The role of cGMP in photosensory and thermosensory transduction in *Dictyostelium discoideum*

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Weak and strong light/heat stimuli induced changes in cyclic guanosine monophosphate (cGMP) levels in vegetative and aggregation competent amoebae and in slug cells of the *Dictyostelium discoideum* strain X22. Mutant strains derived from X22 with mutations in the phototaxis loci *phoA-phoK* fell into four phenotypic classes with respect to cGMP responses to weak and strong light/heat stimuli. These results suggest an intermediary role for cGMP in photosensory and thermosensory processing in slugs and amoebae. The streamer F mutant NP368 which has previously been shown to exhibit a prolonged cGMP response to cAMP, showed a wild-type cGMP response to light and heat. All phototaxis mutant strains with altered cGMP responses to light and heat were unaltered in their cGMP response to cAMP. These results suggest that cAMP and light/heat regulate cGMP via independent pathways. The thermotaxis mutant HPF228 showed altered cGMP responses to heat but not to light stimuli. This suggests that the mutation in HPF228 affects thermosensory transduction before convergence with the phototaxis pathway and the subsequent cGMP response.

Keywords: *Dictyostelium discoideum*, cyclic GMP, cyclic AMP, light and heat stimuli

INTRODUCTION

The lower eukaryote *Dictyostelium discoideum* serves as an exemplary model for the study of cell signalling because of its remarkable life cycle. During the vegetative stage, amoebae are attracted to folic acid and pterin (Pan *et al.*, 1975) which are secreted by their food source, bacteria. When this food source is exhausted, amoebae aggregate to form a multicellular organism (slug) which subsequently forms into a fruiting body, consisting of a droplet of spores supported by a stalk and basal disc (Loomis, 1982).

During the aggregation phase amoebae acquire the ability both to move toward a source of cAMP and to emit cAMP signals (Konijn *et al.*, 1967). The binding of cAMP to cell-surface cAMP receptors elicits various intracellular responses. These include a rapid but transient activation of adenylate and guanylate cyclase (Janssens & van Haastert, 1987; Newell *et al.*, 1988) and transient elevations of inositol triphosphate (IP₃) (Europe-Finner & Newell, 1987; van Haastert *et al.*, 1989) and cyclic GMP

(Wurster *et al.*, 1977; Mato *et al.*, 1977) as well as an uptake of extracellular Ca²⁺ (Wick *et al.*, 1978; Bumann *et al.*, 1986). The cGMP response to cAMP reaches its peak about 10 s after stimulation, returning to basal levels after about 30 s (Mato *et al.*, 1977; Wurster *et al.*, 1977). The decrease in cGMP levels is mainly due to the activity of a specific cGMP phosphodiesterase (Dicou & Brachet, 1980).

The chemotactic signal transduction pathway is thought by some to lead via the G-protein Gα₂, IP₃ liberation, Ca²⁺ release from internal stores and guanylate cyclase activation to cGMP stimulation of Ca²⁺ uptake (Menz *et al.*, 1991). This is based partly on altered cGMP and Ca²⁺ uptake responses by *fgdA* mutants, defective in Gα₂ (Kesbeke *et al.*, 1988; Kumagai *et al.*, 1989), and *stmF* mutants that exhibit low cGMP phosphodiesterase activities (Ross & Newell, 1981; van Haastert *et al.*, 1982). Thus cGMP may act as a second messenger in *D. discoideum* amoebae and may regulate Ca²⁺ uptake by binding to a cation channel as it does in visual transduction in the vertebrate retina (Fesenko *et al.*, 1985). In fact recent evidence has shown cGMP to activate K⁺ channels in the *D. discoideum* membrane (van Duijn & Wang, 1990), resulting in potassium efflux (Aeckerle *et al.*, 1985) and

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Table 1. Genotype of haploid strains of *D. discoideum* used

Strain	Parent	Linkage group*							Reference
		I	II	III	IV	V	VI	VII	
X22	DP4	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>						Williams & Newell, 1976
HPF1	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>			<i>phoK2300</i>			Darcy <i>et al.</i> (1993)
HPF2	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i> <i>phoI2301</i>						P. K. Darcy and others, unpublished
HPF3	HU1628	<i>cyc.A1</i>	<i>acr.A1823</i>	<i>bsg.A5</i>	<i>whiC351</i> <i>phoD2317</i>		<i>man.A2</i>	<i>tsgK21</i> <i>con.A351</i>	Welker & Williams, 1985
HPF5	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i> <i>pho-2303</i>						P. K. Darcy and others, unpublished
HPF6	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>		<i>phoJ2304</i> <i>mtpD2304</i> <i>mig.A2300</i>				P. K. Darcy and others, unpublished
HPF7	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>	<i>phoE2305</i> <i>stp.A2300</i>					P. K. Darcy and others, unpublished
HPF8	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>	<i>mtp.A2300</i>			<i>phoF2306</i> <i>mtpB2301</i>		P. K. Darcy and others, unpublished
HPF9	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>	<i>mtp-2302</i>			<i>phoC2307</i>		P. K. Darcy and others, unpublished
HPF11	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i> <i>pho-2314</i>	<i>phoE2308</i> <i>stp.A2301</i>					P. K. Darcy and others, unpublished
HPF12	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>			<i>phoG2309</i> <i>stpB2302</i>			Darcy <i>et al.</i> (1993)
HPF13	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i> <i>the.A2300</i> <i>migB2302</i>						P. K. Darcy and others, unpublished

Table 1. (cont.)

