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Published in:
Experientia

DOI:
10.1007/BF01944732

Publication date:
1995

 0014-4754/95/121144-11$1.50 + 0.20

Transduction of the chemotactic cAMP signal across the plasma membrane of *Dictyostelium* cells

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Abstract. Aggregating *Dictyostelium* cells secrete cAMP during cell aggregation. cAMP induces two fast responses, the production of more cAMP (relay) and directed cell locomotion (chemotaxis). Extracellular cAMP binds to G-protein-coupled receptors leading to the activation of second messenger pathways, including the activation of adenylyl cyclase, guanylyl cyclase, phospholipase C and the opening of plasma membrane Ca^{2+} channels. Many genes encoding these sensory transduction proteins have been cloned and null mutants of nearly all components have been characterized in detail. Undoubtedly, activation of adenylyl cyclase is the most complex, involving G-proteins, a soluble protein called CRAC and components of the MAP kinase pathway. Null mutants in this pathway do not aggregate, but can exhibit chemotaxis and develop normally when supplied with exogenous cAMP. The pathways leading to the activation of phospholipase C were identified, but unexpectedly, deletion of the phospholipase C gene has no effect on chemotaxis and development, nor on intracellular Ins(1,4,5)P_3 levels; the metabolism of this second messenger will be discussed in some detail. Activation of guanylyl cyclase is G-protein-dependent and essential for chemotaxis. Analysis of a collection of chemotactic mutants reveals that most mutants are defective in either the production or intracellular detection of cGMP, thereby placing this second messenger at the center of chemotactic signal transduction. Analysis of the cAMP-mediated opening of plasma membrane calcium channels in signal transduction mutants suggests that it has two components, one that depends on G-proteins and intracellular cGMP and one that is G-protein-independent.

Key words. Adenylyl cyclase; guanylyl cyclase; G-proteins; inositol phosphates; mutant analysis; phospholiphase C.

Chemotaxis

Growing cells are chemotactically sensitive towards several identified and unidentified compounds secreted by their prey, the bacteria that live in the soil. These compounds include folic acid and pterin[36], but also other chemoattractants that must be secreted by bacteria, because the dominant mutant KI-10 is chemotactically insensitive towards these identified chemoattractants but still moves chemotactically towards bacteria[26]. Chemotaxis and signal transduction during growth will not be discussed in detail in this review. When the bacteria become scarce, starvation induces a very effective cAMP sensory system. Although growing cells do move towards cAMP, cells starved for five hours are about 100-fold more sensitive, because starved cells contain more surface cAMP receptors and more transducing proteins. Cell aggregation is mediated by the interplay of two transducing systems: a relay mechanism that provides coordinated cAMP-induced cAMP secretion thereby activating more distal cells, and a chemosensory mechanism that induces coordinated locomotion in the direction of the cAMP source. The aggregation center initiates cAMP secretion. Therefore, these two mechanisms lead to the outward propagation of cAMP waves inducing the inward attraction of moving cells.

During the passage of a cAMP wave, a cell experiences complex spatial and temporal cAMP gradients. When the wave approaches the cell, it is subjected to a steep spatial gradient and a strong temporal gradient, because the mean cAMP concentration increases with the time. During passage of the rising flank of the wave, both the spatial and temporal gradient have the same directionality, i.e. the highest concentration points to the aggregation center, and the concentration keeps increasing with time. After the maximal cAMP concentration has been attained, both spatial and temporal gradients flip signs: the highest concentration points away from the aggregation center and the mean concentration now decreases with time. Although workers in the field still have different opinions about whether cells respond to spatial or temporal gradients, the behavior of the cells has been undisputed: cells move towards the aggregation center during the risking flank of the cAMP wave, and paralyse or move in a random fashion during the declining flank of the wave and in between the waves. For chemotaxis at least two components are required: movement and orientation. Bacteria can show chemo-

taxis, because the duration but not the direction of movement is biased by the temporal component of the gradient[30]. Thus bacteria do not measure the spatial gradient, and they must move in order to read the gradient. In contrast, immobile *Dictyostelium* cells are still able to orient, as was shown using electroporated cells in Ca^{2+}-buffered solutions[53]. These cells cannot displace themselves, but can still extend pseudopods towards a capillary filled with cAMP with the same accuracy as control moving cells. This experiment not only indicates the differential requirement of locomotion and orientation for intracellular Ca^{2+} (see review by Newell et al.[35]), but also that the cell body does not have to move to show effective chemotaxis. Other experiments[55] suggest that cells do not show chemotaxis when placed in a steep spatial gradient where the concentration decreases everywhere with time, whereas effective chemotaxis is induced by the same spatial gradient when the mean concentration is held approximately constant with time. On the other hand, Varnum-Finney et al.[68] and Wessels et al.[71] suggest that the behavior of *Dictyostelium* cells in natural waves is primarily determined by the temporal dynamics of the wave. Cells establish orientation only during the first 30 s at the front of the wave and orientation is maintained during the remaining rising flank of the wave.

I think that cells use both spatial and temporal clues to orient in a cAMP wave. In such a model, cells respond strongly to a spatial gradient by extending oriented pseudopodia when the mean chemoattractant concentration is increasing with time, whereas cells respond poorly or not at all to spatial gradients (i.e. cells cringe or extend pseudopodia in nearly random directions) when the mean concentration of cAMP is decreasing with time. If the biochemistry of *Dictyostelium* signal transduction has implicit spatial and temporal components, evolution certainly would have sustained and improved these methods of gradient reading, because it would provide a much better means to aggregate and survive starvation. Detection of temporal clues is imposed by the biochemistry of the cGMP response which shows rapid and exact adaptation to constant stimuli (see below). So second messengers are not formed in cells in a test tube when applied twice with the same or a smaller cAMP stimulus. What would be needed to complete the model is a biochemical mechanism that provides directionality to the cell movement.

