Ammonia differentially suppresses the cAMP chemotaxis of anterior-like cells and prestalk cells in *Dictyostelium discoideum*

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A drop assay for chemotaxis to cAMP confirms that both anterior-like cells (ALC) and prestalk cells (pst cells) respond to cAMP gradients. We present evidence that the chemotactic response of both ALC and pst cells is suppressed by ammonia, but a higher concentration of ammonia is required to suppress the response in pst cells. ALC show a chemotactic response to cAMP when moving on a substratum of prespore cells in isolated slug posteriors incubated under oxygen. ALC chemotaxis on a prespore cell substratum is suppressed by the same concentration of ammonia that suppresses ALC chemotaxis on the agar substratum in drop assays. Chemotaxis suppression is mediated by the unprotonated (NH_3) species of ammonia. The observed suppression, by ammonia, of ALC chemotaxis to cAMP supports our earlier hypothesis that ammonia is the tip-produced suppressor of such chemotaxis. We discuss implications of ammonia sensitivity of pst cells and ALC with regard to the movement and localization of ALC and pst cells in the slug and to the roles played by ALC in fruiting body formation. In addition, we suggest that a progressive decrease in sensitivity to ammonia is an important part of the maturation of ALC into pst cells.

1. Introduction

Anterior-like cells (ALC) of *Dictyostelium discoideum*, first defined by Sternfeld and David (1981, 1982), are a population of cells, localized in the prespore region of the slug, that resemble anterior cells (prestalk or pst cells) in exhibiting large neutral red-staining vacuoles. Subsequently, ALC were found to resemble pst cells in several molecular characteristics (Devine and Loomis 1985) but to differ in other characteristics, e.g. presence of a pre-spore antigen in the ALC (Early *et al* 1988). The ALC population is heterogeneous in some characteristics, e.g. of two genes encoding for extracellular matrix proteins, some ALC express only ecmA, some express only ecmB, some express both, and some apparently express neither (Gaskell *et al* 1992; Jermyn and Williams 1991; Jermyn *et al* 1989, 1996).

When the tip of a slug is excised, ALC stream forward from the prespore region and replace the missing pst cells at the new tip (Sternfeld and David 1981, 1982). The presence of an isolated slug tip next to an isolated prespore region suppresses an ALC localization response that Sternfeld and David (1982) interpret as a chemotactic response to cAMP; they conclude that the slug tip emits an inhibitor of the chemotactic response of ALC to cAMP, which results in the concentration of ALC in the slug posterior.

Recently, ALC are seen as part of a progression of cell types in the migrating slug (Sternfeld 1992; Abe *et al* 1994). Prespore cells differentiate into ALC, which differentiate into pstO cells (at the posterior end of the prestalk region), pstA cells at the anterior tip of the prestalk region, and finally pstAB cells (in a central core within the prestalk region); as the slug migrates, the pstAB cells are lost into the slime trail. This process of cell type transformation results in the regulation of migrating slug cell type proportions reported earlier (Raper 1940; Bonner 1957). Sternfeld and David (1982) identified the role of ALC in fruiting body formation as the construction of two supporting cups, one above and

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Abbreviations used: ALC, Anterior-like cells; pst cells, prestalk cells.

one below the sorus or spore mass (also see Jermyn *et al* 1991, 1996; Early *et al* 1993). Sternfeld (1998) showed that the upper cup provides most of the motive force that lifts the sorus up the stalk.

We have shown (Feit *et al* 1990) that adding ammonia to a migrating slug results in a large decrease in the pst cell population and a corresponding increase in the ALC population in the prespore region. The pst cell decrease can be interpreted, in the light of the slug cell type progression (Sternfeld l992), as the result of the blocking by NH_3 of the forward chemotactic movement of the ALC that would ordinarily have compensated for the loss of pstB cells into the slime trail. This ammonia effect led us to propose the hypothesis that NH_3 is the slug tip-produced suppressor of ALC chemotaxis towards a cAMP source.

Our current research addresses three questions that relate to the localization and behaviour of the two cell types (ALC and pst cells) in the migrating slug: (i) Are ALC and pst cells chemotactically sensitive to cAMP in a direct assay? (This part of our study is an attempt to test chemotaxis results for both cell types: pst cells and ALC, using an agar substratum dispersed amoeba drop assay. For comparison, we also include ALC assays on a prespore cell substratum.) (ii) If these cells are chemotactic to cAMP, is the chemotaxis suppressed by NH_3? (This is a test of the hypothesis that NH_3 is the slug tip-produced suppressor of the chemotaxis.) (iii) If NH_3 suppresses cAMP chemotaxis, are the two cell types (ALC and pst cells) equally sensitive to such suppression? (This question relates to the localization of the pst cells in the anterior and ALC in the posterior regions of the slug, respectively.)

