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Ammonia hypersensitivity of slugger mutants of *D. discoideum*

Kathryn Gee, Frances Russell and Julian D. Gross

Department of Biochemistry, Oxford University, South Parks Road, Oxford, UK

SUMMARY

The weak base ammonia inhibits aggregation and culmination of wild-type amoebae of *Dictyostelium discoideum*. Here we have examined its effect on a series of 'slugger' mutants previously assigned to 10 complementation groups, and so-called because they remain as slugs for extended periods. We show that the mutants accumulate normal levels of ammonia and hence may be abnormally susceptible to the ammonia they produce. In agreement with this we find that representatives of the slugger complementation groups are hypersensitive to ammonia inhibition at three clearly recognisable morphological stages of development: aggregation, tip formation and culmination. This finding suggests that a common ammonia-sensitive process underlies each of these developmental events.

Key words: *Dictyostelium*, slugger mutants, ammonia sensitivity

INTRODUCTION

During development starved amoebae of *Dictyostelium discoideum* aggregate in response to periodic cyclic AMP signals and collect into hemispherical mounds of cells. A protruding 'tip' forms at the apex of each mound and the aggregate gradually elongates to form an upright finger-like structure. Depending on environmental conditions, this either transforms directly into a mature fruiting body (a process referred to as 'culmination') or collapses onto the substratum and migrates for a variable period as a 'slug' (Newell et al., 1969).

Schindler and Sussman (1977) have shown that exposure of slugs to an enzyme cocktail that destroys ammonia induces transformation of the slugs into fruiting bodies. Ammonia is produced in large amounts during development as a result of protein catabolism and these workers concluded that accumulation of ammonia is responsible for inducing and maintaining the slug stage. In agreement with this view Bonner et al. (1985) showed that fruiting body formation is favoured by development at low pH and suggested that this was due to a lowering of the concentration of the uncharged form of the weak base, NH_3, relative to the protonated form NH_4^+. Similarly, pH effects have indicated that uncharged ammonia is active in the stimulation of spore formation and inhibition of stalk cell formation by sporogenous mutants (Gross et al., 1983; Riley and Barclay, 1990). Inouye (1988a) showed that culmination can be induced by exposure to atmospheric CO_2 and weak acids, both of which cause cytoplasmic acidification and would therefore decrease the NH_3:NH_4^+ ratio (Inouye, 1988b).

Davies et al. (1993) reported that aggregation of the wild-type strain NC4 is delayed, and that the number of aggregates formed drops progressively as a function of ammonia concentration. Such effects of ammonia on aggregation as well as culmination are consistent with the finding of Williams et al. (1984) that ammonia inhibits the transitory activation of cyclic AMP synthesis following binding of cyclic AMP to cell surface receptors (cyclic AMP 'relay'), and with recent evidence that protein kinase A, is required for aggregation as well as later developmental events (Simon et al., 1989; Firtel and Chapman, 1990; Kay, 1992; Harwood et al., 1992). In order to obtain further insight into the range of developmental processes that are affected by ammonia we have examined its effect, as well as the effect of extracellular pH, on the development of a series of 'slugger' mutants. Slugger mutants are so called because they remain as slugs for abnormally long periods and hence may be hypersensitive to ammonia (Schindler and Sussman, 1979; Newell and Ross, 1982). Thirty-two such mutants have previously been assigned to 10 complementation groups by parasexual techniques (Newell and Ross, 1982).

MATERIALS AND METHODS

Development on filters

Parental strains and their slugger derivatives were grown on SM agar (Sussman, 1987) at 22˚C in the dark in association with *Klebsiella aerogenes* strain OXF1 (Williams and Newell, 1976). The genotypes and developmental characteristics of members of the different slugger complementation groups are described by Newell and Ross (1982). When the growth plates were beginning to clear, after 24-30 hours, they were harvested in Bonner's standard salts solution (SS) (Bonner, 1947). After washing three times by centrifugation at 190 ***g*** for 2 minutes amoebae were resuspended at 10^8 per ml in LPS and pipetted evenly onto the surface of a quartered Millipore HABP04700 filter on top of a Whatman no. 17 filter in a 90 cm Petri dish. The cell density on the filters was 2×10^6 per cm^2. All filters had been boiled in water for 2 minutes, dried in a sterile hood and soaked for several hours before the experiment in LPS (pH 7.3) containing the appropriate concentration of ammonia or other weak base. The Petri dishes were incubated in a humid box in the dark at 22˚C and examined periodically with a Nikon SMZ-2T microscope. Photographs were taken with a Ricoh KR-10M camera using Ilford PANF black and white film.

Development on agar

Cells of parental strains and various slugger mutants were grown and harvested as described above. The cells were resuspended in LPS and plated at 3.5×10^5 cells per cm^2 on Difco Bacto agar buffered at various pH values with LPS (Newell et al., 1969). The plates were incubated in humid boxes over a fluorescent light source and the fraction of aggregates that had fruited was scored after about 50 hours.