The sensitivity of cells in cAMP gradients has been measured in gradients which contain both spatial and temporal components[31]. Calculations of changes in receptor occupancy may give some idea of the cell's problems and abilities to read spatial and temporal gradients. Starved *Dictyostelium* cells contain about 40,000 receptors with a K_d of about 10^{-7} M; the half-time of dissociation of the cAMP-receptor complex is about 1 s, which is therefore taken as the sampling time[59].

Threshold responses are induced within a few minutes by a point source of 10^{-14} mol cAMP applied at a distance of 1 mm from a cell. From these data it can be calculated (see ref. 31) that the mean cAMP concentration around the cell reaches a maximum of 8×10^{-10} M with a maximal spatial gradient of about 4×10^{-12} M/μm and a maximal temporal gradient of about 3×10^{-12} M/s. These data should be compared with the dimensions of a *Dictyostelium* cell of a size of 10 μm moving at a speed of 0.1 μm/s. Combining the threshold concentration of 8×10^{-10} M with the affinity and number of the receptor yields only 317 occupied receptors per cell. The maximal spatial gradient is 4×10^{-12} M/μm, leading to the conclusion that the front half of the cell has only 4 occupied receptors more than the back half of the cell. The increase in the number of occupied receptors due to the maximal temporal gradient of 3×10^{-12} M/s is only 1.2 occupied receptors per second. Finally, the deviation of receptor occupancy due to cell movement (at a speed of 0.1 μm/s) is ± 0.16 occupied receptors per second (the sign depends on whether cells move in the direction of the gradient or in the opposite direction). Clearly, whatever mechanisms *Dictyostelium* cells use to sense and respond to chemotactic signals, at threshold concentrations cells perform at the verge of stochastic and thermal fluctuations.

It has been proposed that cells may measure the spatial gradient by rapid extension of filopodia in random directions and determining which filopodia detect an increasing temporal gradient[11]. One filopodium is relatively small, containing not more than 1% of the cell's volume, and probably not more than 1% of the 40,000 receptors. At the base of the cell the cAMP concentration is 8×10^{-10} M, leading to the mean occupancy of 3.17 receptors per filopodium; extending a filopodium 5 μm away from the cell in the higher cAMP concentration, the receptors may experience 8.2×10^{-10} M cAMP leading to the mean occupancy of 3.25 receptors per filopodium. The dissociation rate of the receptor is about 1 s, which is therefore approximately the sampling time. Obviously stochastic constraints impede either integration of receptor occupancy from several filopodia, or integration over time to discriminate between 3.17 and 3.25 occupied receptors per extended filopodium.

Although these calculations are subject to many uncertainties in the actual distribution of receptors, reaction kinetics, cAMP diffusion constants in the vicinity of the cell surface and what a threshold chemotactic response really means, it seems clear that at cAMP stimuli inducing a detectable chemotactic response only a few hundred of the receptors are occupied with cAMP and less than ten receptors change occupancy either in time or in space within the time needed for the cell to respond. And it is these seemingly sparse values that are so

intriguing in solving the molecular mechanism of chemotaxis.

Receptors

Receptor forms

Extracellular cAMP is detected by surface cAMP receptors. Binding studies reveal different kinetic forms of the receptor during cell aggregation with respect to their affinity and rate of cAMP-receptor dissociation[59]. A large pool of rapidly dissociating A sites converts from a high to a low affinity form during cAMP stimulation (A^H and A^L), whereas a small pool of slowly dissociating receptors displays a reduction of dissociation rate during cAMP stimulation. These different receptor forms have also been detected in membranes where the interconversions from A^H to A^L and from B^S to B^{SS} are induced by guanine nucleotides, suggesting that they originate from the interaction with and activation of G-proteins[60]. Further experiments have provided indications that occupation of A sites is correlated with the activation of adenylyl cyclase, whereas activation of the B sites is proposed to lead to the activation of guanylyl cyclase[57].

Receptor genes

Probably all these receptor forms originate from the same gene product, cAR1[24]. This conclusion is based on the observation that all receptor forms have similar cAMP-binding specificity[65] which is also nearly identical to the specificity of cAR1[19]. Moreover, cAR1 accounts for more than 90% of all cAMP-binding activity during cell aggregation. Although it cannot be excluded that binding forms with very low abundance such as the B sites are encoded by other cAR genes, inactivation of the cAR1 gene leads to the disappearance of both A and B sites[50, 51].

Four genes have been isolated that encode cAMP receptors[18, 29, 43]. These receptors are expressed at different times during *Dictyostelium* development. cAR1 shows the highest expression level and has the highest affinity for cAMP[23]. Inactivation of cAR1 gene expression leads to a defect of cell aggregation. Some residual second messenger responses are still detectable at elevated cAMP concentrations, which under special conditions are sufficient to complete development[15, 47]. Expression of cAR3 partly overlaps with cAR1 expression; this receptor shows a slightly lower affinity for cAMP[19]. Inactivation of the cAR3 receptor does not lead to an obvious aberrant phenotype; however *car1*$^-$/*car3*$^-$ double mutants cannot develop altogether, suggesting that the residual activity in *car1*$^-$ cells is backed up by cAR3[15, 47]. cAR2 and cAR4 are expressed mainly during the multicellular stage and do not play an important role in chemotaxis during cell aggregation[29, 43].