Strain	Parent	Linkage group*							Reference
		I	II	III	IV	V	VI	VII	
HPF14	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>			<i>phoK2311</i>			Darcy <i>et al.</i> (1993)
HPF16	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>		<i>phoJ2313</i> <i>mtpD2305</i> <i>mig.A2301</i>				P. K. Darcy and others, unpublished
HPF17	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i> <i>pho-2314</i>						P. K. Darcy and others, unpublished
HPF228	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>				<i>theB2301</i> <i>migC2303</i>		P. K. Darcy and others, unpublished
HPF229	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i> <i>pho-2319</i>						P. K. Darcy and others, unpublished
HPF230	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i> <i>pho-2318</i>						P. K. Darcy and others, unpublished
HU407	DU584†	<i>cyc.A1</i>		<i>bsg.A5</i>	<i>bwn.A1</i> <i>pho.A2315</i>		<i>man.A2</i>	<i>tsgK21</i> <i>cou.A351</i> <i>phoB2316</i>	Welker & Williams, 1980
HU409	X22	<i>spr.A1</i> <i>tsgE13</i>	<i>whi.A1</i> <i>acr.A1</i> <i>tsgD12</i> <i>cat.A</i>	<i>phoH355</i>					Fisher & Williams, 1982
XP55	DP746†	<i>cyc.A5</i>		<i>bsg.A5</i>					Ratner & Newell, 1978
NP368	XP55	<i>cyc.A5</i>		<i>bsg.A5</i>				<i>stmF406</i>	Ross & Newell, 1981

* *cyc*, resistance to cycloheximide; *spr*, round spores; *whi*, white (non-yellow) spores; *tsg*, temperature sensitive for growth at 27 °C; *acr*, resistance to acriflavin; *cat*, catalase deficient; *bsg*, unable to grow on a *Bacillus subtilis* lawn; *bwn*, production of brown pigment; *man*, α -mannosidase deficient; *pho*, impaired slug phototaxis; *cou*, coumarin sensitivity; *mtp*, multiple tip formation; *stp*, short or absent stalks; *mig*, short slug migration.

† Parental haploids of the following diploids are: DU584 (XP55 and NP187); DP746 (X22 and NP194).

membrane hyperpolarization (van Duijn & Wang, 1990). Despite the attractiveness of this scenario, aspects of it remain controversial as Milne & Coukell (1991) failed to find any alterations in cAMP-stimulated Ca^{2+} uptake in *stmF* or *fgdA* mutants, including *fgdA* gene disruptants. The phenotype of *stmF* mutants has also been suggested to implicate cGMP in cell elongation and myosin association with the Triton-insoluble cytoskeleton (Liu &

Newell, 1988) as well as myosin phosphorylation perhaps via cGMP-regulated kinases (Berlot *et al.*, 1987).

Fisher *et al.* (1984) proposed a model of signal processing in slugs and amoebae, in which signals from the cAMP receptor, photoreceptor and thermoreceptor converge at or before the regulation of cGMP. Therefore, light and heat signals, like cAMP, should induce changes in cGMP

levels upon stimulation. We show here that weak and strong light/heat stimuli induce changes in cGMP levels in both amoebae and slugs of the strain X22 which is wild-type for phototaxis. Characterization of wild-type strains, phototaxis mutants and a *stmF* mutant in relation to cGMP and behavioural responses suggests independent pathways for cAMP and light/heat regulation of cGMP.

METHODS

Strains and culture conditions. All strains of *Dictyostelium discoideum* were grown in association with *Klebsiella aerogenes* on nutrient SM agar at 21.5 °C. The origins and genotypes of strains are given in Table 1.

cAMP-induced cGMP response in *Dictyostelium* wild-type strain X22 and phototaxis mutants. Amoebae of the parental strain X22 and phototaxis mutants were grown in association with *K. aerogenes* on nutrient SM agar for 48 h at 21 °C. Amoebae were harvested from plates, washed free of bacteria by

centrifugation and resuspended at 2.5×10^8 cells ml^{-1} in phosphate buffer (0.1 mM $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 6.0). The amoebal suspension was spread over eight phosphate agar plates (10^8 cells per plate) and left to dry for 15 min. Amoebae from two such plates for each developmental time (0, 2, 4 or 6 h) were harvested, washed twice with phosphate buffer and diluted to a density of 1.3×10^8 cells ml^{-1} . Samples (90 μl) of this amoebal suspension were added to Eppendorf tubes containing 10 μl 1 μM cAMP. A control tube was set up containing 90 μl of cell suspension to which 10 μl phosphate buffer had been added. After cAMP stimulation of cells for 0, 10 and 30 s, the reaction was stopped by the addition of 100 μl ice-cold 3.5% (w/v) perchloric acid. After thorough mixing, the tubes were stored at -20 °C overnight. Prior to being assayed for cGMP, cell lysates were thawed on ice and centrifuged for 3 min. A portion (180 μl) of supernatant was placed into labelled Eppendorf tubes and to these 80 μl 16.8% (w/v) potassium hydrogen carbonate was added to neutralize the acid. After 15 min incubation on ice, the tubes were centrifuged for 1 min and cGMP levels of phototaxis mutants and wild-type strain X22 were measured using the Amersham cyclic GMP assay kit as previously described (Mato *et al.*, 1977).

Measurement of cGMP responses to light and heat stimuli.