Measurement of ammonia

Ammonia was assayed using an ammonia gas sensing probe (EDT model ISE 321, EDT Research, 14 Trading Estate Road, London NW10 7LU), which responds to the partial pressure of ammonia gas in solution. A standard curve of E versus the log of the concentration of ammonia was produced using standard solutions of ammonium chloride in water.

After 20 or 40 hours the lower pads that had supported filters carrying developing cells were soaked in distilled water for 10 minutes to allow the ammonia to diffuse from the pads. For each assay, 5 ml of the resulting solution was put in a vial, 0.5 ml 1 M NaOH was added and the liquid was constantly stirred with a magnetic bar. The ammonia probe was placed into the solution and the voltage recorded once the reading had settled (after about 5 minutes). Lower ammonia concentrations were assayed first to minimise the required response times.

RESULTS

Ammonia production

One explanation for the prolonged slug migration characteristic of slugger mutants is that they produce abnormally large quantities of ammonia. In order to examine this possibility we compared ammonia production in the slugger mutants with that of the four parental strains from which they are derived (Newell and Ross, 1982). After 20 and 40 hours' incubation the ammonia released into the lower pad was taken up in water and measured with an ammonia-sensitive electrode (see Materials and Methods). In each case the amounts at 20 hours were approximately 75% of those at 40 hours. The averages of three independent measurements at 40 hours for each strain are given in Table 1. In the parental strains between 1 mM and 3 mM ammonia accumulates in the lower pads. This is equivalent to 10-30 μmoles per 10^8 cells, similar to the figure obtained by Schindler and Sussman (1977) for developing NC4 amoebae.

Examination of the ammonia production levels in Table 1 indicates that slugger mutants do not accumulate significantly higher levels of ammonia than their corresponding parental strain, and so presumably are hypersensitive to the normal levels of ammonia that they accumulate within aggregates (see below). Ammonia production has previously been shown to be normal in mutants KY3 (group D), JC2 (group C) and JC4 (group C) (Schindler and Sussman, 1979; Sussman et al., 1978).

Ammonia hypersensitivity of culmination in slugger mutants

Inhibition of culmination by ammonia has been studied in the wild-type strain NC4 by Schindler and Sussman (1977) and Davies et al. (1993). The former reported inhibition of culmination by concentrations of ammonia in excess of 16 mM when aggregates were incubated on filters over buffer in Lucite chambers. Davies et al. (1993) found that inhibition required

Table 1. Summary of properties of slugger mutants

Strain (group, parent)		Concentration of ammonia at 40 hours (mM)	% of aggregates that fail to culminate	Sensitivity of aggregation to ammonia	% of aggregates without tips
NC4		1.47±0.45	0	–	1
NP187		1.12±0.16	0	–	23
XP55		1.85±0.43	0	–	0
X22		1.30±1.08	0	–	0
NP419	(A, XP55)	1.25±0.58	100	1.14	64
NP446	(A, NP187)	1.16±0.33	100	2.74	7
NP407	(B, XP55)	1.41±0.17	92	1.61	9
NP445	(B, NP187)	1.15±0.17	88	4.85	17
JC2	(C, NC4)	1.69±0.26	91	1.28	25
JC4	(C, NC4)	1.52±0.89	98	*	14
NP351	(C, X22)	1.68±0.74	100	*	27
KY3	(D, NC4)	1.24±0.28	3	1.83	40
NP392	(E, XP55)	2.16±0.73	2	1.22	11
NP422	(E, NP187)	1.79±0.18	27	1.71	78
NP440	(F, NP187)	1.72±0.31	96	0.99	100
NP441	(F, NP187)	1.63±0.42	100	3.60	31
NP452	(F, NP187)	2.23±0.78	100	2.35	70
NP409	(G, XP55)	1.53±0.14	27	3.04	38
NP410	(G, XP55)	1.86±0.17	71	2.01	55
NP449	(H, NP187)	1.20±0.23	100	2.91	25
NP435	(I, NP187)	1.43±0.13	83	1.46	70
NP439	(J, NP187)	1.06±0.11	84	1.06	55

The amount of ammonia produced by developing aggregates after 40 hours on filters in the absence of added ammonia was measured as described in Materials and Methods. Data are from 3 to 7 experiments for each strain, mean ± s.d. The percentage of aggregates that failed to culminate was measured after 40 hours of development on filters at pH 7.3 in the absence of added ammonia. These figures include tipless aggregates. The "sensitivity of aggregation to ammonia" is presented as the ratio of the ammonia concentration required to reduce the number of aggregates formed by 50% in the parental strain to that required in the mutant. (*This value is unavailable for these strains as the concentration of ammonia required to produce this effect exceeds the highest concentration of ammonia to which the mutants were exposed, 50 mM.) The percentage of aggregates without tips was scored after 40 hours on filters at the highest concentration of ammonia at which aggregates form.