Mutagenesis of cAR1

cAR1 has high affinity for cAMP whereas cAR2 binds this ligand with low affinity[19]. To find the parts of the proteins that are responsible for the difference in these cAMP-binding constants, Kim and Devreotes[22] used a very elegant technique to prepare random chimeras of cAR1 and cAR2 by placing both cDNAs in tandem separated by a small multiple cloning site in a bacterial plasmid. The plasmid is double-digested with multiple restriction enzymes in this multiple cloning site and transformed without ligation to bacteria which are grown under selection. To allow growth of the bacteria, the vector must be religated, which is most cases occurs by recombination between homologous cAR1 and cAR2 sequences, thereby creating a large pool of chimeras. Using a set of chimeras, it was demonstrated that the portion of the protein responsible for the low affinity of cAR2 resides mainly in the second extracellular loop. Interestingly, there are only five amino acid differences between cAR1 and cAR2 in this area[22].

In most G-protein-coupled receptors the third intracellular loop is essential for interaction with G-proteins. Based on this notion, the N-terminal eight amino acids of this loop were randomly mutagenized in *Dictyostelium*[4]. Fifteen cAR1 mutants were obtained that all showed cAMP-binding activity; none of them was a dominant negative receptor mutant. Eleven mutants showed more or less wild-type phenotype, whereas four mutants were virtually inactive; there was no bias to mutation of one of the eight amino acids in these inactive receptors[4].

Upon prolonged stimulation of *Dictyostelium* cells with cAMP, signal transduction to adenylyl and guanylyl cyclase ceases due to adaptation. These two cyclases probably adapt by independent processes, because they show very different kinetics and temperature sensitivities[58]. Adaptation of adenylyl cyclase correlates very well with the cAMP-induced phosphorylation of the C-terminal tail of cAR1[69]. This part of the cAR1 protein contains 18 serines grouped in four clusters that are potentially phosphorylated in the basal or the cAMP-stimulated state. In an extended study Harald et al.[16] mutated all serines in different clusters, and demonstrated that the cAMP stimulus induced the addition of approximately two phosphates to cluster 1 and one phosphate to cluster 2. When a mutant cAR1 lacking all phosphorylation sites was expressed in cells lacking the endogenous cAR1 gene, cells showed essentially normal chemotaxis, pulsatile cell aggregation and development, suggesting that phosphorylation of the receptor is not essential for its normal function (Harald, Valkema, Van Haastert and Devreotes, unpublished observations). In these cells the activation of adenylyl and guanylyl cyclase is transient with similar kinetics as in cells expressing wild-type receptors. Possibly the kinetics of recovery

of adenylyl cyclase stimulation may be different with cAR1 receptors that cannot be phosphorylated. Prolonged stimulation of *Dictyostelium* cells results in alteration of the receptor leading to a loss of cAMP-binding (LLB) and internalization and degradation of the receptor (down-regulation). LLB still occurs in the absence of second messenger production, but no longer occurs in the phosphorylation-deficient cAR1 mutants[63]. For down-regulation of the receptor these phosphorylation sites are essential as well as the activation of several second messenger pathways, including the activation of adenylyl cyclase[63]. In conclusion, under normal conditions cAR1 mediates chemotaxis, but its phosphorylation regime appears to be of minor importance.

G-proteins

Gα-subunits

Early studies on the binding of cAMP to membranes provided strong evidence for the presence of heterotrimeric G-proteins by showing that guanine nucleotides altered the affinity of the cAMP-receptor, and that cAMP increased GTPase activity and GTP-binding to membranes (see ref. 48). *Fgd*A mutants previously characterized by Coukell et al.[7] were soon found to be defective in these properties[20]. Upon the genetic identification of two Gα-subunits[39], it appeared that *fgd*A mutants showed mutations in the Gα2 gene[25]. A null mutant of Gα2 does not aggregate, shows no chemotaxis, and does not respond to extracellular cAMP. Presently eight Gα-subunits have been identified. The phenotype of *gα2*-null mutants is unprecedented; deletion of other Gα-subunits give only subtle phenotypes[72]. For chemotaxis it is relevant to mention that deletion of Gα4 impairs chemotaxis towards folic acid, but not towards cAMP[13]. Deletion of other Gα-subunits may affect later development, but seems to have little effect on chemotaxis, and is therefore not further discussed here.

Upon cAMP stimulation, Gα2 becomes transiently phosphorylated on a serine residue[12]. Since Gα2 plays such an essential role in signal transduction, the phosphorylated serine was identified as ser-113, and mutated. Expression of this mutated Gα2-subunit in *gα2*-null cells restored all defects, including activation of adenylyl cyclase, chemotaxis and development[5].