2. Materials and methods

2.1 *Growth and harvesting of amoebae and formation of migrating slugs*

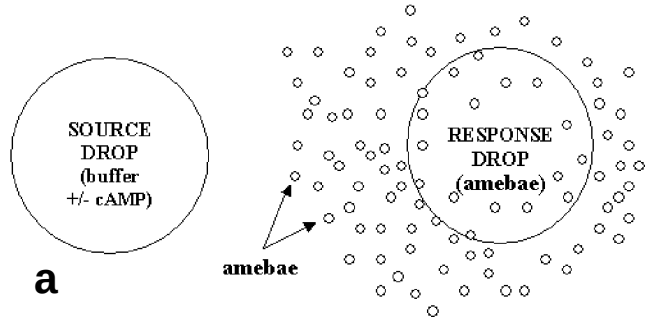
Stocks of *D. discoideum* (strain NC-4) were maintained on nutrient agar (Bonner l967) with *Escherichia coli* B/r as the food source. For experiments, the amoebae were grown in liquid culture at 22°C for 72 h using the method of Sussmann (1961) with the substitution of *E. coli B/r* for *Enterobacter aerogenes.* Flasks were shaken at 100 rpm in an EnvironShaker 3597 at 22°C. Amoebae were harvested by centrifugation on a Sorvall SP/X angle centrifuge for 3 min at 310 g (1500 rpm) and washed one time with 17 mM Na/K phosphate buffer, pH 6·6. The amoebae were suspended in 2 ml of the buffer, to which was added 0·1 ml of neutral red dye (Sigma, 0·02 mg/ml in 17 mM Na/K phosphate buffer, pH 6·6). The suspension was immediately diluted to 12 ml by addition of the same buffer (procedure modified from Sternfeld and David 1982). The amoebae were washed twice more with the buffer before resuspension in the buffer as a thick paste at a final concentration of about 1×10^8 cells/ml. The paste of amoebae was deposited in a line across the center of the surface of 2% Difco Bacto-Agar in Petri dishes (15 × 100 mm diameter, plastic). The dishes were covered with aluminum foil and incubated at 17°C; slugs began emerging from the lines of amoebae within 24 h.

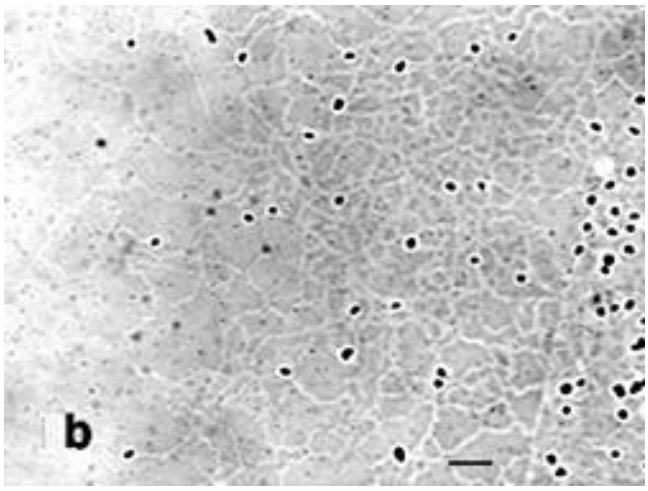
2.2 *Drop assays for chemotaxis of individual ALC and pst cell amoebae toward cAMP sources*

The red-stained anterior tips (the prestalk region) and the red-stained rear-guard cell regions at the extreme posterior ends of the neutral red-stained slugs were removed from each slug by use of the tip of a B-D 24 g inoculating needle and an eyelash attached to a Pasteur pipette. The isolated prespore regions served as a source of ALC and the isolated prestalk regions served as a source of pst cells. For each test of chemotaxis, four excised prespore or prestalk masses were placed in a 10 µl drop of 17 mM Na/K buffer of specified pH on the surface of 2% plain agar dissolved in 17 mm Na/K buffer, pH 6·6 in a 100 × 15 mm Petri dish. Such drops are referred to as "response drops" (figure 1). The excised cell masses were crushed and mixed together using a hair loop and a heat-rounded tip of a thin glass rod. A 10 µl "source" drop of 1×10^{-6}M cAMP (adenosine-3′,5′-cyclic monophosphate-sodium salt, Sigma) in buffer was placed, using a plastic template, at a distance of 3 mm from the "response drop" (figure 1a). The Petri dishes were wrapped in aluminum foil and incubated at 22°C for 48 h, after which the plates were examined for movement of ALC or pst cell amoebae outside of the response drops (figure 1b, c). The isolated prestalk masses contain only pst cells; the prespore masses contain both ALC and prespore cells. Since the ALC in the prespore masses have red-stained autophagic vacuoles and the prespore cells do not, we were able to determine that the emerging cells that we measured were in fact ALC.

