A Role for G-proteins and Inositol Phosphate Signalling in *Dictyostelium discoideum* Slug Behaviour

By P. K. DARCY AND P. R. FISHER*

Microbiology Department, La Trobe University, Bundoora 3083, Melbourne, Australia

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Phototaxis by *Dictyostelium discoideum* slugs was disoriented in the presence of pertussis toxin, suggesting a role for pertussis-toxin-sensitive G-proteins in slug behaviour. The decrease in orientation accuracy was dependent on the concentration of this bacterial toxin. Pertussis toxin did not significantly affect slug thermotaxis, while cholera toxin had no significant effects on either slug phototaxis or thermotaxis. The addition of micromolar concentrations of lithium salts (LiCl or LiBr) resulted in disorientation of both phototaxis and positive thermotaxis by slugs. The corresponding sodium salts had no effect on slug behaviour, confirming that the effects were specific to lithium ions. The effects of lithium ions suggest a role for inositol phosphates as possible second messengers in *Dictyostelium* slug behaviour. Except for slight impairment of thermotaxis by D-sphingosine, we found no effects on slug behaviour of either phorbol 12-myristate 13-acetate or sphingolipids. We thus found little pharmacological evidence for a role in slug signal transduction of the diacylglycerol branch of the inositol lipid pathway.

INTRODUCTION

The life cycle of the cellular slime mould *Dictyostelium discoideum* presents unique opportunities to study eukaryotic cell and molecular biology at both the unicellular and multicellular levels of organization (Loomis, 1982). During aggregation by chemotaxis to form the multicellular state, *Dictyostelium* amoebae process chemoattractant (cAMP) signals in a manner analogous to higher eukaryotes via activation of G-proteins and increased intracellular concentrations of the second messengers cGMP, inositol-trisphosphate (IP₃) and Ca²⁺ (McRobbie, 1986; Janssens & van Haastert, 1987; Newell *et al.*, 1988; Gerisch, 1987).

Although less widely studied, sensory transduction in the multicellular 'slug' stage of the *D. discoideum* life cycle seems to be based on the same molecular processes as in the unicellular amoeboid stage (Fisher *et al.*, 1984). This leads to the expectation that G-proteins and phosphatidylinositol lipid metabolism might play a role in transmembrane signalling in *D. discoideum* slugs. The striking effects on slug orientation behaviour of fluoride ions have been known for some time (Dohrmann *et al.*, 1984a) and, as is the case in other organisms (Sternweis & Gilman, 1982), may be explained by the action of aluminofluoride on G-proteins involved in processing behavioural signals in slugs. We report here that pertussis toxin, a specific inhibitor of G-proteins in higher eukaryotes (Stryer & Bourne, 1986), impairs slug orientation during phototaxis (but not thermotaxis).

In *D. discoideum*, as in other organisms, Li⁺ ions specifically disrupt phosphoinositide signalling by inhibiting the regeneration of inositol via dephosphorylation of IP₃ and other inositol phosphates (Worley *et al.*, 1988; Leitz & Muller, 1986; van Lookeren Campagne *et al.*, 1988). We show here that Li⁺ ions impair slug orientation during both phototaxis and positive thermotaxis, consistent with a possible role for IP₃ as an intracellular second messenger in slug behaviour.

Abbreviations: IP₃, inositol trisphosphate; PIP₂, phosphatidylinositol bisphosphate; PMA, phorbol 12-myristate 13-acetate.

METHODS

Chemicals. D-Sphingosine, sphingosylphosphorylcholine, lithium and sodium salts, and cholera toxin were obtained from Sigma. Pertussis toxin was supplied by Dr P. Marley in vehicle buffer [50% (w/v) glycerol, 0.2% (w/v) NaHCO_3 , 1.46% (w/v) NaCl , 0.18% (w/v) L-lysine] from the Commonwealth Serum Laboratories, Melbourne, Australia.

Strains and culture conditions. *D. discoideum* strain X22 was grown in association with *Klebsiella aerogenes* on nutrient SM agar at 21.5 °C as previously (Fisher *et al.*, 1981; Fisher & Williams, 1982).

Phototaxis and thermotaxis experiments. These were done as previously (Fisher *et al.*, 1981; Fisher & Williams, 1982). Amoebae were washed free of bacteria and resuspended in Bonner's salt solution (per litre: 0.6 g NaCl , 0.75 g KCl , 0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) at a density of about 2.5×10^8 amoebae ml^{-1} . A 10 μl volume of the amoebal suspension was spread over a 1 cm $\times$ 1 cm origin in the centre of each water agar plate supplemented with the pharmacological agent being tested.