Gβ-subunit

Dictyostelium cells contain a single Gβ-subunit, which shows strong homology with Gβ-subunit from other organisms[28]. The paradigm for G-protein functioning is that the activated receptor induces the exchange of GDP to GTP in the $\alpha\beta\gamma$ complex leading to the dissociation of the complex in α-GTP and $\beta\gamma$, both of which may transduce the signal to the effector. Furthermore, both the α- and $\beta\gamma$-complex are essential to activate either subunit; thus in a cell it is expected that an α-subunit cannot be activated without the $\beta\gamma$-complex, and vice versa the $\beta\gamma$-complex cannot be activated without an α-subunit. Since *Dictyostelium* cells have only one β-subunit, it is expected that its deletion will block signal transduction via all heterotrimeric G-proteins. Indeed, *gβ*-null cells do not show chemotaxis to any chemoattractant and lack the activation of adenylyl and guanylyl cyclase by both cAMP and folic acid (Wu et al., personal commun.). Cells do not aggregate, and special treatment with cAMP pulses that rescues some mutants has no effect in these cells.

Adenylyl cyclase and intracellular cAMP

Adenylyl cyclase genes and proteins

Two genes encoding proteins with adenylyl cyclase activity have been identified[38]. One gene (ACA) predicts a protein that has the topology of mammalian adenylyl cyclases: two domains of six transmembrane-spanning segments separated by a putative catalytic domain and an additional C-terminal catalytic domain. The deduced amino acid sequence of the second gene (ACG) predicts a protein which spans the membrane only once and has one putative catalytic domain. Expression of ACA is maximal during cell aggregation, whereas expression of ACG is only detectable in spores and during germination.

Inactivation of the ACA gene leads to the impairment of cell aggregation[38]. Cells still show chemotaxis towards a capillary filled with cAMP, and addition of cAMP to these cells in a regime that wild-type cells would experience during normal cell aggregation leads to the expression of many cAMP-induced genes as well as to the formation of small fruiting bodies (see ref 41). These results suggest that the main function of ACA is to generate cAMP as a first messenger. However, the experiments do not preclude an essential function of cAMP in processes that are not essential for cell aggregation and development. For instance, in wild-type cells prolonged stimulation with cAMP leads to the down-regulation of cAR1; this process does not occur in *aca*$^-$ cells, and is rescued by a cell-permeable cAMP analog[56]. Inactivation of the ACG gene does not lead to a noticeable phenotype during cell aggregation and spore formation or spore germination. Although ACA and ACG are regulated by very different mechanisms (see below), expression of ACG in *aca*$^-$ cells rescues to a large extent the aggregation-minus phenotype of *aca*$^-$ cells[38].

Regulation of adenylyl cyclase (scheme 1)

Extracellular cAMP stimulates ACA about 20-fold; half-maximal stimulation occurs at about 5 nM cAMP. Stimulation via the receptor is transient and prestimulus activities are recovered within about 5 min. Interestingly, cAR-deletion mutants have revealed that adapta-

Scheme 1. Regulation of adenylyl cyclase.

Component	Activation	Inhibition
Receptor	cAR1 or cAR3	cAR1
G-protein	Gα2 and Gβ in vivo Gβ in vitro any Gα in vitro	pertussis toxin substrate
Regulator	CRAC MAP kinase	
Questions	direct activator is βγ translocation CRAC to membrane regulation MAP kinase by receptor why no activation by folic acid role phosphorylation cAR1 site of inhibition by caffeine	

tion of adenylyl cyclase absolutely requires cAR1, whereas activation of adenylyl cyclase can be mediated by both cAR1 and cAR3[40]. In membranes, GTPγS stimulates ACA to a similar extent as cAMP in intact cells[52,61]. Stimulation by cAMP or GTPγS requires a soluble protein called CRAC. In cells that have adapted to the cAMP stimulus, GTPγS no longer stimulates adenylyl cyclase[52,61].

Mutant studies indicate that in vivo the G-protein Gα2 is essential for activation of ACA. However, in vitro GTPγS-mediated activation of ACA is essentially normal in *gα2*$^-$ cells (20). In cells with a deletion of the Gβ-subunit both the cAMP-mediated activation in vivo as well as the GTPγS-mediated activation in vitro are lost (Wu et al., personal commun.). The most straightforward explanation is that ACA is activated by the βγ-complex; in vivo this complex is liberated specifically from the Gα2βγ-complex by the cAMP receptor, whereas in vitro it can be liberated by GTPγS from any Gαβγ-complex. The role of the CRAC protein is emerging. It has a pleckstrin homology domain suggesting that it may transduce signals via protein-protein interactions[14,27]. Interestingly, the protein binds to the membrane upon stimulation of wild-type cells with cAMP; this translocation still occurs in mutants lacking ACA, but not in mutants lacking the Gβ-subunit (Devreotes et al., unpublished observations). The simplest explanation is that CRAC forms a sensory bridge between the receptor-activated Gβγ-complex and the adenylyl cyclase ACA.

Recently it was observed that mutants lacking a MAP kinase (ERK2) are specifically defective in the activation of ACA[46]. Guanylyl cyclase and chemotaxis are relatively unaltered in these mutants. Cells contain normal levels of G-protein subunits, CRAC, and ACA, but by mechanisms not yet understood coupling between receptor and ACA does not occur in cells lacking ERK2.