For each plate, the distance migrated by the 5 farthest amoebae moving towards and the 5 farthest amoebae moving away from the source drop was recorded with the 10 × objective of a Nikon TMS inverted phase contrast microscope using digital video microscopy (Scion Image 1·62). To avoid including possible atypical movement of the outermost cells in the analysis of the data, we eliminated the distance value of the four outermost cells and used only the value of the fifth cell.

The differences between the distances amoebae moved toward and the distances they moved away from the source drop were analysed for significance by use of a paired *t*-test (Data Desk). Amoebae were considered to





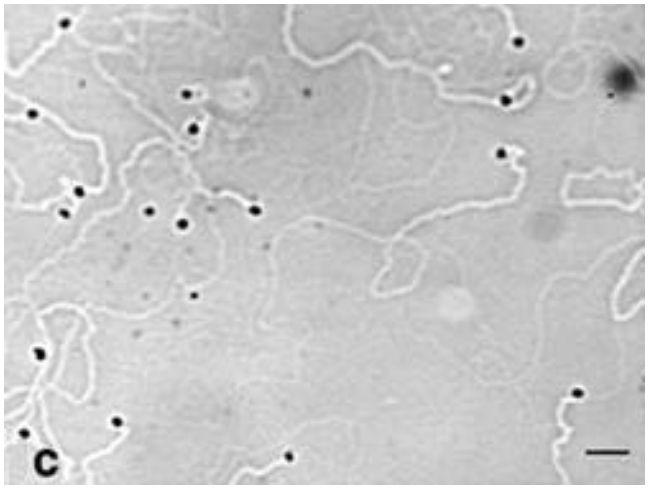


Figure 1. *Dictyostelium discoideum* cAMP chemotaxis drop assay. (**a**) Arrangement of "source" (buffer + 1×10^{-6} M cAMP) and "response" (amoeba) drops 3 mm apart on the agar surface in a 100 × 15 mm Petri dish. Amoebae are represented as small circles moving toward and away from the source drops. (**b**) ALC moving out of the response drop, which is positioned 3 mm from a source drop containing 1×10^{-6} M cAMP. The edge of response drop is at the right side (bar = 20 µm). (**c**) ALC moving toward a cAMP source drop, showing more clearly the trails that the amoebae leave behind on the agar surface (bar = 20 µm).

show a positive chemotactic response to cAMP if there was a significant difference in their movement (at a significance level of 0·05), and no significant difference in controls in which source drops lacked cAMP.

2.3 *Drop assays for ammonia inhibition of ALC and pst cell chemotaxis to cAMP*

The tests for ammonia inhibition of chemotaxis were performed as described above, except that the agar substratum contained specified concentrations of NH_4Cl in Na/K buffer, pH 6·6 and results of the tests were compared to results of control tests in which the agar contained equivalent concentrations of NaCl. In these inhibition tests, both experimental and control source drops contained 1×10^{-6} M cAMP. A given concentration of NH_4Cl was considered to inhibit chemotactic response if there was no significant toward and away movement of amoebae (at a significance level of 0·05) in plates in which the agar contained that concentration of NH_4Cl, and if there was a significant difference in the movement in the corresponding NaCl control plates.

2.4 *Testing for ALC chemotaxis to cAMP and for ammonia suppression of such chemotaxis in submerged, oxygenated isolated slug posteriors suspended in 0·17 mM Na/K buffer, pH 6·6 or 7·5*

The red-stained anterior tips (the prestalk region) and the red-stained rear-guard cell regions at the extreme posterior ends of the neutral red-stained slugs were removed as in the drop assay procedure. Each isolated prespore mass was placed into a separate suspension drop of 17 mM Na/K phosphate buffer of specified pH; all drops except chemotaxis response controls contained 1×10^{-6} M cAMP (procedure adapted from Sternfeld and David 1981). For tests of ammonia inhibition, the drops also contained specified concentrations of NH_4Cl or, in controls, NaCl. The isolated slug posteriors were crushed with a small glass ball at the end of a pulled glass microneedle to mix the stained and unstained cells within the masses before they rounded up. Each drop was positioned within a glass ring sealed with high vacuum stopcock grease to the bottom of a 15 × 35 mm diameter Petri dish. The dishes were placed into a glove box lined with moistened paper towels and agitated on an Eberbach reciprocating shaker at 100 oscillations/min while moisture-saturated 100% oxygen was circulated through the box.