To measure cGMP responses to light an experimental chamber (light box) was designed as shown in Fig. 1. The box contained a laterally placed light source in the form of a 15 W tungsten bulb placed in a metal reflector that protruded on the outside of the box to allow heat dissipation to the surroundings. The interior of the box was painted white to scatter light internally, and a 3 cm thick 5% (w/v) CuSO_4 solution was placed in front of the light source to reduce infra-red radiation. Eppendorf pipettes each loaded with 100 μl 3.5% (w/v) perchloric acid were inserted into the lid of the box, so that the tip of each pipette extended into the corresponding Eppendorf tube. The cells were allowed to dark-adapt for 30 min and were then exposed to a low (30 lx) or high (65 lx) light intensity for various time intervals (0, 30 s, 1 min, 5 min and 30 min) at which time 100 μl of perchloric acid was released into the suspension from the corresponding Eppendorf pipette. The cells were then frozen and stored at -20 °C overnight before radioimmunoassay for cGMP (Amersham). The cell suspensions were not aerated during the course of the experiment because of the technical difficulty of doing so without introducing light or temperature changes. In separate control experiments, amoebae were found by phase contrast microscopy to be morphologically normal and motile after periods of up to 1 h under similar conditions.

During the course of the experiments we discovered that cell suspensions exposed to light under the conditions described above also underwent slow warming owing to heat transfer from the tungsten lamp, despite the precautionary use of the CuSO_4 filter. For the 30 lx stimulus this warming was measured by thermistor to be 0.017 °C min^{-1} and for the 65 lx stimulus it was 0.033 °C min^{-1} . Throughout this paper cells exposed to light under these conditions are described as having been given a combined light/heat stimulus. In experiments where it was necessary to deliver a light stimulus in the absence of any warming, the rack of Eppendorf tubes containing the cells was placed in a 21 °C water bath within the light box. Under these conditions we confirmed by thermistor that no measurable warming took place over periods of 2 h with even the highest light intensities used. The term 'light stimulus' is reserved throughout this paper for stimuli delivered under these conditions.

To investigate the effects of a heat stimulus, in the absence of an

Fig. 1. Light box used to examine the cGMP responses to light and heat stimuli in the parental strain X22 and phototaxis/thermotaxis mutants. For experimental procedures, see Methods. The interior of the box was painted white to help reflect and scatter the light inside providing a more uniform light distribution. A 5% (w/v) CuSO_4 solution was placed in front of the light source to reduce infra-red radiation. The light source was a 240 V, 15 W tungsten bulb. For measurement of responses to light only, the Eppendorf tubes were placed in a 21 °C water bath (clear container).

accompanying light stimulus, we placed the rack bearing the Eppendorf tubes into a temperature-controlled water bath that allowed precisely regulated warming (and cooling) rates which were checked by thermistor. Cells were incubated at constant temperature (21 °C) in the dark for 30 min and then exposed to strong (0.033 °C min⁻¹) and weak (0.017 °C min⁻¹) heat stimuli corresponding to the heat generated by the low and high light intensities tested previously. After appropriate time intervals (0, 30 s, 1 min, 5 min and 30 min), 100 µl 3.5% (w/v) perchloric acid was released into the corresponding cell suspension as for light stimuli. The cell suspensions were frozen and stored overnight before radioimmunoassay for cGMP levels.

Developmental regulation of cyclic GMP responses to a combined light/heat stimulus. To test whether the cGMP responses to combined light/heat stimuli were developmentally regulated, amoebae from strain X22 were grown in association with *K. aerogenes* on nutrient SM agar for 48 h at 21 °C. Amoebae were then harvested from plates, washed free of bacteria by centrifugation and resuspended at 5×10^8 cells ml⁻¹ in phosphate buffer. The amoebal suspension was spread over eight phosphate agar plates (10⁸ cells ml⁻¹) and left to dry for 15 min. Amoebae from two such plates for each developmental time (0, 2, 4 or 6 h) were harvested, washed twice with phosphate buffer and resuspended at 2×10^8 cells ml⁻¹. Portions (100 µl) of each amoebal suspension for each developmental time were placed into each of five Eppendorf tubes which were placed into a rack contained in a light box (Fig. 1). The cells were incubated in the dark for 30 mins and their cGMP response to a weak (30 lx/0.017 °C min⁻¹) or strong (65 lx/0.033 °C min⁻¹) light/heat stimulus was measured as described above.

To test the response to weak and strong light/heat stimuli by X22 slug cells, amoebae from parental strain X22 were grown in association with *K. aerogenes* on nutrient SM agar for 48 h at 21 °C. Amoebae were harvested from plates, washed free of bacteria by centrifugation and resuspended at 5×10^8 cells ml⁻¹ in SS as previously described (Fisher *et al.*, 1981). Portions (100 µl) of this cell suspension were spread over a 5 cm × 1 cm strip in the centre of 10 charcoal plates. These plates were incubated overnight in black PVC boxes with a hole drilled in the side as described previously (Fisher & Williams, 1982). Slugs (which had just begun to migrate) were harvested, disaggregated by passage several times through an Eppendorf tip, washed three times with SS and resuspended at 2×10^8 cells ml⁻¹. Portions (100 µl) of this cell suspension were placed into each of five Eppendorf tubes which were placed into a rack contained in a light box (Fig. 1). The cGMP response of the disaggregated slug amoebae to weak (30 lx/0.017 °C min⁻¹) and strong (65 lx/0.033 °C min⁻¹) light/heat stimuli was measured as above.