Compared to ACA, much less is known about the regulation of ACG. Since ACG is only expressed in spores, which are difficult to investigate biochemically, most information has been obtained from cells that express ACG in *aca*$^-$ cells during growth (Valkema et al., unpublished results). In membranes the activity of ACG is not enhanced or inhibited by guanine nucleotides, suggesting that this enzyme is not directly regulated by G-protein subunits. In cells cAMP leads to a significant but modest threefold activation of ACG activity; stimulation of ACG requires tenfold higher cAMP concentrations than the activation of ACA. It is inhibited by the receptor antagonist (Rp)-cAMPS, and shows the specificity of surface receptors and not of cAMP-dependent protein kinase. Prolonged stimulation with cAMP leads to transient activation of ACG, suggesting that activation of this cyclase is also subject to adaptation. Interestingly, receptor-mediated activation of ACG does not require CRAC, because it still occurs in *crac*$^-$ cells expression ACG. Presently it is not known whether G-protein subunits are required for receptor-mediated activation of ACG. Clearly the mechanisms of activation of ACA and ACG are very different. However, since both ACA and ACG are stimulated by cAMP in a transient way, both enzymes may relay the cAMP stimulus and therefore mediate cell aggregation.

Intracellular cAMP and cAMP secretion

The major part of the produced cAMP during cell aggregation is secreted in the medium where it diffuses and activates neighboring cells. This mechanism of cAMP secretion is still largely unclear. During cell aggregation intracellular cAMP probably does not play a very pronounced role. This may be very different for multicellular differentiation as is discussed in the chapter by Reymond et al.[41].

Phospholipase C and inositol phosphate metabolism

Phospholipase C gene and protein

One gene encoding phosphatidylinositol-specific phospholipase C (Dd-PLC) has been identified[8]. Mammalian cells possess three classes of PLC isozymes. PLCβ is regulated by G-proteins; PLCγ contains SH2 and SH3 domains and is activated by tyrosine kinase receptors. Regulation of enzymes of the PLCδ class is largely unknown; it has been suggested that intracellular Ca^{2+} is largely responsible for altering enzyme activity. The deduced primary sequence of Dd-PLC places it in the class of PLCδ isozymes. Cells with an inactivated Dd-PLCδ gene have lost all detectable PLC activity[9]. Extended searches by PCR or low stringency hybridization have not given any indication of another PLC gene.

The deduced primary sequence of Dd-PLCδ reveals a N-terminal segment of about 300 amino acids followed by the conserved A and B domains of about 150 amino acids each. The C-terminal domain shows the strongest homology with enzymes of the PLCδ class. The func-

tion of the N-terminal 300 amino acids is unknown; all PLC isozymes have a N-terminal segment before the A domain, all about 300 amino acids in length but with very different primary sequences. In rat PLCδ 135 amino acids can be deleted from the N-terminus without loss of activity[10], but this is probably not the case in *Dictyostelium* where deletion of 44 amino acids leads to inactivation of the enzyme (Drayer et al., unpublished observations). The conserved A and B segments are thought to form collectively the catalytic domain. In *Dictyostelium* PLC these segments are interspersed by a segment containing a sequence that is predicted to be a strong EF-hand Ca^{2+}-binding domain. Mutations of those amino acids that would chelate the Ca^{2+} ion lead to reduced PLC activity. Deletion of small parts of the C-terminal segment of Dd-PLC destroys enzyme activity; a similar observation was made for rat PLCδ[10].

Regulation of phospholipase C (scheme 2)

Dd-PLCδ is strictly Ca^{2+}-dependent with half-maximal stimulation at about 0.5 µM Ca^{2+} (ref 1, and Drayer et al., unpublished observations). In cells cAMP stimulates PLC enzyme activity about twofold; this activation does not require cAR1, and probably also not cAR3[2]. Some cAMP analogues that were previously shown to be chemotactic antagonists inhibit PLC activity; this inhibition requires cAR1 expression. In a cell free extract GTPγS stimulates PLC activity. Stimulation of PLC by cAMP in cells or by GTPγS in lysates is lost in mutants with a deletion of the Gα2 gene, suggesting that activation of PLC is mediated by an unknown cAMP receptor and G2. Inhibition of PLC by cAMP antagonists is lost in cells with a deletion of the Gα1 gene, suggesting that PLC inhibition is mediated by cAR1 and G1[2].

Although the regulation of PLC is known in detail, as is its enzymatic function in the production of Ins(1,4,5)P_3 and DAG, the real function of PLC is still unclear because deletion of the Dd-PLC gene causes no phenotype[9]. Cells grow and show normal locomotion, chemotaxis and multicellular development. Experiments investigating a stronger dependency on extra- or intracellular Ca^{2+}, such as sensitivity to EGTA or caffeine, do not show a difference between *plc*$^-$ and control cells (Drayer et al., unpublished observations). Even more unexpectedly, cells without detectable PLC activity have essentially normal levels of Ins(1,4,5)P_3. Clearly, cells have other ways to make Ins(1,4,5)P_3. Does this redundancy mean that Ins(1,4,5)P_3 (and thus PLC) is very important, or is Ins(1,4,5)P_3 one of the many inositolphosphate metabolites that has no role in signal transduction?

Scheme 2. Regulation of phospholipase C.