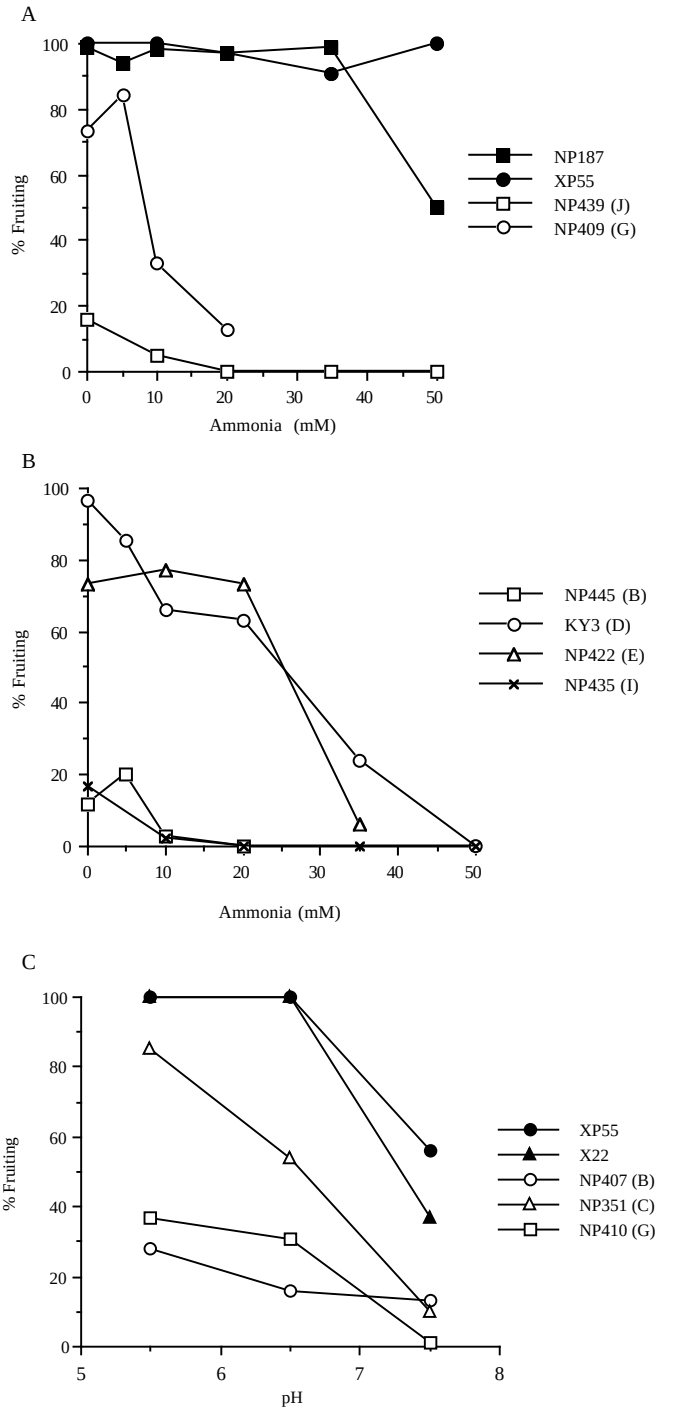

Fig. 1. The ammonia hypersensitivity of culmination in slugger mutants. Cells were washed, plated out for development on filters and scored after 40 hours as described in Materials and Methods. Structures that had a distinct spore-head and a stalk were counted as fruiting bodies, whether or not viable spores were produced. The percentage of aggregates that had fruited after 40 hours is shown. (A and B) Development on filters. The results are means of 3 to 16 experiments for each strain at each concentration with duplicate plates for each experiment. The group to which the slugger strain belongs is shown in parenthesis. Aggregates of the other B group mutant (NP407) and E group mutant (NP392) examined culminated eventually at the highest concentration of ammonia at which aggregates formed. There was negligible fruiting in members of the groups not shown. (C) Effect of extracellular pH on fruiting on agar. Each point represents a mean of 3 to 11 determinations. Standard deviations are omitted for clarity.

concentrations of ammonia between 50 and 100 mM when aggregates were incubated on filters resting on buffer-soaked support pads. Fig. 1A (filled symbols) shows the response of two of the four parental strains to concentrations of ammonia up to 50 mM under the present conditions, i.e. incubation on Millipore filters with LPS at pH 7.3. It can be seen that these concentrations had no effect on fruiting in XP55 while in NP187 about 50% of aggregates failed to culminate in the presence of 50 mM ammonia. The other two parental strains, NC4 and X22, behaved like XP55 (not shown).