Phototaxis by streamer F mutant NP368 and parental strain XP55. Phototactic behaviour by the streamer F mutant NP368 and parental strain XP55 was examined by toothpicking amoebae ($\sim 10^6$) from each strain onto several water agar plates containing 0.5% (w/v) activated charcoal. Each plate was placed in an individual black PVC box with a 2 mm hole drilled in one side and incubated for 48 h at 21.5 °C in a lighted room as described previously (Fisher *et al.*, 1981). After incubation, slug trails were transferred to clear PVC discs and stained with Coomassie Blue to obtain a permanent record of the result (Fisher *et al.*, 1981). Start and end points of trails from each disc were digitized using a Summagraphics M1201 digitizing tablet connected to a Force Microforce 2 computer. Estimates of the accuracy of phototaxis were calculated by means of directional statistics as described previously (Fisher *et al.*, 1981; Fisher & Williams, 1982).

RESULTS

cAMP-induced cGMP responses of wild-type and mutant amoebae

During this study we examined the cGMP response to cAMP (1 µM) in wild-type strains NC4 and X22, and phototaxis mutants altered in loci (*phoA-phoK*). Cells were used 6 h after starvation, the time point at which cells are aggregation competent. All strains tested showed a transitory increase in cGMP peaking at 10 s and returning towards basal levels by 30 s after cAMP stimulation. Fig. 2(a) shows, as an example, results for two mutant strains only (HPF5 and HPF6). These results are consistent with cAMP-induced cGMP responses found previously in *D. discoideum* wild-type strains (van Haastert *et al.*, 1982; Wurster *et al.*, 1977; Mato *et al.*, 1977; Ross & Newell, 1981). Since the phototaxis mutants showed wild-type cGMP responses to cAMP, we suggest that the gene products from the *pho* loci *phoA-phoK* do not participate in cAMP regulation of cGMP.

Fig. 2. Cyclic-AMP-mediated cGMP accumulation in (a) wild-type strain X22 (●), phototaxis mutants HPF5 (■) and HPF6 (▲), and (b) wild-type strain XP55 (●) and streamer F mutant NP368 (■). Amoebal cells of wild-type, phototaxis mutant strains and the streamer F mutant were stimulated after 6 h starvation with 1 µM cAMP for 0, 10 or 30 s. Mean values and SD are shown for data from two independent experiments that have been normalized relative to the basal cGMP level at the beginning of stimulation.

Fig. 3. Cyclic GMP response of disaggregated slug amoebae (a) and vegetative amoebae (b) of strain X22 to weak (■) and strong (●) light/heat stimuli. cGMP levels were normalized with respect to the basal level at the beginning of stimulation and averaged over several experiments. (a) Slugs (which had just begun to migrate) were harvested and mechanically disaggregated. The cells were then washed and resuspended at a concentration of 2×10^8 cells ml^{-1} and cGMP responses to a weak and strong stimulus of light/heat were measured. Normalized cGMP levels were averaged from two experiments.

Previously, streamer mutants altered in the *stmF* locus were isolated and showed alterations in their cGMP response to cAMP (Ross & Newell, 1981). In these strains cGMP concentrations reached a higher level and declined more slowly than in the parental strain XP55 (Ross & Newell, 1981). We confirmed these results here for the streamer F mutant NP368 (Fig. 2b).

Wild-type cGMP responses to combined light/heat stimuli

Having shown that none of our phototaxis mutants were altered in cGMP responses to cAMP, we decided to determine whether *D. discoideum* amoebae responded to light and heat stimuli with changes in cGMP levels as was predicted by Fisher *et al.* (1984). Initial experiments failed to reveal significant cGMP responses to combined light/heat stimuli within 30 s of the onset of stimulation. However, subsequent experiments over longer periods of stimulation revealed both increases and decreases in cGMP levels depending on the intensity of the stimulus (Fig. 3). It is possible that these sign reversals in cGMP responses may be related to sign reversals in the behaviour of both slugs and amoebae (Fisher *et al.*, 1984).

To determine whether cGMP responses to light/heat stimuli were developmentally regulated, we first compared the responses of vegetative (Fig. 3a) and disaggregated slug amoebae (Fig. 3b). In each case, after a strong stimulus (65 lx , $0.033 \text{ }^\circ\text{C min}^{-1}$) the cGMP levels increased to a maximum after about 1 min and returned to basal levels within 5–30 min. A weaker stimulus (30 lx , $0.017 \text{ }^\circ\text{C min}^{-1}$) led to a decrease in cGMP levels to a minimum after about 1 min, returning to starting levels after 5–30 min stimulation. Measurement of cGMP responses to both strong and weak light/heat stimuli during the first 6 h of differentiation to aggregation competence revealed that amoebae at all stages responded similarly (Fig. 4). It is during this period that cGMP responses to cAMP stimuli are acquired (Mato *et al.*, 1977; Wurster *et al.*, 1977). Unlike the more rapid cGMP responses to cAMP, the cGMP responses to light/heat stimuli are not developmentally regulated. This parallels the behavioural responses in that both slugs and amoebae are phototactic and thermotactic, and the slug behavioural mutants that have been tested also exhibit altered amoebal behaviour (Fisher *et al.*, 1984).

cGMP responses to light/heat stimuli by amoebae of slug phototaxis mutants

Having shown that wild-type amoebae exhibit cGMP responses to light/heat stimuli and that these responses are not developmentally regulated, we examined the responses of vegetative amoebae of our slug phototaxis

(b) Vegetative amoebal cells of strain X22 were grown, harvested and diluted to a concentration of 5×10^8 cells ml^{-1} . These cells were exposed to a weak or strong stimulus of light/heat and assayed for cGMP. Mean values of normalized cGMP levels from two experiments are shown.