Plates were incubated for phototaxis experiments in the light at 21.5 °C for 48 h in individual black PVC boxes with a 2 mm hole drilled in the side as previously (Fisher *et al.*, 1981; Fisher & Williams, 1982). For thermotaxis plates were incubated for 72 h in individual black PVC containers (without holes) on the surface of an insulated aluminium heat bar at temperatures ranging from 1 to 8 (arbitrary units referred to henceforth as T1, T2...T8). The heat bar was the same design as that used previously, for which the temperature gradient at the agar surface under the same conditions was 0.2 °C cm^{-1} , with temperatures at the centres of the plates ranging from 14 °C to 28 °C (Fisher & Williams, 1982).

Quantitative behavioural measurements. Slug trails were transferred to clear PVC discs and stained with Coomassie Blue as previously 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 and thermotaxis were calculated by means of directional statistics as previously (Fisher *et al.*, 1981; Fisher & Williams, 1982).

The statistical methods were based on the von Mises distribution, an analogue for directional data of the familiar bell-shaped normal distribution. The von Mises distribution has two parameters – a mean direction μ and a concentration parameter κ that measures the concentration of individual directions around the mean. In no case was the mean direction of travel significantly different from the direction towards the light source in phototaxis, towards the warmth in positive thermotaxis or towards the cold in negative thermotaxis. κ was used in this work, as previously (Fisher *et al.*, 1981; Fisher & Williams, 1982), as a measure of the accuracy of slug orientation in phototaxis and thermotaxis. κ ranges from a value of 0 in the case of no orientation in any particular direction to ∞ in the case of perfect orientation. We followed the previously used convention of assigning negative values of κ to significant negative thermotaxis (Fisher & Williams, 1982).

RESULTS

G proteins may play a role in slug behaviour

In higher eukaryotes fluoride ions combine with trace amounts of aluminium to activate the α subunits of G_s , G_i , G_o and transducin (Sternweis & Gilman, 1982; Stryer & Bourne, 1986). In *D. discoideum* fluoride alters both phototaxis and thermotaxis by slugs (Dohrmann *et al.*, 1984a), perhaps by interacting with G-protein(s). Since pertussis and cholera toxins specifically ADP-ribosylate G-proteins in higher eukaryotes, we examined *D. discoideum* slug behaviour in the presence of these toxins. The results in Figs 1 and 2(a) show that the accuracy of slug phototaxis decreased as pertussis toxin concentrations increased from 20 ng ml^{-1} to 200 ng ml^{-1} . This suggests the possible involvement of a pertussis-toxin-sensitive G-protein in photosensory transduction in slugs.

Thermotaxis by *D. discoideum* slugs is maximally positive at temperatures near the growth temperature (T4 to T5) and becomes less accurate at higher and lower temperatures, finally becoming insignificant and then negative around T2 and T8 (Fisher & Williams, 1982). Even at the highest concentration tested (200 ng ml^{-1}) pertussis toxin had little effect on slug thermotaxis at any temperature on the heat bar (Fig. 3). Neither slug phototaxis nor thermotaxis were significantly altered by the presence of cholera toxin at concentrations up to 1 $\mu\text{g ml}^{-1}$ (Figs 2b and 3).

Inositol phosphates may play a role in slug behaviour

In *D. discoideum* amoebae, as in other organisms, IP3 triggers release of intracellular bound calcium which in turn appears to regulate the accumulation of cGMP and the cytoskeleton (Newell *et al.*, 1988; Europe-Finner & Newell, 1985, 1986a, b, 1987). Since Li^+ ions disrupt IP3

Fig. 1. Phototaxis by slugs of strain X22 in the presence or absence of pertussis toxin. Amoebae (2.5×10^6) were inoculated onto a 1 cm^2 origin in the centre of a water agar plate (a) with or (b) without 200 ng pertussis toxin ml^{-1} . After 48 h incubation at 21.5°C slug trails were transferred to PVC discs, stained and digitized. The direction of the light source was from the top of the figure and digitized trails are plotted from a common origin. Bar, 1 cm .

Fig. 2. Effect of pertussis and cholera toxins on the accuracy of phototaxis by X22 slugs. The amoebae were inoculated onto water agar containing (a) pertussis or (b) cholera toxin at the indicated concentrations. Other details as for Fig. 1. Bars represent 90% confidence limits.