After 1·5–2 h of incubation, the rounded-up slug posteriors were examined under the microscope and scored for chemotactic response to cAMP [movement of the red-stained ALC to the periphery (figure 2a) versus no response (figure 2b)]. Statistical significance of the

chemotactic response (significant ALC movement to the periphery), and of ammonia suppression of the chemotactic response (lack of significant ALC movement to the periphery) was analysed by c^2 analysis (Data Desk).

3. Results

3.1 *Chemotactic response of ALC and pst cells in the drop assay*

Both ALC and pst cells show a chemotactic response to cAMP, based on the following observations: When 1×10^{-6} M cAMP is present in the source drops, both cell types show a significant movement toward the drops compared with their movement away. No significant difference between toward and away movement is seen in controls, in which source drops lack cAMP (table 1).

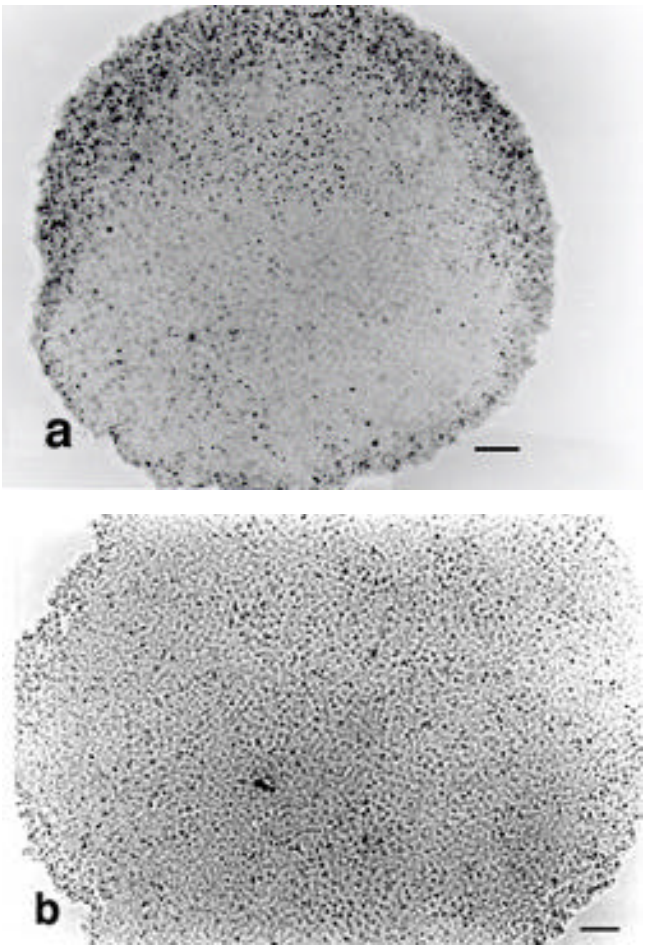


Figure 2. Squashes of isolated *Dictyostelium discoideum* slug prespore regions (prestalk and rearguard regions have been removed) incubated in an oxygenated 17 mM Na/K phosphate buffer, pH 6·6. (**a**) The buffer contains 1×10^{-6} M cAMP. Most of the ALC (visualized by neutral red staining of their autophagic vacuoles) have moved to the periphery of the rounded prespore mass (bar = 20 µm). (**b**) Control squash incubated in buffer lacking cAMP. The ALC are dispersed through the slug posterior (bar = 20 µm).

3.2 *Inhibitory effect of ammonia on the chemotactic response of ALC and pst cells in the drop assay*

The validity of the drop assay is supported by these observations (tables 1, 2):

In every case in which cAMP is present in the source drop, except when the higher concentrations of ammonia are present in the agar substratum, the difference in movement of amoebae toward the source drop compared to movement away from the source drop is significant (49/49 cases). In every case in which cAMP is not present in the source drop, the difference is not significant (29/29 cases).

The chemotactic response of ALC to cAMP is suppressed by 1×10^{-3} M NH_4Cl ($NH_{3\ unprotonated} = 2 \times 10^{-6}$ M), but not by 5×10^{-4} M NH_4Cl ($NH_{3\ unprot} = 1 \times 10^{-6}$ M) (table 2). In each case, comparison is made with the appropriate control lacking ammonia; in these experiments, NaCl control source drops contain 1×10^{-6} M cAMP. Amoebae in all these NaCl controls show chemotaxis toward cAMP, i.e. a significant difference in movement toward and away from the source drops.