Fig. 4. Cyclic GMP response to a weak (■) or strong (●) stimulus of light/heat in amoebae of strain X22 at developmental times (a) 0 h, (b) 2 h, (c) 4 h and (d) 6 h. Amoebae were exposed to a weak or strong stimulus of light/heat after developing on phosphate-buffered agar plates (about 10^8 cells per plate) for each of the specified developmental times. Means of normalized cGMP levels from two experiments are shown.

Table 2. cGMP responses of phototaxis mutants to a combined light/heat stimulus

Complementation group	Mutant	Mutant allele	Parental strain	Phenotypic class*
<i>pboA</i> †	HU407	<i>pboA2315</i>	DU584	III
<i>pboB</i>		<i>pboB2316</i>		
<i>pboC</i>	HPF9	<i>pboC2307</i>	X22	III
<i>pboD</i>	HPF3	<i>pboD2317</i>	X22	III
<i>pboE</i>	HPF7	<i>pboE2305</i>	X22	I
	HPF11	<i>pboE2308</i>	X22	
<i>pboF</i>	HPF8	<i>pboF2306</i>	X22	III
<i>pboG</i>	HPF12	<i>pboG2309</i>	X22	III
<i>pboH</i>	HU409	<i>pboH355</i>	X22	IV
<i>pboI</i>	HPF2	<i>pboI2301</i>	X22	IV
<i>pboJ</i>	HPF6	<i>pboJ2304</i>	X22	I
	HPF16	<i>pboJ2313</i>	X22	III
<i>pboK</i>	HPF1	<i>pboK2300</i>	X22	III
	HPF14	<i>pboK2311</i>	X22	III
Unassigned‡	HPF5	<i>pbo-2303</i>	X22	II
	HPF17	<i>pbo-2314</i>	X22	IV
	HPF230	<i>pbo-2318</i>	X22	II
	HPF229	<i>pbo-2319</i>	X22	I

* See text for details.

† *pboA* and *pboB* are both required to express the *pbo* mutation in HU407 and therefore belong to the same phenotypic class with respect to cGMP responses to light/heat.

‡ *pbo* mutations in these mutants map to linkage group II and have not as yet been assigned to any complementation group.

Fig. 5. Cyclic GMP response to weak (■) and strong (●) light/heat stimuli in vegetative amoebae of the parental strain X22 (a) and one phototaxis mutant from each of the four phenotypic classes: (b) HPF229, phenotypic class I; (c) HPF5, phenotypic class II; (d) HPF3, phenotypic class III; (e) HPF2, phenotypic class IV. Vegetative amoebae of all strains were exposed to a weak or strong light/heat stimulus for 0, 0.5, 1, 5 or 30 min and assayed for their cGMP response. Cyclic GMP levels were normalized to 100% with respect to the zero time point. Mean values with SD from two experiments are shown.

mutants. The basal cGMP levels prior to stimulation were similar to wild-type for all of the mutants, averaging about 0.5 pmol per 10^7 vegetative amoebae. However, the cGMP responses to weak and strong light/heat stimuli were, in most cases, different from wild-type so that the phototaxis mutants could be placed into four phenotypic classes (Table 2).

Phenotypic class I. Wild-type cGMP response to both weak and strong light/heat stimuli. Phototaxis mutants HPF6, HPF7, HPF11 and HPF229 showed cGMP responses to weak and strong light/heat stimuli similar to those of the parental strain X22. Fig. 5(b) shows results for one of the mutants, HPF229. Cyclic GMP levels decreased after a weak light/heat stimulus and increased after a strong light/heat stimulus. These results suggest that the gene products of mutant *pho* alleles in these strains participate in sensory transduction downstream of the point at which cGMP levels are regulated.

Phenotypic class II. Cyclic GMP responses to both weak and strong light/heat stimuli abolished. Strains HPF5 and HPF230 showed no significant cGMP response to either weak or strong light/heat stimuli. Fig. 5(c) shows the results for the mutant strain HPF5. Thus mutant *pho* alleles in these strains encode gene products that act in the sensory transduction chain before cGMP.

Phenotypic class III. Cyclic GMP responses to a strong light/heat stimulus impaired. Strains belonging to this phenotypic class showed wild-type cGMP responses to a weak light/heat stimulus (i.e. decrease in cGMP) but showed either a decrease (HPF1, HPF14, HPF16) or no significant change (HPF3, HPF8, HPF9, HU407) in cGMP levels after a strong light/heat stimulus. The cGMP response to a light/heat stimulus of strain HPF3 representing this phenotypic class is shown in Fig. 5(d). The gene products of mutant *pho* alleles from these strains participate in sensory transduction upstream from cGMP.

Phenotypic class IV. Inverted cyclic GMP responses to both weak and strong light/heat stimuli. Strains belonging to this phenotypic class showed inverted cGMP responses to weak and strong light/heat stimuli. Cyclic GMP levels decreased after a strong light/heat stimulus and increased after a weak light/heat stimulus with maximal cGMP responses occurring after 1 min. This inverted cGMP response was observed in strains HPF2, HPF17 and HU409. Fig. 5(e) shows results for the mutant strain HPF2. These results suggest that the gene products of mutant *pho* alleles from these strains also participate in sensory transduction upstream from cGMP.