Fig. 3. Effect of pertussis and cholera toxins on thermotaxis by X22 slugs. Amoebae ($2.5 \times 10^6 \text{ cm}^{-2}$) were inoculated onto unsupplemented water agar (□), or water agar containing 200 ng pertussis toxin ml^{-1} (●), or $1 \mu\text{g}$ cholera toxin ml^{-1} (■). Plates were incubated for 72 h on the heat bar as described in Methods, with agar surface temperatures (arbitrary units) ranging from 1 at the cold end to 8 at the warm end. After incubation, slug trails were transferred to PVC discs and stained with Coomassie Blue before digitizing. Bars represent 90% confidence limits.

Fig. 4. Effect of lithium salts on the accuracy of phototaxis by X22 slugs. The cell density was 2.4×10^6 amoebae cm^{-2} . The agar contained at the indicated concentrations (a) LiBr (■) or NaBr (●), (b) LiCl (■) or NaCl (●). Other details as for Fig. 1.

Fig. 5. Effect of lithium salts on thermotaxis by X22 slugs. Amoebae ($2.4 \times 10^6 \text{ cm}^{-2}$) were inoculated onto unsupplemented water agar (○) or water agar containing (a) 5 mM-LiCl (■) or NaCl (●), (b) 5 mM-LiBr (■) or NaBr (●). Other details as for Fig. 3.

metabolism, we examined the effects of lithium salts on *D. discoideum* slug behaviour. Micromolar concentrations of LiBr and LiCl impaired both slug phototaxis (Fig. 4a, b) and positive thermotaxis (Fig. 5a, b), while the corresponding sodium salts had no effect. Negative thermotaxis at T2 was significantly stronger in the presence of Li^+ ions, while the temperature at which slugs switched from positive to negative thermotaxis was shifted towards the growth temperature (Fig. 5). As in the case of phototaxis, the effects of Li^+ ions on thermotaxis were concentration dependent (e.g. Fig. 6a, b, c), with half-maximal inhibition occurring at concentrations of $500 \mu\text{M}$ or less. Like sensory transduction mutations and other pharmacological agents (Fisher & Williams, 1982; Dohrmann *et al.*, 1984a, b), Li^+ had least effect on thermotaxis at high temperatures (T8) (Fig. 5).

As well as IP3, hydrolysis of phosphatidylinositol bisphosphate (PIP_2) releases diacylglycerol which, in other eukaryotes, also plays a second-messenger role by activating protein kinase C in the cell membrane (Kikkawa & Nishizuka, 1986). Since the lysosphingolipids D-sphingosine and sphingosylphosphoryl choline inhibit protein kinase C in human platelets (Hannun & Bell, 1987), while phorbol esters such as phorbol 12-myristate 13-acetate (PMA) activate it (Kikkawa & Nishizuka, 1986), we examined whether these compounds affected slug behaviour. Except for impairment of positive thermotaxis by D-sphingosine (Fig. 7), no significant difference was detected in slug behaviour in the presence or absence of phorbol ester (up to $5 \mu\text{M}$) or lysosphingolipids (up to $100 \mu\text{M}$) (not shown). These results provide little pharmacological evidence for but do not exclude the possibility that the diacyl glycerol branch of the inositol phospholipid pathway is important in intracellular signalling in slugs.

Fig. 6. Concentration dependence of the effect of lithium on slug thermotaxis at (a, d) T2, (b, e) T3 and (c, f) T5. Details as for Fig. 5 except that the agar contained Li⁺ (●) or Na⁺ (■) at the indicated concentrations as either the chloride salt (a, b, c) or the bromide salt (d, e, f).

Fig. 7. Influence of D-sphingosine on the accuracy of thermotaxis by X22 slugs. The cell density was 2.5×10^6 amoebae cm^{-2} on water agar with (■) or without (□) 80 μ M D-sphingosine. Other details as for Fig. 3.

DISCUSSION

The use of pharmacological agents provides an initial approach to studying the potential importance of specific target molecules and pathways in intracellular signalling in slugs. EGTA, for example, has a profound effect on slug phototaxis and thermotaxis (Dohrmann *et al.*, 1984a),

consistent with an important role in intracellular signalling in slugs for either transmembrane Ca^{2+} fluxes or IP₃ mobilization of intracellular Ca^{2+} . In a similar vein, the data presented in this paper, combined with earlier work, suggest the possible involvement of G-proteins and IP₃ metabolism in signal transduction in slugs.