Component	Activation	Inhibition
Receptor	cARx	cAR1
G-protein	Gα2	Gα1
Regulator	Ca^{2+} required for PLC activity	
Questions	role Gβ	
	localization of PLC	

Intracellular IP3 and inositol phosphate metabolism

The metabolism of inositol phosphates is very complex and probably only partly elucidated. The scheme combines experiments on metabolic phosphorylation and dephosphorylation routes in vitro with experiments on the metabolism of [^{3}H]inositol and [^{32}P]orthophosphate in vivo. Four metabolic subroutes can be recognized. The first part is found in all organisms: inositol is incorporated into phospholipids that are hydrolysed by PLC producing Ins(1,4,5)P_3 which is hydrolysed to inositol; the only difference from other organisms is an extra Ins(1,4,5)P_3 1-phosphatase in *Dictyostelium*[64]. The second part is the sequential phosphorylation of inositol to $InsP_6$; the $InsP_3$ isomer has been identified as Ins(3,4,6)P_3, which does not release Ca^{2+} [49]. The third subroute is the sequential phosphorylation of Ins(1,4,5)P_3 in a nucleus-associated fraction (Van der Kaay et al., unpublished observations), whereas the fourth subroute is the dephosphorylation of Ins(1,3,4,5,6)P_5 to Ins(1,4,5)P_3 (Van Dijken et al., unpublished observations). This last route probably mediates Ins(1,4,5)P_3 formation in cells lacking PLC activity, because *plc*$^-$ cells have significantly reduced levels of Ins(1,3,4,5,6)P_5 [9]. Interestingly, this enzyme is Ca^{2+}-dependent with half-maximal activity at 0.9 µM Ca^{2+}. Inactivation of this enzyme should reveal whether Ins(1,4,5)P_3 has an important function in *Dictyostelium*.

Guanylyl cyclase

Regulation of guanylyl cyclase (scheme 3)

The gene encoding guanylyl cyclase has not yet been cloned. A chemotactic mutant (see below) has been identified that shows strongly reduced guanylyl cyclase activity; together with other mutants in cGMP metabolism, this provides a clue to the function and regulation of cGMP as second messenger. Stimulation

Scheme 3. Regulation of guanylyl cyclase.

Component	Activation	Inhibition
Receptor	cAR1 (or cAR3)	blockade of G-protein activation
G-protein	Gα2 and Gβ in vivo?	
Regulator	soluble protein	Ca^{2+} on guanylyl cyclase
	mutation in KI-10	kinase on guanylyl cyclase
		cGMP-mediated stimulation of cGMP-phosphodiesterase
Questions	mechanism of adaptation	

of cells with extracellular cAMP or folic acid leads to a transient tenfold increase of cGMP levels with a maximum at 10 s and recovery of basal levels within 30 s. In contrast to the activation of adenylyl cyclase, which is found to be inhibited by many mutations and many drugs, activation of guanylyl cyclase by cAMP in vivo is very robust. This may indicate that many components are involved in the activation of adenylyl cyclase as is being uncovered by the essential role of CRAC, Gβ and MAP kinase, whereas only a few components participate in guanylyl cyclase regulation. The cGMP response is strongly reduced in *car1*$^-$ cells and absent in *car1*$^-$/*car3*$^-$ double mutants, indicating that in wild-type cells cAMP-mediated activation of guanylyl cyclase is mediated predominantly by cAR1[15,47]. In cells with a deleted Gβ gene, activation of guanylyl cyclase by both cAMP and folic acid is lost (Wu et al., personal commun.). Inactivation of Gα2 impairs stimulation of guanylyl cyclase by cAMP but not by folic acid[20], whereas inactivation of Gα4 leads to a loss of folic acid-induced cGMP response without affecting the cAMP-mediated response[13]. These experiments reveal that G2 and G4 mediate the cAMP- and folic acid-mediated activation of guanylyl cyclase. It is possible that the Gα-subunits activate guanylyl cyclase. Alternatively, guanylyl cyclase may be activated by the common Gβ that is specifically liberated from G2 by cAMP and from G4 by folic acid. *Dictyostelium* guanylyl cyclase is strongly inhibited by Ca^{2+} ions; inhibition is cooperative with a Hill coefficient of 2.3 and half-maximal inhibition at about 50 nM Ca^{2+} [17,66]. Experiments with permeabilized cells and computer simulations suggest that Ca^{2+}-mediated inhibition of guanylyl cyclase plays an important role in regulation of the maximal cGMP response[67]. The regulation of *Dictyostelium* guanylyl cyclase activity by Ca^{2+} is similar to the regulation of this enzyme in the vertebrate eye[23].

In cell lysates Mg^{2+}-dependent guanylyl cyclase activity is membrane bound, but requires a protein from the cytosol to show full activity[45]. Guanylyl cyclase activity in cell lysates is strongly reduced in conditions where protein kinase activity is high[44]. The role of these components for the receptor-mediated cGMP response in vivo is presently unclear and awaits identification of mutants defective in these potentially regulatory components.

Intracellular cGMP

Only a small amount of produced cGMP is secreted. The major part is degraded by a cGMP-specific phosphodiesterase that is encoded by the *stm*F gene[6,42,62]. Mutation of this gene results in a strongly enhanced cGMP accumulation that lasts for several minutes. Intracellular cGMP is detected by a cGMP-binding protein[37] that may have cGMP-dependent protein kinase activity[70]. The role of this protein as transducer of the cGMP accumulation is not known, because mutants have not yet been identified. The function of cGMP was investigated using the *stm*F mutant with a defective cGMP phosphodiesterase. In this mutant cAMP-mediated association of conventional myosin heavy chain with the Triton-insoluble cytoskeleton is greatly prolonged, whereas the association of actin with the same cytoskeleton is as in control cells (see ref. 35 for a further discussion of the role of cGMP on the distribution and phosphorylation of myosin heavy and light chains). These conclusions are confirmed using KI mutants defective in the activation of guanylyl cyclase (KI-10) or that lack guanylyl cyclase activity (KI-8); these mutants do not show the association and phosphorylation of myosin, whereas the association of actin with the cytoskeleton is essentially normal (see ref. 35, and Kuwayama et al., unpublished observations). Experiments with mutant *stm*F have also revealed that Ca^{2+} uptake is mediated by intracellular cGMP, since uptake is strongly prolonged in this mutant[32]. However, this conclusion is not directly supported by experiments with other mutants showing normal Ca^{2+} uptake without cGMP accumulation (see below).