Since cGMP responses to a combined light/heat stimulus were altered in three distinct phenotypic classes of

Fig. 6. Cyclic GMP response to a weak (■) or strong (●) combined stimulus of light/heat (a) or to separate light (b) and heat (c) stimuli in vegetative amoebae of strain X22. Stimuli were delivered as described in Methods. Cyclic GMP responses to separate light and heat stimuli in X22 were similar to cGMP responses after combined light/heat stimuli of equivalent strength. Mean values with SD from two experiments are shown.

phototaxis mutants, we suggest that cGMP is involved in photosensory and thermosensory processing in *D. discoideum*. Furthermore cAMP and light/heat signals must regulate cGMP via at least partly separate pathways, since cAMP-induced cGMP responses were unaltered in these phototaxis mutants.

Cyclic GMP response to separate light and heat stimuli in the parental strain X22 and thermotaxis mutant HPF228

All experiments described so far involved the use of a combined light/heat stimulus (see Methods). Since all phototaxis mutants were impaired also in thermotaxis, it was not surprising that cGMP responses to combined stimuli were altered in most strains. However, it remained possible that the responses by *D. discoideum* amoebae to combined stimuli were, in fact, responses to only one or other of the two stimuli involved. To test this possibility, we measured cGMP responses to separate light and heat stimuli of equivalent intensity to those involved in the combined stimuli used in earlier experiments. In the parental strain X22 separate light and heat stimuli resulted in similar cGMP responses (Fig. 6). Weak light (30 lx) and heat ($0.017^{\circ}\text{C min}^{-1}$) stimuli led to decreased cGMP levels, while strong light (65 lx) and heat ($0.033^{\circ}\text{C min}^{-1}$) stimuli resulted in increased cGMP levels. In each case the responses were similar to those after an equivalent combined stimulus.

Since HPF228 was isolated as a thermotaxis mutant and was unaltered in phototaxis (P. K. Darcy and others, unpublished), we also examined the effect of separate light and heat stimuli on cGMP levels in this strain. Cyclic GMP responses to weak and strong light stimuli in

HPF228 were similar to those of wild-type strain X22 (Fig. 7), however the cGMP response to heat was inverted compared with the response in the parental strain X22. Cyclic GMP levels increased after a weak heat stimulus and decreased after a strong heat stimulus (Fig. 7). These results suggest that the mutation in HPF228 lies in the thermosensory pathway before convergence with the photosensory pathway. Although the cGMP response in HPF228 to a combined light/heat stimulus was similar to that of the parental strain X22, the magnitude of the cGMP response to a strong combined light/heat stimulus was slightly decreased (Fig. 7).

Cyclic GMP response to separate light and heat stimuli in the thermotaxis mutant HPF13

One mutant in our collection, HPF13, was isolated originally as a phototaxis mutant but shown subsequently to be impaired strongly in thermotaxis and only slightly in phototaxis (P. K. Darcy and others, unpublished). Since HPF13 was found to be more strongly altered in thermotaxis than phototaxis we examined the effect of separate light and heat stimuli on cGMP levels in this strain. Our results showed that light and heat had different effects on cGMP levels (Fig. 8). Cyclic GMP responses to light stimuli were inverted relative to those of the wild-type, the response to a weak heat stimulus was similar to wild-type and the response to a strong heat stimulus was abolished with cGMP levels declining instead of increasing under these conditions (Fig. 8). These results suggest that two independent mutations in HPF13 may be affecting phototaxis and thermotaxis separately. Genetic analysis (data not shown) partly confirms this conclusion in that the *pho* mutation could not be mapped, despite normal segregation of all marked linkage groups and clear

Fig. 7. Cyclic GMP response to a weak (■) and strong (●) combined stimulus of light/heat (a) and to separate light (b) and heat (c) stimuli in vegetative amoebae of the thermotaxis mutant HPF228. Mean values with SD from two experiments are shown.

Fig. 8. Cyclic GMP response to a weak (■) and strong (●) combined stimulus of light/heat (a) and to separate light (b) and heat (c) stimuli in vegetative amoebae of the thermotaxis mutant HPF13. Cyclic GMP responses in HPF13 to a combined light/heat stimulus and to a heat stimulus were similar, i.e. cGMP levels decreased in response to both strong and weak stimuli, but cGMP responses to light were inverted relative to those of wild-type strains, exhibiting an increase with a weak stimulus and a decrease with a strong stimulus. Mean values with SD from two experiments are shown.

mapping of the mutant thermotaxis locus (*theA2300*) to linkage group II. All segregants from crosses involving HPF13 were wild-type for phototaxis, irrespective of their thermotaxis phenotypes, so that there was no detectable Pho^- phenotype linked to *theA2300*, despite a Pho^- phenotype in the original mutant. It would be interesting to test cGMP responses to light and heat stimuli in further experiments with selected haploid segregants from crosses involving HPF13.

Cyclic GMP responses to light and heat stimuli by the streamer F mutant NP368 and the parental strain XP55

Since the streamer F mutant NP368 shows altered cGMP responses to cAMP, we examined cGMP responses to light and heat stimuli in this strain. The cGMP responses to light and heat stimuli were unaltered compared to the parental strain XP55 and other wild-type strains (Fig. 9).

Fig. 9. Cyclic GMP response to a weak (■) and strong (●) stimulus of light/heat in vegetative amoebae of the parental strain XP55 (a) and the streamer F mutant NP368 (b). Mean values with SD from two experiments are shown.