Pertussis and cholera toxins and fluoride ions (as aluminofluoride) are known in other organisms to affect intracellular signalling by acting on G-proteins. The dramatic effects of fluoride ions on slug behaviour were reported previously (Dohrmann *et al.*, 1984a). While slug behaviour was indifferent to the presence of cholera toxin, pertussis toxin significantly impaired phototaxis (Figs 1 and 2). In higher eukaryotes cholera toxin activates G_s but does not affect the other classes of G protein. Pertussis toxin and fluoride on the other hand are less specific in that they act on several different kinds of G protein (Kikkawa & Nishizuka, 1986). The greater specificity of cholera toxin might explain why it fails to affect slug phototaxis whereas both fluoride ions and pertussis toxin do. Leichtling *et al.* (1981) identified a 42 kDa G_s -like GTP-binding protein in *D. discoideum* that can be ADP ribosylated by cholera toxin. Our results indicate that this protein is unlikely to be important in signal transduction in slugs. Alternatively it may be inaccessible *in vivo* to cholera toxin because, for example, of the inability of the toxin to penetrate the *D. discoideum* membrane.

Pertussis toxin affects slug phototaxis much less dramatically than does fluoride (Dohrmann *et al.*, 1984a). While both slug phototaxis and thermotaxis are altered in the presence of fluoride, the effects on thermotaxis are less pronounced (Dohrmann *et al.*, 1984a). If the same were true for pertussis toxin, it might be difficult to detect a difference between thermotaxis in the presence and absence of the toxin. Alternatively, pertussis-toxin-sensitive G-protein(s) might be involved in slugs only in phototransduction, at a point before the signalling pathway converges with that for thermotaxis (Fisher *et al.*, 1984). Taken together, the effects of fluoride and pertussis toxin indicate that G-proteins transduce extracellular signals in *D. discoideum* slug behaviour. This conclusion is consistent with mounting evidence for G-protein signalling in the unicellular amoeboid stage of the *D. discoideum* life cycle (Newell *et al.*, 1988; Ludérus *et al.*, 1988; Snaar-Jagalska *et al.*, 1988; Kesbeke *et al.*, 1988; Europe-Finner *et al.*, 1988).

In mammalian cells G-proteins can activate hydrolysis of phosphatidylinositol biphosphate, releasing IP₃ into the cytosol and diacylglycerol in the membrane. Both products act as second messengers, IP₃ by mobilizing intracellular bound Ca^{2+} and diacyl glycerol by activating protein kinase C. PMA, a protein kinase C activator, was shown recently to inhibit by up to 50% the first spike of the cAMP-stimulated light-scattering change in aggregation-competent amoebae (Thiery *et al.*, 1988). *In vitro*, PMA reduces an ATP- and Ca^{2+} -dependent decrease in the number of cAMP receptors in membrane preparations from *D. discoideum* amoebae (Ludérus *et al.*, 1988).

We found little pharmacological evidence for diacylglycerol and protein kinase C signalling in slugs and, in particular, found no effect of PMA on slug behaviour, or indeed on aggregation and later development (not shown). However, our results do not exclude a role for protein kinase C in either aggregating amoebae or slugs. Indeed, it remains possible that the effects of D-sphingosine on thermotaxis are mediated by inhibition of protein kinase C. There is considerable evidence from other sources that extracellular cAMP signals might regulate slug tip position, and by this means slug behaviour, in a manner analogous to the way they determine the location of aggregation centres earlier in development (Fisher *et al.*, 1984). The effects of PMA on the cAMP responses of amoebae may be insufficient to markedly influence either aggregation or the behaviour of the multicellular slugs.

Li^+ ions perturb IP₃-signalling by inhibiting phosphatases that dephosphorylate the IP₃ and regenerate inositol for formation of new PIP₂. Several *D. discoideum* inositol phosphate phosphatases are inhibited *in vitro* by Li^+ with half-maximal concentrations ranging from 0.24 mM (Ins(1,4,5)P₃ phosphatase) to 2.5 mM (van Lookeren-Campagne *et al.*, 1988). Our observation that similar concentrations of Li^+ specifically impair slug phototaxis and positive thermotaxis suggests that IP₃ is an intracellular second messenger in slug behaviour. This is consistent with the mounting evidence for IP₃ signalling in the responses of aggregating amoebae to cAMP (Newell *et al.*, 1988; Europe-Finner & Newell, 1985, 1986a, b, 1987).

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