We have examined the development of representatives of all of the slugger complementation groups on Millipore filters with LPS at pH 7.3. Mutants belonging to groups A, C, F and H failed to form any fruits under these conditions (Table 1). On the other hand, members of groups B, D, E, G, I and J did form significant numbers of fruits and in these cases it was possible to test whether culmination is abnormally sensitive to ammonia. The results in Fig. 1A (open symbols) and Fig. 1B show that the fruiting of certain mutants in each of groups B, D, E, G, I and J is hypersensitive to ammonia. In general these mutants culminated less on agar at pH 7.3 than on filters at the same pH, and their culmination was stimulated at low pH (Fig. 1C) where the concentration of free ammonia should be greatly reduced (Schindler and Sussman, 1977; Bonner et al., 1985; Newell and Ross, 1982). Mutants belonging to groups A, F and H failed to fruit even at pH 5.5, and there was some stimulation of fruiting in group C mutants, by low pH.

Taken together these results indicate that the slugger phenotype of members of at least six of the slugger complementation groups, groups B, D, E, G, I and J, is due to hypersensitivity to ammonia. Schindler and Sussman (1979) have previously demonstrated hypersensitivity of culmination to ammonia in mutant KY3 (group D).

Inhibition of aggregation

Davies et al. (1993) reported that aggregation of the wild-type strain NC4 is delayed and that the number of aggregates formed is progressively reduced as a function of ammonia concentration. Similar results were obtained in the present experiments, with the parental strains XP55 and NP187 being somewhat more sensitive than the other two parental strains (not shown).

In order to test whether aggregation of the slugger mutants is hypersensitive to ammonia, the mutants and their parents were allowed to develop on Millipore filters in the presence of various concentrations of ammonia. Under control conditions the onset of aggregation of slugger mutants in all groups except C is retarded when compared to that of the corresponding parental strain. In all groups except C and J aggregation was delayed by an additional 2-6 hours relative to that of the corresponding parent by concentrations of ammonia from 10-50 mM (not shown). The number of aggregates formed also dropped more sharply as a function of ammonia concentration in representatives of the same groups than in the parental strains. Fig. 2 illustrates the effect on aggregation in mutant