Cyclic GMP levels decreased after a weak light or heat stimulus and increased after a strong light or heat stimulus as observed for vegetative amoebae of wild-type strain XP55. These results further strengthen the hypothesis

that cAMP and light/heat signals regulate cGMP via independent pathways.

Phototactic behaviour by streamer F mutant NP368 and parental strain XP55

The accuracy of orientation (κ) of slug phototaxis in the streamer F mutant NP368 and parental strain XP55 was measured as described in Methods. The accuracy of orientation in slug phototaxis at low cell density on water agar was found to be unaltered in NP368 [accuracy of phototaxis (κ) with 90% confidence limits = 32 (18, 50)] compared with the accuracy of orientation of slug phototaxis in the parental strain XP55 [accuracy of phototaxis (κ) with 90% confidence limits = 34 (20, 52)].

DISCUSSION

The results presented here provide good evidence for a role for cGMP in photosensory and thermosensory processing in *D. discoideum*. Not only do light and heat stimuli alter cGMP levels consistent with an intermediary role for cGMP in sensory transduction, but most phototaxis and thermotaxis mutants show alterations in their cGMP responses to these stimuli.

The phototaxis mutants fell into four phenotypic classes with respect to cGMP responses to weak and strong light/heat stimuli. Most of the phototaxis mutants were altered in their cGMP response to a combined stimulus of light and heat, however one class of mutants showed a wild-type cGMP response similar to the cGMP response to light/heat in the parental strain X22. These results suggest that some phototaxis mutant gene products participate in sensory transduction upstream from cGMP and some downstream from cGMP. Fig. 10 shows two possible pathways incorporating cAMP and light/heat regulation of cGMP and behaviour. These pathways also include possible sites of action of *pho* loci in some mutants.

In model A, pathways from the photoreceptor and thermoreceptor converge (Fisher *et al.*, 1981) to regulate cGMP. The pathways leading to behavioural and cGMP responses diverge at a point after the convergence of the photoreceptor and thermoreceptor. Mutants altered in slug phototaxis and thermotaxis but unimpaired in their cGMP responses would be affected in the part of the pathway leading to behaviour but not to cGMP (e.g. HPF7). *pho* mutations from mutants altered in both behaviour and light/heat regulation of cGMP would act at a site before the divergence of pathways leading to cGMP and behaviour (e.g. HPF1). An alternative model shown in pathway B shows cGMP regulating the behavioural response. *pho* mutations from mutants which are unaltered in cGMP responses, but impaired in slug phototaxis and thermotaxis would act downstream from cGMP (Fig. 10). *pho* mutations from mutants that are altered in both cGMP and behaviour would act upstream from cGMP.

To elucidate further how cGMP and behaviour may be connected in *D. discoideum* signal processing we examined the phototactic behaviour of the streamer F mutant

Fig. 10. Two possible pathways (A and B) incorporating cAMP and light/heat regulation of cGMP and behaviour. In both models, folate and cAMP converge prior to regulation of cGMP levels via the *stmF* product. Neither model attempts to explain the differences in cGMP responses amongst mutants altered in both phototaxis and thermotaxis as well as in the cGMP responses (see text).

NP368. Since this strain carries a mutation that affects cGMP phosphodiesterase activity in crude extracts (Ross & Newell, 1981; Coukell & Cameron, 1986), we might expect phototaxis to be impaired if cGMP regulates this behaviour (pathway B, Fig. 10). The accuracy of orientation in slug phototaxis was found to be unaltered in NP368 compared to the parental strain XP55.

The lack of impairment of phototaxis in NP368 slugs suggests that the cGMP-specific phosphodiesterase does not play a role in responses to light and heat but is replaced for these stimuli by the other known cyclic nucleotide phosphodiesterase in this organism. This less specific enzyme degrades both cGMP and cAMP (van Haastert *et al.*, 1982). Under this alternative hypothesis cGMP degradation after light or heat stimulation would

depend on the slower breakdown by the less specific phosphodiesterase and would be unaltered in *stmF* mutants. This was in fact observed, leading us to conclude that cAMP and light/heat stimuli regulate cGMP via different pathways (Fig. 10). One of the several ways in which this could occur would be if the cGMP-specific phosphodiesterase were regulated by an inhibitory subunit that dissociates from the holoenzyme after stimulation by cAMP in wild-type cells but not in *stmF* mutant cells. The inactivity of the holoenzyme in crude extracts from *stmF* mutants (Coukell & Cameron, 1986) could be consistent with either altered catalytic or regulatory subunits (e.g. an inhibitory subunit that fails to dissociate from the holoenzyme during extraction from mutants).

The conclusion that cAMP and light/heat stimuli regulate cGMP via different pathways is strengthened by the fact that all phototaxis mutants with altered cGMP responses to light and heat were unaltered in their cGMP response to cAMP. Our results are also consistent with the proposed model of signal processing in *Dictyostelium* slugs (Fisher *et al.*, 1984), which shows separate convergent pathways involving cAMP and light/heat regulation of cGMP. In both pathways increases in cGMP concentration could be mediated primarily by activation of guanylate cyclase, these increases being limited not only by the activity of one or other phosphodiesterase but also by the extent and duration of cyclase activation.

