pkaC null cells. Data represent or are representative of at least 3 independent experiments.
Fig. 6. Gradient sensing in pkaC null cells. (A) Live imaging of Raf1(RBD)-GFP in cells, treated with 10 μM LatB, upon changes of cAMP gradient orientation. Two examples of pkaC null cells are shown (Cell 1 and Cell 2). Data are representative of at least 3 independent experiments. *, indicates the position of the cAMP-filled micropipette. Scale bars, 10 μm. (B) Quantification of the relative fluorescence intensity of membrane-localized Raf1(RBD)-GFP in cells shown in (A), for the sides alternatively closest and
opposite to the positioned micropipette (Sides A and B; top graphs) and the lateral sides (Sides 1 and 2; bottom graphs), as indicated in the graphs legend.
Fig. 7. The expression of cAR1 and its role in RasG, Rap1, and TORC2 activation in pkaC null cells.

(A) Expression of cAR1, Ras, Rap1, Pia, PKB, and PKBR1 in vegetative (0 h) and in cells pulsed with cAMP for 5.5 h, detected by immunoblotting using protein-specific antibodies. (B) cAR1-GFP expression in carA and pkaC null cells, revealed by GFP immunoblotting. (C) Chemotaxis phenotype of cAR1-GFP expressing carA and pkaC null cells migrating in an exponential cAMP gradient created by a point source. Images
of Differential Interference Contrast (DIC) and fluorescence (GFP), showing expressed cAR1-GFP, are shown. Right, traces for a subset of the migrating cells. *, position of the cAMP-filled micropipette. Scale bars, 50 μm. (D) cAMP-induced RasG and Rap1 activation. Active RasG (Ras-GTP) and Rap1 (Rap1-GTP) were pulled-down with GST-Raf1(RBD) and GST-RalGDS(RBD), respectively, and revealed by anti-Pan-Ras and Rap1 immunoblotting. (E) cAMP-induced phosphorylation of PKB’s and PKBR1’s hydrophobic motif (HM^P; TORC2 site). CB, Coomassie blue staining. Data are representative of at least 3 independent experiments.
Fig. 8. Proposed model for the role of PKA in regulating the chemoattractant signal transduction pathways in *Dictyostelium*. Whereas PKA controls gene expression during development, including that of cAR1, and this probably explains part of the observed...
severe chemotaxis phenotypes of \textit{pkaC} null cells, our study suggests that PKA is also likely to play a direct role in controlling the directional migration of cells. In light of our findings, we propose that PKA controls chemotaxis, in part, by spatially and temporally regulating the activation of RasG, Rap1, and TORC2.
Fig. S1. (A) Chemotaxis phenotype of HBW2 (WT) and HBW1 (pkaC⁻) cells migrating in an exponential cAMP gradient created by a point source. Right, traces for a subset of the migrating cells. *, position of the cAMP-filled micropipette. Scale bars, 50 μm. (B) cAMP-induced TORC2-mediated phosphorylation of PKB and PKBR1 at their respective hydrophobic motif (HM²). CB, Coomassie blue staining. Data are representative of at least 3 independent experiments.
**Fig. S2.** cAMP-stimulated phosphorylation of PKA substrates in WT cells treated with the PKA pharmacological inhibitor H89 or its vehicle (DMSO) was detected by immunoblot using a phospho-PKA substrate antibody (P-PKAS).
**Fig. S3.** Pia expression was assessed by immunoblot using cell lysates from WT and \( pkaC \) null cells compared to \( piaA \) null cells to validate our custom-made antibody.
Supplementary movies

**Movie 1.** Wild-type cells migrating in an exponential gradient of cAMP created by a point source (micropipette). Corresponds to the chemotaxis analysis of wild-type cells in Fig. 1B, 1C.
Movie 2. *pkaC* null cells exposed to an exponential gradient of cAMP created by a point source (micropipette). Corresponds to the chemotaxis analysis of *pkaC* null cells in Fig. 1B, 1C.
**Movie 3.** Imaging of Lifeact-GFP in wild-type cells migrating in an exponential gradient of cAMP. Position of the micropipette is indicated with a blue dot. Corresponds to data presented in Fig. 2A.
Movie 4. Imaging of Lifeact-GFP in pkaC null cells exposed to an exponential gradient of cAMP. Position of the micropipette is indicated with a blue dot. Corresponds to data presented in Fig. 2A.
Movie 5. Imaging of Lifeact-GFP in wild-type cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 2B.
Movie 6. Imaging of Lifeact-GFP in *pkaC* null cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 2B.
**Movie 7.** Imaging of PHcrac-GFP in wild-type cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 3E.
**Movie 8.** Imaging of PHcrac-GFP in *pkaC* null cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 3E.
**Movie 9.** Imaging of Raf1(RBD)-GFP in wild-type cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 5D.
Movie 10. Imaging of Raf1(RBD)-GFP in pkaC null cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 5D.
Movie 11. Imaging of RalGDS(RBD)-GFP in wild-type cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 5G.
**Movie 12.** Imaging of RaGDS(RBD)-GFP in pkaC null cells uniformly stimulated with 5 μM cAMP. cAMP was added at 3 sec (third frame). Corresponds to data presented in Fig. 5G.
**Movie 13.** Imaging of Raf1(RBD)-GFP in wild-type cells treated with 10 μM LatB upon changes in cAMP gradient orientation. Position of the micropipette is indicated with a blue dot. Corresponds to data presented in Fig. 6.
Movie 14. Imaging of Raf1(RBD)-GFP in \textit{pkaC} null cells treated with 10 μM LatB upon changes in cAMP gradient orientation. Position of the micropipette is indicated with a blue dot. Corresponds to data presented in Fig. 6, \textit{pkaC} null cell 1.
Movie 15. Imaging of Raf1(RBD)-GFP in *pkaC* null cells treated with 10 μM LatB upon changes in cAMP gradient orientation. Position of the micropipette is indicated with a blue dot. Corresponds to data presented in Fig. 6, *pkaC* null cell 2.
**Movie 16.** cAR1-GFP expressing *carA* null cells migrating in an exponential gradient of cAMP created by a point source (micropipette). Corresponds to data presented in Fig. 7C.
**Movie 17.** cAR1-GFP expressing *pkaC* null cells exposed to an exponential gradient of cAMP created by a point source (micropipette). Corresponds to data presented in Fig. 7C.