The chemotactic response of pst cells to cAMP is suppressed by 5×10^{-6} M NH_4Cl ($NH_{3\ unprot} = 1 \times 10^{-5}$ M), but not by 1×10^{-3} M NH_4Cl ($NH_{3\ unprot} = 2 \times 10^{-6}$ M) (table 2). As with ALC, comparisons were made with the appropriate controls lacking ammonia and these controls show chemotaxis toward cAMP.

3.3 *Chemotactic response of ALC, in submerged oxygenated prespore masses, to a gradient of cAMP*

The oxygenated submerged prespore masses were scored for distribution of the stained ALC to the periphery versus non-peripheral distribution of the cells. ALC, under the assay conditions used, redistribute to the periphery of the rounded-up slug prespore masses (figure 1) when exposed to a gradient established by immersing the posteriors in 1×10^{-6} M cAMP (table 3). Control slugs immersed in buffer lacking cAMP do not show such a redistribution of ALC to the periphery. Following the interpretation of Sternfeld and David (1981), who designed this assay, we consider these results to be evidence of chemotactic response to cAMP.

3.4 *Inhibitory effect of ammonia on the chemotactic response of ALC, in submerged oxygenated prespore masses, to a gradient of cAMP*

In isolated prespore masses submerged in oxygenated 0·17 mM Na/K buffer, treatment with 1×10^{-3} M NH_4Cl ($NH_{3\ unprot} = 2 \times 10^{-6}$ M) suppresses the chemotactic response of ALC to a gradient of cAMP, while 5×10^{-4} M NH_4Cl ($NH_{3\ unprot} = 1 \times 10^{-6}$ M) does not. Comparisons are made to controls lacking NH_4Cl in the pH 6·6 buffer; these controls show a significant chemotactic response.

3.5 *pH dependence of the inhibitory effect of ammonia on the chemotaxis of ALC to cAMP in submerged oxygenated prespore masses*

In isolated prespore masses submerged in oxygenated 0·17 mM Na/K buffer, treatment with 5×10^{-4} M NH_4Cl ($NH_{3\ unprot} = 1 \times 10^{-6}$ M) suppresses the chemotactic response of ALC at pH 7·5 but not at pH 6·5 (table 5). At each pH, controls lacking NH_4Cl in the submersion buffer show a significant chemotactic response to 1×10^{-6} M cAMP.

Table 1. Chemotactic response of ALC and pst cells to 1×10^{-6} M cAMP.

	Distance of amoeba movement (mm)			
Treatment	Toward cAMP	Away from cAMP	*n*	*P*
ALC				
1×10^{-6} M cAMP in the source drop	1·54	0·55	15	0·001*
Buffer, no cAMP in the source drop (control)	0·64	0·67	15	0·277
pst cells				
1×10^{-6} M cAMP in the source drop	1·18	0·833	14	0·0001*
Buffer, no cAMP in the source drop (control)	1·22	1·21	14	0·895

Both source and response drops are placed on 17 mm Na/K buffer agar, pH 6·6.
P = Probability that the difference in distance moved toward the drop and the distance moved away from the drop is due to chance alone (null hypothesis), based on a paired *t*-test.
*Significant movement (chemotaxis) toward the source drop, using a significance level of 0·05.

Table 2. Effect of NH_4Cl on cAMP chemotaxis of ALC and pst cells.

	Distance of amoeba movement (mm)			
Treatment	Toward cAMP	Away from cAMP	*n*	*P*
ALC				
1×10^{-3} M NH_4Cl in the agar	1·71	1·62	5	0·340
1×10^{-3} M NaCl in the agar (control)	2·00	1·41	5	0·015*
5×10^{-4} M NH_4Cl in the agar	1·18	0·84	5	0·025*
5×10^{-4} M NaCl in the agar (control)	1·48	0·92	5	0·009*
Pst cells				
5×10^{-3} M NH_4Cl	1·79	1·52	5	0·054
5×10^{-3} M NaCl in the agar (control)	2·35	1·31	5	0·013*
1×10^{-3} M NH_4Cl	2·26	1·69	5	0·01*
1×10^{-3} M NaCl in the agar (control)	1·45	0·71	5	0·0054*

Both source and response drops are placed on 17 mm Na/K buffer agar, pH 6·6. NH_4Cl or NaCl are dissolved in the agar substratum.
All treatments include 1×10^{-6} M cAMP in the source drop.
P = Probability that the difference in distance moved toward the drop and the distance moved away from the drop is due to chance alone (null hypothesis), based on a paired *t*-test.
*Significant movement (chemotaxis) toward the source drop, using a significance level of 0·05.

Table 3. Chemotactic response to cAMP of ALC in isolated prespore masses submerged in oxygenated 17 mm Na/K buffer, pH 6·6.