Although not shown in Fig. 10, Fisher *et al.* (1984) proposed that signal-'strength'-dependent sign reversals in phototaxis and thermotaxis may be controlled by a region of the pathway termed 'the sign reversal generator'. This region consists of mutually opposed feedback loops that, perhaps via Ca^{2+} , regulate cAMP (positive loop) and STF (Slug Turning Factor) (negative loop) secretion (Fisher *et al.*, 1984). Cyclic AMP and STF were proposed to play the respective roles of a slug tip activator and inhibitor whose activities control slug behaviour and are modulated by incoming signals from the photoreceptor and thermoreceptor. Signal-strength-dependent changes in the balance between tip activation and tip inhibition after stimulation were suggested to account for sign reversals in slug behaviour (Fisher *et al.*, 1984). In the parental strain X22 we observed sign reversals in the cGMP response to weak and strong light/heat stimuli which may indicate that the cGMP response lies downstream of or is part of the sign reversal generator. *pho* mutations whose site of action lies upstream of or in this region may alter the interaction between the cAMP and STF loops and provide an explanation for the inverted cGMP responses to weak and strong light/heat stimuli observed in HPF2, HPF17 and HU409.

Others of our mutants exhibited either a loss of all cGMP responses to light/heat stimuli, or a specific loss of the increase in cGMP after strong stimuli. Clearly the sites of action of these mutant alleles must also be upstream of or in the region where cGMP levels are regulated. Fisher *et al.* (1984) suggested that as well as cGMP, Ca^{2+} might play a pivotal role in the sign reversal generator. Without additional characterization of our mutants in relation to

Ca²⁺ (and other) responses, we are unable to specify more precisely the different regions of the signal transduction pathway that might correspond to our different mutant classes. Some of the mutants show abnormalities in fruiting body morphology that seem to be associated with the mutant *pho* loci (P. K. Darcy and others, unpublished), but these do not correlate simply with the phenotypic classes identified here in relation to cGMP responses.

During the course of this study, the effect of light and heat on cGMP levels in HPF13 and HPF228 produced several interesting results. Since HPF228 is altered in thermotaxis only, we examined the effect of separate light and heat stimuli on cGMP levels in this strain. The prediction that cGMP responses to heat but not light stimuli would be altered in this mutant was fulfilled. The mutation in HPF228 affects thermosensory transduction before convergence with the phototaxis pathway and the subsequent cGMP response. Although HPF13 was originally isolated as a phototaxis mutant (P. K. Darcy and others, unpublished), it appeared to be more strongly impaired in thermotaxis than phototaxis. In subsequent mapping studies we were able to map the thermotaxis mutation to linkage group II (P. K. Darcy and others, unpublished). However cGMP responses by HPF13 amoebae to separate light and heat stimuli were altered, each in a different manner. This indicates that perhaps separate mutations affect phototaxis and thermotaxis in HPF13. This is consistent with the mapping studies in which we were unable to map a *pho* mutation from HPF13 to any of the marked linkage groups and the Pho⁻ phenotype was not linked to the The⁻ phenotype.

pho mutations from strains HPF6 and HPF16 were found to map to the same linkage group and to belong to the same complementation group (*phoJ*) (P. K. Darcy and others, unpublished). However, cGMP responses to a combined light/heat stimulus were different in these two strains. HPF6 showed a wild-type cGMP response to light/heat whereas the cGMP response to strong light/heat stimuli was impaired in HPF16. Possible explanations include (i) an additional mutation in HPF16 affects cGMP but not behavioural responses and (ii) an additional mutation in HPF16 mapping to the same linkage group as *phoJ* affects both cGMP and behavioural responses. Further experiments are required to test these possibilities.

Although the pathways illustrated in Fig. 10 remain incomplete our results do support a role for cGMP in photosensory and thermosensory transduction in *D. discoideum*. It would be interesting in further experiments to test the effect of folate on cGMP responses in the phototaxis mutants. Streamer F mutants show an alteration in the cGMP response to folate as well as to cAMP (van Haastert *et al.*, 1982), although they show normal cGMP responses to light/heat stimuli. The two chemosensory pathways therefore converge prior to regulation of cGMP levels via the *stmF* gene product. We may therefore expect cGMP responses to folate, like those to cAMP, to be unaltered in the phototaxis mutants. It would also be interesting to test the effect of light, heat, cAMP and folate on inositol triphosphate (IP₃) and Ca²⁺

responses in these phototaxis mutants. One hypothesis may be that signals from the photoreceptor and thermoreceptor converge and regulate the second messenger IP₃ via the activation of G-protein(s). Cyclic AMP, folate and light/heat activation of IP₃ may release Ca²⁺ from non-mitochondrial stores leading to transient formation of cGMP and polymerization of actin (Europe-Finner & Newell, 1986, 1987; Small *et al.*, 1986). Our results indicate that in the chemotransduction pathway but not in the phototransduction or thermotransduction pathways, the increase in cGMP is limited by activation of the cGMP phosphodiesterase via the *stmF* gene product. Cyclic GMP may in turn regulate Ca²⁺ uptake (Menz *et al.*, 1991) either by binding to a cation channel as in the vertebrate retina (Fesenko *et al.*, 1985) or by activating a cGMP-dependent protein kinase (Wanner & Wurster, 1990) that catalyses a modification of the cation channel (Menz *et al.*, 1991). To confirm this hypothesis the continued study of signal processing mechanisms in slug behavioural mutants may give a clearer picture, unifying all potential signalling components involved.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Research Council. We are grateful to Mr K. Darcy for drawing Fig. 1.

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Received 9 September 1993; revised 15 February 1994; accepted 18 February 1994.