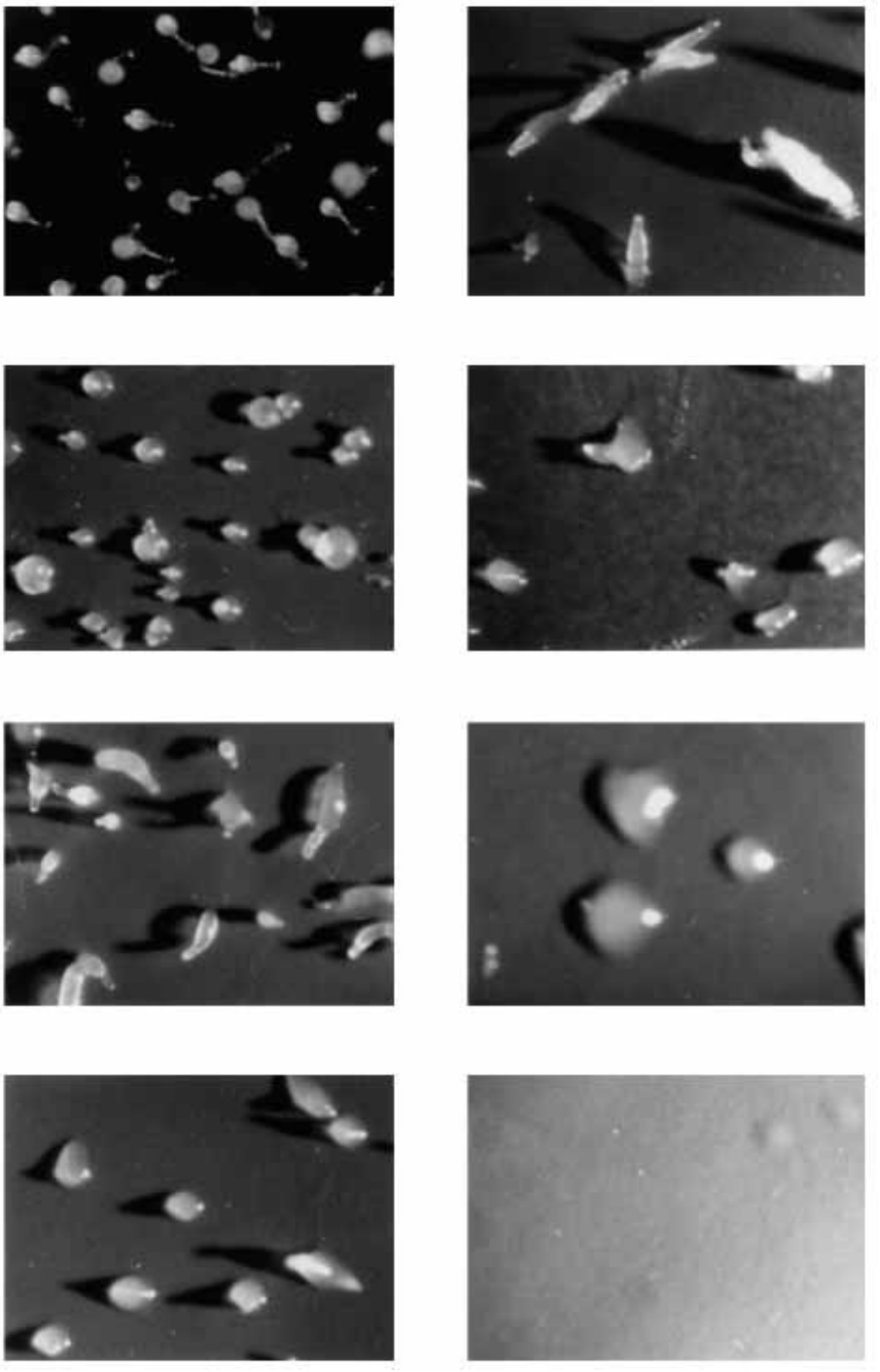

Fig. 2. The effect of ammonia on the development of mutant KY3 and its parent NC4. Cells were plated for development onto filters with 0, 20 mM, 35 mM and 50 mM ammonia and photographed after 15 hours. Left-hand column shows NC4, right-hand column shows KY3, ammonia concentration increases from top to bottom. The long side of each photograph is 8 mm.

KY3 and its parent NC4 and results with representatives of groups A, B and H are presented in Fig. 3 together with their parental strain, NP187. Taken together these findings demonstrate that mutants in at least eight of the ten slugger complementation groups display hypersensitivity to ammonia with regard to inhibition of aggregation.

Inhibition of tip formation

Aggregates of three of the parental strains, NC4, X55 and X22, formed in the presence of ammonia produced tips and first fingers in the normal way. However in the fourth parental strain, NP187, about 20 per cent of the aggregates formed in the presence of 50 mM ammonia failed to generate tips (Fig. 4A). We examined tip formation in all of the slugger mutants in the presence and absence of ammonia (Table 1, column 4). Representatives of all 10 groups formed a substantial proportion of tipless aggregates in the presence of ammonia. Some examples are presented in Fig. 4B, and Fig. 5 illustrates the appearance of tipless aggregates in strain NP452 (F). Formation of tipless aggregates was generally much more pronounced in the mutants than in the corresponding parental strains. The exceptional cases (such as strains NP455 and

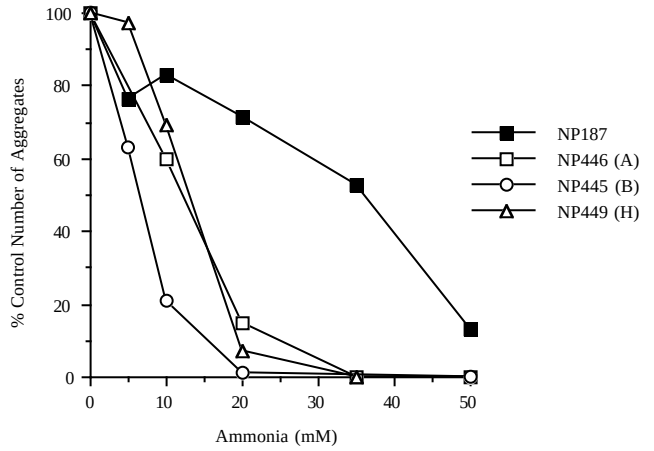

Fig. 3. Reduction of aggregate number by ammonia in NP187 and three slugger derivatives. The number of aggregates in a given area was scored after 40 hours. Results are mean of 3 to 16 experiments for each strain at each concentration of ammonia with duplicate filters in each experiment. Standard deviations are omitted for clarity.