Treatment	Isolated posteriors with ALC at the periphery	c^2	P
1×10^{-6} M cAMP + buffer	68·8% (n = 45)	26·23	0·01*
pH 6·6 buffer only (control)	11·9% (n = 42)		

P = Probability that the movement of ALC to the periphery of isolated prespore masses independent of exposure to the exogenously-generated gradient of cAMP, as determined by c^2 analysis.
*Difference in the fraction of isolated prespore masses with ALC localized at the periphery relative to the control is significant at the 0·05 confidence level.

Table 4. Effect of NH_4Cl on chemotaxis of ALC to cAMP in isolated prespore masses submerged in oxygenated 17 mm Na/K buffer, pH 6·6.

Treatment	Isolated posteriors with ALC at the periphery	c^2	P
1×10^{-3} M NH_4Cl*	28·0% (n = 25)	17·89	0·01*
5×10^{-4} M NH_4Cl	70·8% (n = 24)	0·01	0·92
pH 6·6 buffer (control)	73·3% (n = 101)		

All treatments include 1×10^{-6} M cAMP in the 17 mM Na/K buffer in the source drops.
P = Probability that the movement of ALC to the periphery of isolated prespore masses is independent of exposure to the exogenously-generated gradient of cAMP, as determined by c^2 analysis.
*Difference in the fraction of isolated prespore masses with ALC localized at the periphery relative to the control is significant at the 0·05 confidence level.

Table 5. pH dependence of the effect of NH_4Cl on ALC chemotaxis to cAMP in isolated prespore masses submerged in oxygenated 0·17 mM Na/K buffer.

Treatment	Isolated posteriors with ALC at the periphery	c^2	P
pH 6·6			
5×10^{-4} M NH_4Cl[a]	70·8% (n = 24)	0·01	0·92
pH 6·6 buffer (control)[a]	73·3% (n = 101)		
pH 7·5			
5×10^{-4} M NH_4Cl*	41·2% (n = 165)	4·32	0·05*
pH 7·5 buffer (control)	62·6% (n = 131)		

All treatments include 1×10^{-6} M cAMP in the buffer.
P = Probability that the movement of ALC to the periphery of isolated prespore masses is independent of exposure to the exogenously-generated gradient of cAMP, as determined by c^2 analysis.
*Difference in the fraction of isolated prespore masses with ALC localized at the periphery relative to the control is significant at the 0·05 confidence level.
[a]Same data as in table 4.

4. Discussion

4.1 *Chemotactic response of ALC and pst cells to cAMP*

Both pst cells and ALC respond chemotactically to 1×10^{-6} M cAMP. Pst cells show this chemotactic response to cyclic AMP in drop assays on an agar substratum (table 1). ALC show the response both as isolated cells moving on an agar substratum in the drop assay (table 1) and when moving on a substratum of prespore cells in submerged prespore isolates (table 3). These findings confirm results of three studies that involved movement of neutral red stained cells on a prespore cell substratum: Sternfeld and David (1981) reported cAMP chemotaxis of ALC and of "stained cells" in a mixture of ALC and pst cells, Matsukuma and Durston (1979) described chemotaxis of pst cells toward cAMP, and Traynor *et al* (1992) reported that ALC respond chemotactically to basal sources of cAMP in cAMP-depleted cell masses. Our observation that pst cells on an agar substratum are chemotactic to cAMP agrees with the report of Mee *et al* (1985) that density gradient- separated pst cells (or perhaps a mixture of pst cells and ALC) are chemotactic to cAMP on agar surface assays.

4.2 *The role of ammonia in cell type patterning*

With regard to cell patterning in the slug, ammonia has been suggested to play a role as a key signal and regulatory molecule in a source/sink model (Cotter *et al* 1993), and in an activator/inhibitor model (Inouye 1990). Ammonia stimulates prespore cell gene expression and suppresses prestalk gene expression (Oyama and Blumberg 1986; Gross *et al* 1983; Bradbury and Gross 1989) Ammonia inhibits DIF accumulation (Naeve *et al* 1983, 1988) and stalk cell maturation (Inouye 1988) and promotes spore cell formation (Gross *et al* 1981, 1983; Town 1984; Riley and Barclay 1986; Riley *et al* 1989) Stalk cell maturation is triggered by ammonia depletion (Wang and Schaap 1989; Schaap 1991; Dominov and Town 1986).