Calcium uptake

Regulation of calcium uptake (scheme 4)

Stimulation of cells with cAMP or folic acid leads to the uptake of Ca^{2+} ions[3]. This uptake has been investigated in many different mutants with apparently contradictory conclusions. In mutants lacking cAR1, strongly reduced Ca^{2+} uptake is recorded, whereas uptake is very high in vegetative cells overexpressing any of the cAR receptors, suggesting that each cAR can mediate Ca^{2+} uptake[33]. In *stm*F cells with enhanced cGMP response due to a defect in the cGMP phosphodiesterase, prolonged uptake of Ca^{2+} has been measured using Ca^{2+} electrodes[32]. The conclusion that cGMP mediates Ca^{2+} uptake is not confirmed using other mutants: transformants lacking Gα2 or Gβ show a normal cAMP-mediated uptake of Ca^{2+} provided that they express sufficient cAMP receptors; however, these cells have no detectable cGMP response[34]. Furthermore

Scheme 4. Regulation of Ca^{2+}uptake.

Component	Activation	Inhibition
Receptor	any cAR	?
G-protein	no G-protein required	
Regulator	unknown	
Questions	part of Ca^{2+} uptake may be mediated by intracellular cGMP, and is thus dependent on cAMP-mediated cGMP production (see scheme 2).	

cAMP-mediated Ca^{2+} uptake is still present in mutant KI-8 which has no guanylyl cyclase activity (Kuwayama et al., unpublished observations). Thus cAMP may stimulate multiple Ca^{2+} uptake mechanisms, one G-protein and cGMP-dependent, and another independent of G-proteins. Alternatively the different conclusions may be related to the different methods used to measure Ca^{2+} uptake. Experiments with *stm*F were performed with extracellular Ca^{2+} electrodes that measure the balance between uptake and secretion, whereas all other experiments use $^{45}Ca^{2+}$ uptake assays, that probably only measure the uptake component. Thus it is possible that intracellular cGMP does not stimulate Ca^{2+} uptake, but inhibits Ca^{2+} secretion.

Chemotactic mutants

Dictyostelium cells are sensitive to many different chemoattractants, including cAMP during cell aggregation and folic acid and pterin during growth. These chemoattractants are detected by different surface receptors, which may be coupled to different G-proteins[13,20,21]. It is expected that somewhere in the signal transduction cascade to directed cell locomotion the signals from different receptors merge into one pathway. Indeed, it has been suggested that the same pool of guanylyl cyclase is activated by both cAMP and folic acid, because cells that are simultaneously sensitive to both chemoattractants do not show additivity of cGMP stimulation[54]. Based on these observations Kuwayama et al.[26] set up a screen for hunting mutants that are defective in the common transduction pathway shared by cAMP and folic acid. Cells were mutagenized and about 10,000 survivors were inspected for the absence of cell aggregation, since it is generally accepted that chemotaxis to cAMP is essential for cell aggregation. The 243 aggregation-defective mutants were individually assayed for chemotaxis to cAMP and folic acid using the very efficient semi-quantitative agar cutting assay for chemotaxis. It appeared that 51 mutants did not show chemotaxis to cAMP, 21 mutants did not show chemotaxis to folic acid, and 10 mutants were identified that did not respond to either chemoattractant. These KI mutants were investigated genetically and biochemically in detail. One mutant (KI-3) appears to be the only false positive, because it shows only slightly reduced chemotaxis; all other mutants are severely defective, indicating the power of the selection method. Genetic analysis revealed that all mutants are recessive except KI-10. Interestingly this dominant mutant does not show chemotaxis to cAMP, folic acid and pterin, but responds normal to bacteria, yeast extract and human urine; this must mean that these broad spectrum sources contain still more unidentified chemoattractants. Further genetic analysis demonstrates that all mutants can complement each other. Thus no complementation group was found containing more than one allele[26]. This is unexpected, since another mutant hunt to *fgd* mutants starting with a similar number of aggregation minus mutants yielded ten *fgd* mutants which were placed in only five complementation groups[7]. Possibly this large difference between KI and *fgd* mutants is just due to the statistics of small numbers, or it may be that the defective gene products of the KI mutants act in very subtle ways; for instance, two alleles of a gene encoding a protein that functions as a dimer may complement each other. Cloning the mutated genes should establish whether each complementation group belongs to a different gene. This match is important, because it provides information on the number of genes that are involved in chemotaxis. If all complementation groups belong to different genes, the mutant hunt is far from over and many interesting mutants are still to be found.