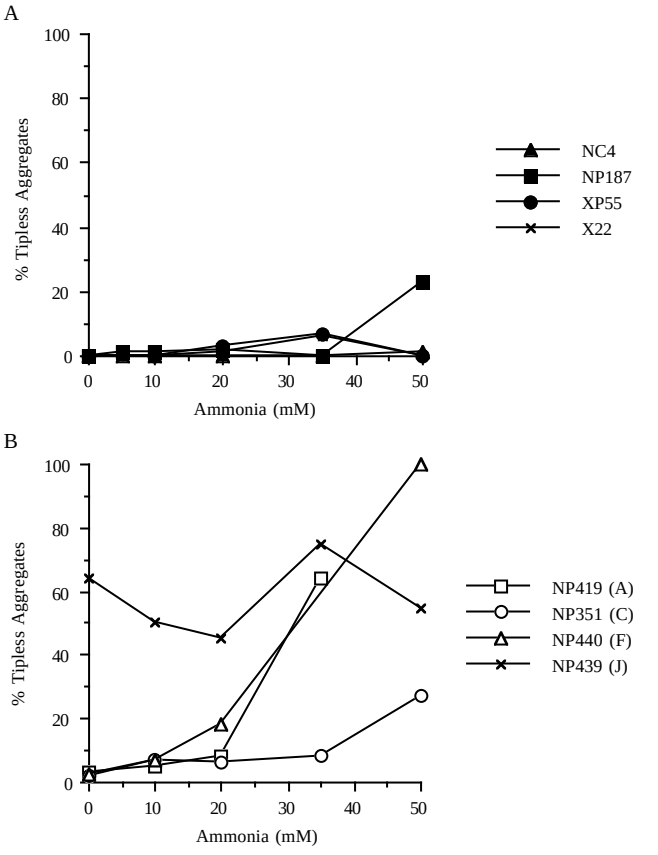

Fig. 4. Ammonia-hypersensitivity of tip formation in slugger mutants. Results are means of 3 to 16 experiments for each strain at each concentration of ammonia with duplicate filters in each experiment. Standard deviations are omitted for clarity. (A) Parental strains; (B) slugger strains showing typical responses.

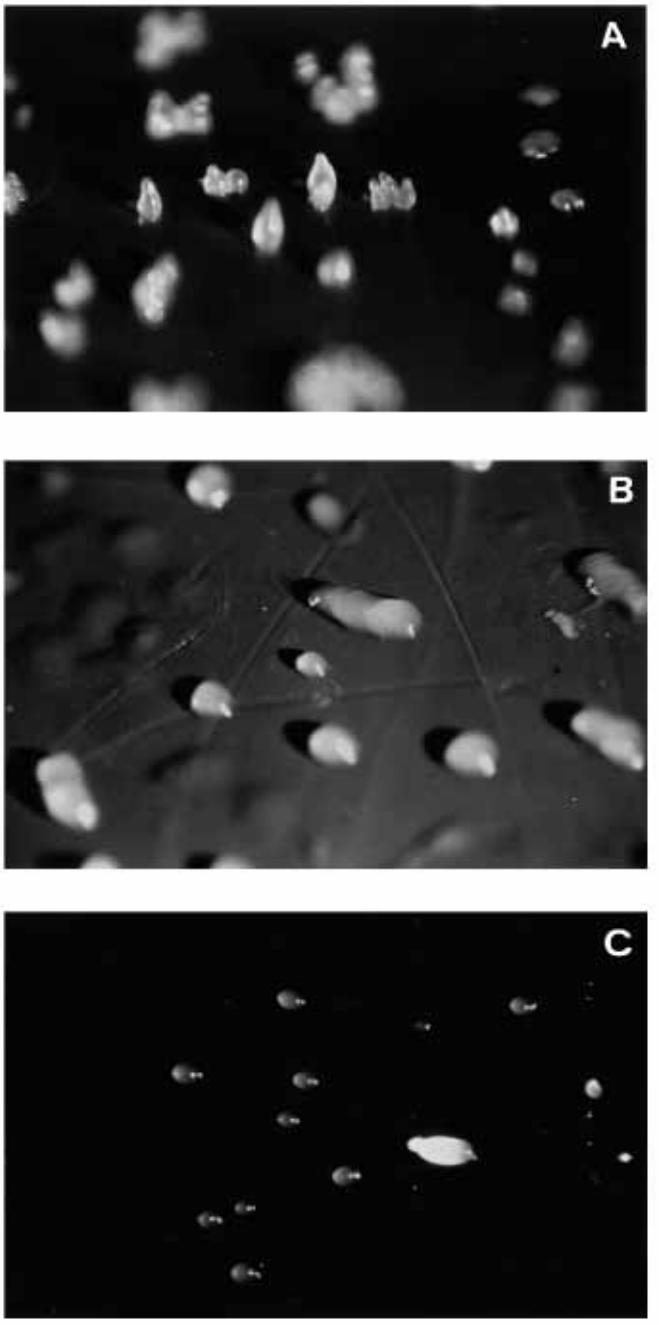

Fig. 5. Failure of tip formation in slugger mutant NP452. Cells of strain NP452 (group F, parent NP187) were plated on filters with LPS (pH 7.3) with and without ammonia. Photographs were taken after 18.5 and 38 hours of development. By 18.5 hours tips had formed on aggregates not exposed to ammonia (A), but not on those with 20 mM ammonia (B). The majority of the aggregates on 20 mM ammonia remained tipless after 38 hours (C). The long edge of each photograph is 8 mm.