Several mutants affect cell type progression and cell patterning in *Dictyostelium*. For example, Wariai (Wri) null mutants, produce an increase in the pstO compartment of the slug at the expense of the prespore region, while the pstA and pstAB compartments remain unaltered (Han and Firtel 1998). Expression of the *wri* gene in the pstA cells apparently regulates the differentiation and proportion of pstO cells. Similarly, slugger mutants, with low proportions of pst cells relative to prespore cells, show increased sensitivity to ammonia (Sussman *et al* 1978). Cell patterning mutants could be valuable tools in dissecting the role that cellular mechanisms of resistance of chemotaxis to suppression by ammonia play in the progression of cell types.

4.3 *The inhibitory effect of ammonia on the chemotactic response of ALC and pst cells toward cAMP: Implications for the localization of the ALC and pst cell types in the migrating slug*

Ammonia suppresses the chemotactic response of both ALC and pst cells to cAMP compared to NaCl controls, but pst cells require a higher concentration of ammonia for suppression of the response (table 2). Amoebae in the slug show chemotactic response to cAMP relay waves emanating from the tip (Siegert and Weijer 1992). The slug tip is a source of ammonia due to high levels of ammonia production resulting from deamination during autophagic breakdown of proteins and nucleic acids (Gregg *et al* 1954; Wright and Anderson 1960). A diffusible substance emitted by the slug tip suppresses the forward chemotaxis of ALC but not of pst cells (Sternfeld and David 1981). If ammonia is the tip-emitted diffusible inhibitor of chemotaxis, the relative lack of sensitivity of pst cells compared to ALC with regard to ammonia chemotaxis suppression could account for the localization of the pst cells in the anterior and the ALC in the posterior, prespore region of the slug.

4.4 *Is NH_3 or NH_4^+ responsible for the suppression of the ALC chemotactic response to cAMP?*

The pH dependence of the suppression effect enables us to assess which species of ammonia, the protonated (NH_4^+) or the unprotonated (NH_3) species, is the active agent. The two species of ammonia are in equilibrium and an decrease in pH shifts the ratio in favour of the NH_4^+ species due to protonation of the uncharged species. The relationship is given by the equation:

$$(NH_4^+)/(NH_3) = 10^{9{\cdot}3 - pH}.$$

For a given concentration of NH_4Cl, the concentration of unprotonated NH_3 is 7·9-times as high at pH 7 as it is at pH 6·6. The observation that the chemotactic response of ALC on a prespore cell substratum is suppressed by 1×10^{-4} M NH_4Cl at pH 7·5, but not by the same concentration at pH 6·6 (table 5) supports the hypothesis that the unprotonated (NH_3) species of ammonia is the effective agent of suppression of the chemotactic response of the ALC to cAMP.

The activity of the unprotonated (NH_3) rather than the protonated (NH_4^+) species of ammonia accords with earlier findings on ammonia suppression of the transition from migration to fruiting (Schindler and Sussman 1977), on ammonia bleaching of acid autophagic vacuoles (Yamamoto and Takeuchi 1983), on ammonia promotion of differentiation in submerged cell masses (Sternfeld and David 1979) and on ammonia promotion of spore cell differentiation (Gross *et al* 1981, 1983; Naeve *et al* 1983, 1988; Riley *et al* 1989; Riley and Barclay 1990).

4.5 *How does ammonia suppress the chemotactic response of ALC and pst cells?*

The greater effectiveness of unprotonated ammonia (NH_3) compared to the ammonium ion (NH_4^+) in suppression of the chemotactic response of ALC may relate to the relative permeability of the cell membrane of the amoebae to the uncharged form, and to the effectiveness of neutral ammonia in alkalinization of the cytoplasm (Inouye 1988).

The inhibitory action of ammonia on the chemotactic response of the ALC may be mediated by the ability of ammonia to suppress the intracellular accumulation of cAMP (Schindler and Sussman 1979) and its ability to affect the levels of extracellular cAMP as well (Schindler and Sussman 1979; Thadani *et al* 1977**;** Riley and Barclay 1990). Ammonia may act through alkalinization of intracellular acid compartments, with a resultant decrease in Ca^{2+} sequestration by a Ca^+/H^+ antiport; prolonged Ca^{2+} transients in response to cAMP stimulation may impact on processes essential to chemotaxis (Gross *et al* 1988; Davies *et al* 1993).

Alterations in uptake or sequestration of calcium may play an important role in the progression of cell types from prespore cells to ALC to pst cells. Increase in free or cytosolic calcium (Ca_i^{2+}) favours differentiation of amoebas into pst cells (Cubitt *et al* 1995, 1998; Azhar *et al* 1996).