Biochemical analysis of the KI mutants revealed that most mutants show a normal activation of adenylyl cyclase and phospholipase C[26]. This confirms the reverse experiments with mutants deleted in adenylyl cyclase or phospholipase C which show normal chemotaxis. cAMP-mediated Ca^{2+} uptake is significant in all but one mutant. In mutant KI-1 cAMP does not lead to the enhanced uptake of $^{45}Ca^{2+}$ ions, but instead to inhibition of $^{45}Ca^{2+}$ uptake; this mutant shows a nearly normal cGMP response (ref. 26, and Kuwayama et al., unpublished observations). In all other mutants, we observed an altered production or detection of cGMP. Mutant KI-8 has a strongly reduced guanylyl cyclase activity. Mutant KI-10 has normal basal guanylyl cyclase activity which, however, cannot be stimulated by cAMP or folic acid. Mutants KI-2 and KI-7 show a delayed cGMP response with a maximum at 20 s after stimulation versus 10 s in wild-type cells; this difference is not due to altered cGMP phosphodiesterase activity[26]. The delayed cGMP response may seem only a small difference, but we have never seen this before in dozens of experiments with mutants or drugs which may affect the maximum of the response but not the timing of the maximum. Mutants KI-4, 5, 6, and 9 do show a cAMP-mediated cGMP response, but only at elevated stimulus concentrations[26]. Since vegetative wild-type cells respond to cAMP with only a very small cGMP response but do show significant chemotaxis to cAMP, the small cGMP response of starved mutant cells cannot be the only explanation for the complete absence of chemotaxis towards cAMP.

Dictyostelium cells contain a cGMP-binding protein that may have cGMP-dependent protein kinase activity[37,40]. Binding of cGMP shows two kinetic forms, fast association/dissociation with low affinity (F-form) and slow association/dissociation with high affinity (S-form). In wild-type cells oligonucleotides promote the

S-form of the cGMP binding protein[37]. The cGMP-binding protein of mutants KI-4 and 5 is already in the S-form. In contrast, the cGMP-binding proteins of mutants KI-2, 6 and 7 is more in the F-form (Kuwayama et al., unpublished observations). The observation that cGMP-binding activity is altered in several chemotactic mutants establishes the central role of this second messenger in chemotaxis. The strongly diminished activation of guanylyl cyclase in mutants KI-4 and 5 with an altered cGMP-binding activity is intriguing, because it suggests that a component functioning downstream of cGMP also plays an important role upstream of cGMP at the level of guanylyl cyclase regulation.

Conclusions

In this review our current information about the regulation of the four second messenger systems adenylyl cyclase, phospholipase C, guanylyl cyclase and Ca^{2+} uptake, is summarized. There could be several other second messenger systems in *Dictyostelium* that either have not been investigated (such as uptake of protons and potassium ions) or that are presently unknown. Nevertheless, the mechanisms activating these second messengers are emerging. Activation of all messenger enzymes depends on surface receptors. Secondly, most transduction pathways possess both stimulatory and inhibitory branches. Whereas the inhibitory branches all depend on cAR1, the unexpected finding is that stimulation of adenylyl cyclase and phospholipase C may be mediated by other cARs. Ca^{2+} uptake is the only second messenger response that does not absolutely depend on G-proteins. This is shared with other cAMP-induced effects, such as phosphorylation of cAR1 and loss of ligand binding that can also occur in the absence of functional G-proteins. Activation of adenylyl cyclase, guanylyl cyclase and phospholipase C all depend on G-proteins. In this respect G2 is most important, but it is not clear whether in all cases the $G\alpha 2$-subunit mediates the stimulatory effect, or whether the $G\beta\gamma$-complex that is specifically released from G2 by the surface cAMP receptor is also involved; point mutations in the proposed effector domains of $G\alpha 2$ and $G\beta$ should establish this.

From the different schemes of the regulation of the four second messenger pathways it is not easy to construct one scheme starting from the receptor and ending at the different effector enzymes. This leads to the question of whether concepts of a unified signal transduction network must be used to understand signal transduction. In other words, does binding of cAMP to a specific cAR1 receptor molecule lead to the activation of all second messenger enzymes, or are the components grouped in functional units based on specific second messenger enzymes? Adenylyl cyclase may be associated in a patch of the membrane with G2, cAR1 receptor kinases and other molecules; binding of cAMP to that specific cAR1 molecule leads only to the activation of the adenylyl cyclase molecule in that patch. In other patches cAR1 may be associated with guanylyl cyclase and its regulatory proteins, and activation of that cAR1 protein only stimulates guanylyl cyclase in the patch and no other second messenger enzymes. Such models could explain how some components such as the stimulatory G2 can be shared by different second messenger pathways, whereas the receptors that stimulate G2 may be different for adenylyl cyclase and phospholipase C. In the long run when we understand more about the regulation of guanylyl cyclase, we may also understand how spatial information on the distribution of cAMP outside the cell is transduced and retained inside the cell. Finally these sensory transduction domains could easily explain the heterogeneity of the kinetics of cAMP-binding to A and B sites on cells, even though these sites are encoded by the same gene: cAR1 in patches transducing to adenylyl cyclase may have A-site kinetics, whereas cAR1 in patches transducing to guanylyl cyclase may have B site kinetics.

Mutant analysis clearly demonstrates that the second messenger cGMP and probably Ca^{2+} ions play a pronounced role in chemotaxis. Intracellular cAMP may not be important at all, whereas the function of PLC-derived second messengers ($Ins[1,4,5]P_3$, DAG and Ca^{2+}) are not clear, because these second messengers appear not to be formed exclusively from PLC. Unfortunately, the genes encoding proteins producing, degrading or detecting cGMP have not been cloned. Identification of these genes either by creating new REMI mutants or by complementation of the existing chemotactic mutants should allow us to unravel the early steps in the transduction of chemotactic signals from receptor to second messengers. This should then be connected with the wealth of information on the regulation of the locomotion apparatus that is rapidly emerging. Indeed, *Dictyostelium* may be the first eukaryotic system where chemotaxis is understood at a molecular level.

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