NP466) were among those in which aggregation itself was particularly sensitive. From Fig. 4 it can be seen that the mutant NP439 (J) also formed substantial numbers of tipless aggregates in the absence of added ammonia. This was also true of several mutants belonging to groups B, F and G. When developing on agar at pH 7.3 rather than on filters most of the slugger mutants examined (but not their parents) formed significant numbers of tipless aggregates in the absence of added ammonia. Formation of such aggregates was generally greatly

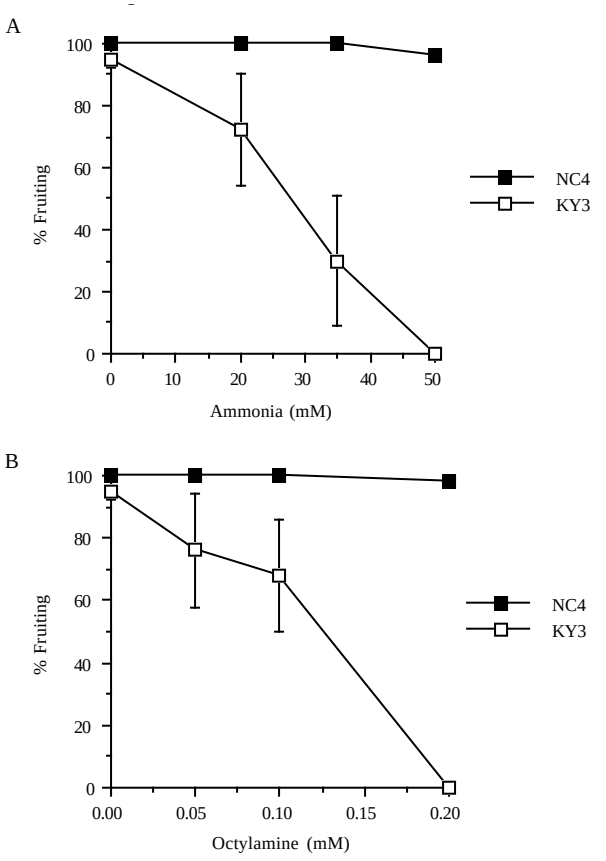

Fig. 6. Hypersensitivity of culmination to ammonia and octylamine. Cells of the strain KY3 and its parent NC4 were plated onto filters with LPS (pH 7.3) containing various concentrations of ammonia or octylamine. Aggregates were scored as in Fig. 1. The results are mean ± s.e.m. of 5 experiments in each case with duplicate filters in each experiment; (A) ammonia and (B) octylamine.

reduced at pH 5.5 (not shown), lending support to the idea that endogenously generated ammonia was responsible.

Hypersensitivity to octylamine and ammonia

Davies et al. (1993) have shown that each of several primary alkylamines examined resembles ammonia in inhibiting aggregation and culmination, and that their potency is related to their chain length and hence to their lipid solubility. To test whether the hypersensitivity of the slugger mutants is restricted to ammonia we compared the effects of ammonia and octylamine, the most effective of the alkylamine weak bases, on aggregation and culmination of several slugger mutants and their parental strains (NP440, NP441, NP452, KY3, with NP187, NC4). In each case the mutants were hypersensitive to both of the weak bases with respect to each parameter examined: time of aggregation, number of aggregates formed and culmination. The hypersensitivity of culmination of strain KY3 (group D) to ammonia and octylamine is shown in Fig. 6.

Nature of the aggregation defect

We have tested the ability of four slugger strains to co-aggregate with an equal number of parental cells in the presence of concentrations of ammonia that inhibit aggregation of the slugger mutants on their own. Aggregates formed in mixtures at a time when the sluggers on their own had not aggregated were dissociated and the individual amoebae were plated clonally to score their genotypes.

In three cases (KY3(D), NP441(F) and NP452(F)) the proportion of slugger amoebae in the mixed aggregates formed in the presence of ammonia varied between 30% and 60% and was similar to the proportion in mixtures formed in the absence of added ammonia. This finding suggests that these mutants can respond to cyclic AMP signals generated by wild-type amoebae and that the hypersensitivity of their aggregation to weak bases is due to a defect in signal production. The same result has been reported for cells expressing a dominant-negative form of the regulatory subunit of protein kinase A: such cells are aggregation-defective on their own but can co-aggregate with wild-type cells (Harwood et al., 1992). Aggregates formed by mixtures of a fourth slugger mutant (NP410 (G)) with its parent contained only 7 per cent mutant amoebae in the presence of ammonia compared to 3 per cent in its absence. In this case the mutant defect may be cell autonomous.