The levels of total Ca^{2+} in the prestalk region of the slug are higher than those in the prespore region (Maeda and Maeda 1973; Azhar *et al* 1996). The increase in calcium level occurs in both sequestered Ca^{2+} and in Ca_i^{2+} (Azhar *et al* 1996; Tirlapur *et al* 1991; Cubitt *et al* 1995). Observations by Cubitt *et al* of calcium spikes with a calcium doubling time of about 1 min suggest the existence of fast calcium waves (Jaffe 1995), which may be involved in

chemotactic response movement. These observations on both free and sequestered calcium accord well with the proposal that resistance to chemotaxis suppression is mediated by increased ability to sequester calcium, since bursts of free calcium may be needed for generation of fast calcium waves during chemotaxis. We assume that such periodic unloading is consistent with relatively high cytosolic calcium.

4.6 *A suggestion that as ALC mature into pst cells, they decrease their sensitivity to ammonia, with implications for* Dictyostelium *development*

We propose that, as ALC cells differentiate from prespore cells and develop towards becoming pst cells, part of their maturation process may involve a progressive loss of sensitivity to ammonia suppression of the chemotactic response. The resulting spectrum of degrees of ALC sensitivity to ammonia would explain why ALC are concentrated in the prespore mass yet at any given time a fraction of the ALC are moving into the prestalk zone (Sternfeld 1992). Since the slug tip is an ammonia source, our proposal would explain why a larger fraction of the ALC enters the anterior of a slug when the tip has been excised (Raper 1940; Bonner 1957; Sternfeld and David 1981, 1982). The spectrum of degrees of ALC ammonia sensitivity would also explain the deficit in pst cells when elevated ammonia concentrations are applied to the slug (Feit *et al* 1990), since these levels would block ALC that would otherwise have been able to move into the prestalk zone. Sternfeld and David (1981) found that in a mixture or ALC and pst cells, the pst cells relocated to the front and the ALC to the rear, but if the attachment of fresh anterior tissue is delayed for 1·5 h, the ALC relocate to the front; this is in accord with our proposal of a progressive decrease in ALC sensitivity to ammonia.

If ALC decrease in sensitivity to ammonia as they mature, we would expect a concentration, at the anterior end of the prespore region, of ALC that are the most chemotactic and the most ready to transform into pst cells. According to Pogge-von Strandman and Kay (1990), the new anterior region of a regenerating slug posterior is formed predominantly from cells in the front of the prespore region. These ALC cells would transform initially into pstO cells (Jermyn *et al* 1991, 1996; Sternfeld 1992; Abe *et al* 1994); this idea is supported by the observation that the *ecmA* gene in both ALC and pstO cells is turned on by a distal promoter, while the *ecmA* gene in pstA cells is turned on by a more proximal promoter (Early *et al* 1993).

The progressive desensitization to ammonia begun in the slug posterior may continue in the slug anterior even after the ALC have transformed into pst cells. Early *et al* (1995) observed that pstA cells move twice as fast toward a source of cAMP as pstO cells do. This difference in movement may reflect decreased sensitivity to ammonia suppression of chemotoaxis as cells transform from pstO cells to pstA cells. Also, in 10% of cases in which pstA and pstAB cells were transplanted into slug posteriors, these cells formed new slug tips, but pstO cells, which are transitional between ALC and pstA cells, did not form new tips (Buhl and MacWilliams 1991). If we assume that the ability of cells to form a new tip requires chemotactic responsiveness, these results also suggest that the progressive desensitization to ammonia may continue in the pst cells of the slug anterior.

4.6 *Relation of our findings to* Dictyostelium *fruiting body formation*

The evidence that we have presented for suppression of the chemotactic response of the ALC to cAMP supports our hypothesis that the tip-emitted suppressor of this response (Sternfeld and David 1981) is ammonia. This implies that ammonia, by inhibiting chemotaxis to cAMP, maintains a population of ALC in the posterior of the migrating slug.

When fruiting is initiated, ammonia production at the slug tip decreases (Sussman *et al* 1978; Wilson and Rutherford 1978; Schindler and Sussman 1979; Wang and Schaap 1989) and ALC move to the front and rear of the prespore region to contribute to the upper and lower cups of the sorus (Sternfeld and David 1981, 1982; Early *et al* 1993; Jermyn and Williams 1991, 1996; Dormann *et al* 1996; Bichler and Weijer 1994). Based on the sensitivity of ALC to ammonia, we suggest that the decrease in slug ammonia levels may allow localized chemotactic activity of ALC that results in formation of the two cups. The phenomenon of released local chemotaxis of ALC may be comparable to the formation of stripes of stained ALC in the slug posterior when the ambient ammonia concentration is lowered enzymatically (Feit *et al* 1990). The decreased slug ammonia level and a resultant increase in ALC chemotaxis to cAMP could also allow the ALC of the upper cup to play their role in the raising of the spore mass up the stalk.

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