DISCUSSION

It has been shown previously that ammonia inhibits both aggregation (Siegert and Weijer, 1989; Davies et al., 1993; Williams et al., 1984) and culmination (Schindler and Sussman, 1977) in wild-type amoebae. The present results extend the evidence for the role of an ammonia-sensitive process or processes in development. We have shown that all three clearly recognisable morphological stages of *Dictyostelium* development, aggregation, tip formation and culmination, can be inhibited by ammonia in wild-type (non-slugger) strains and that each of these stages is abnormally susceptible to ammonia inhibition in many of the slugger mutants. It is possible therefore that a common ammonia-sensitive process underlies each of these developmental events. The slugger mutants are presumably arrested at the slug stage rather than earlier in development because ammonia can accumulate to high levels at this stage due to its retention by the relatively impermeable slime sheath.

Previously, Schindler and Sussman (1979) showed that the slugger D mutant KY3 could be induced to re-enter the fruiting mode by application of a glutamate dehydrogenase enzyme cocktail designed to lower the ambient ammonia concentration. Newell and Ross (1982) reported that the only additional mutants that could be induced to fruit by this procedure, and hence were hypersensitive to ammonia, were representatives of slugger E and slugger G. The difference between this observation and our finding that members of all slugger mutant groups are hypersensitive to ammonia by other criteria can probably be ascribed to the fact that mutants of groups D, E and G are those that are least defective in fruiting (see Table 1) and which may therefore be most readily induced to fruit by ammonia depletion.

Our observations of the different slugger mutants are in general agreement with the report of Newell and Ross (1982) that members of the same complementation group display common characteristics with respect to slug morphology, migration and fruiting behaviour. In addition the aggregation of almost all the mutants was more delayed in response to

ammonia than was that of the parental strains (see Results). Nevertheless, a striking feature of our findings is the large amount of variation between different mutants within the same complementation group with regard to quantitative measures of ammonia sensitivity (Table 1). As the products of the genes altered in the slugger mutants are likely to be implicated in essential developmental processes (see below) the mutations probably involve alteration rather than loss of function. Hence some of the allelic variation may reflect differences in the extent of the mutant defect. Other variations may be due to subtle differences in the genetic background of these nitrosoguanidine-induced mutants.

Recent experiments suggest that weak bases exert their effects by interfering with acidification of an intracellular compartment, and that they do so by accumulating in this compartment where they become protonated and act as 'proton shuttles' (Davies et al., 1993). This view is based on the observation that the potency of different weak bases is a function of their hydrophobicity, as in the case of neutralisation of mammalian lysosomes (Poole and Ohkuma, 1981) and on the finding that biologically effective doses of weak bases substantially raise the pH of an acidic early endosomal compartment while having no discernible effect on cytosolic pH (Brénot et al., 1992; Davies et al., 1993). That an intracellular compartment is the target for weak base action in the slugger mutants is indicated by our finding that mutations in each of three different slugger groups increases sensitivity to the hydrophobic weak base octylamine in parallel with that to ammonia (Fig. 6).

Binding of cyclic AMP to cell-surface receptors leads to a transitory activation of adenylyl cyclase and accumulation and subsequent release of cyclic AMP (Devreotes, 1982). Williams et al. (1984) have presented evidence that concentrations of ammonia similar to those employed in the present work progressively reduce the amplitude of this response, and Schindler and Sussman (1979) have shown that adenylyl cyclase activation is hypersensitive to ammonia inhibition in one of the slugger mutants, KY3 (group D). Acidification of the weak-base-sensitive intracellular compartment may therefore be essential for efficient activation of adenylyl cyclase (see Davies et al., 1993, for discussion). This could be required for intercellular cyclic AMP signalling at each stage of development, or for activation of cyclic AMP-dependent protein kinase which has been shown to be required for aggregation (Simon et al., 1989; Firtel and Chapman, 1990), as well as for culmination (Harwood et al., 1992; Kay, 1992). Our finding, that members of at least two slugger complementation groups can co-aggregate with parental cells in the presence of concentrations of ammonia that inhibit aggregation of the mutants on their own, supports the idea that ammonia interferes with intercellular signalling, at least during aggregation.

There is evidence that, in addition to its effect on adenylyl cyclase activation, ammonia selectively inhibits DIF-dependent gene expression on the stalk cell pathway (Gross et al., 1983; Bradbury and Gross, 1989; Wang et al., 1990). Such an effect may contribute to the inhibition of tip formation and culmination by weak bases.

Some of the 10 complementation groups of slugger mutants examined here may have acidification defects similar to those displayed by recently isolated endocytosis mutants (Bof et al., 1992). Others may instead be partially defective in additional cellular components required for efficient activation of adenylyl cyclase, or they may harbour lesions in protein kinase A itself. Any such defects could conceivably render development of the mutants abnormally sensitive to interference with acidification by weak base. A striking example of such synergy has recently been reported by Hopper et al. (1993), who showed that a strain expressing a dominant-negative form of the regulatory subunit of protein kinase A under the control of a prespore-cell-specific promoter is hypersensitive to ammonia.

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