Ammonium phosphate in sori of *Dictyostelium discoideum* promotes spore dormancy through stimulation of the osmosensor ACG

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The sori of *Dictyostelium discoideum* (strains SG1, SG2, NC4 and V12) contained more than 100 mM ammonium phosphate. Glutamine synthetase (GS), which could remove ammonia from the sorus, was not present in 2-d-old dormant spores but enzyme activity returned to vegetative levels after spore germination. Based on mRNA blotting, the activity of this enzyme in germinating spores appeared to be transcriptionally controlled. At the same time that GS activity was increasing, ammonia was released from germinating spores. Exogenous ammonium ions at a concentration of 28 mM did not block germination nor modulate GS activity in nascent amoebae. It was concluded that the transcription and translation of GS is not environmentally regulated but is an integral part of the germination process, preparing nascent amoebae for vegetative growth. An exogenous concentration of 69 mM ammonium phosphate could maintain dormancy in spores of strains SG1 and SG2 for at least a week in the absence of any other inhibitory component from the sori. The inhibition was reversible at any time either by dilution or by washing the spores free of the ammonium ion. Spores of strain *acg*[−] were not inhibited by 100 mM ammonium phosphate. A model is presented in which GS in prespore cells serves as a sink for ammonia to allow the osmotically sensitive adenylyl cyclase aggregation protein (ACA) to activate protein kinase A (PKA) to induce fruiting-body formation. After fruiting-body formation is complete, the decline in GS and ACA activities in developing spores is offset by their replacement with the osmotically and ammonia-stimulated adenylyl cyclase osmosensor for germination (ACG). Ammonia and discadenine may act as separate signals to synergistically activate PKA by stimulating ACG activity while inhibiting cAMP phosphodiesterase activity in fully dormant spores.

Keywords: *Dictyostelium discoideum*, glutamine synthetase, spore dormancy, ammonia inhibition, adenylyl cyclases

INTRODUCTION

Ammonia is produced throughout multicellular development by protein degradation using powerful lysosomal enzymes (North & Cotter, 1991; Bonner, 1993; North *et al.*, 1996), various transaminases (Krivanek & Krivanek, 1965), and glutamate dehydrogenases (Wright

& Bard, 1963; Pamula & Wheldrake, 1990, 1991, 1992, 1996). About half of all the protein accumulated by vegetative cells of *Dictyostelium discoideum* is consumed in construction of the final fruiting body (see Loomis, 1975). The use of protein as a metabolic fuel for development releases large quantities of ammonia, which can regulate cell determination; thus 33 mM (NH₄)₂SO₄ inhibits transcription of prestalk genes but stimulates transcription of prespore genes (Oyama & Blumberg, 1986), and concentrations of ammonia from 50 to 100 mM reversibly block aggregation, tip formation and culmination (Davies *et al.*, 1996). Evidence

Abbreviations: ACA, adenylyl cyclase for cellular aggregation; ACG, adenylyl cyclase osmosensor for regulation of spore germination; GDH, glutamate dehydrogenase; GS, glutamine synthetase; MSX, L-methionine sulfoximine; PKA, protein kinase A;

that prestalk cells produce most of the ammonia postaggregation, while prespore cells become rich in specific amino acids such as glutamine, glutamate and asparagine, has led to a hypothesis called the ammonia source and sink model (Cotter *et al.*, 1992). In this, a portion of the ammonia produced by prestalk cells (source) is consumed by prespore cells (sink) in the production of specific amino acids (Cotter *et al.*, 1992). The TCA cycle of mitochondria should have different functions in the two cell types: in the prestalk cells, to consume glutamate and aspartate producing carbon dioxide and ammonia; in prespore cells to produce glutamate and aspartate using precursors derived from glycogen. Glutamine production would require that glutamine synthetase (GS) be active in consuming glutamate and additional ammonia in prespore cells, and indeed, it is present in vegetative cells. Its specific activity increases during differentiation and it is highly localized in prespore cells (Dunbar & Wheldrake, 1995, 1997a). Its activity is maximal at culmination, and declines during formation of the fruiting body (Dunbar & Wheldrake, 1995, 1997a).

The fruiting body consists of an upright column of dead stalk cells supporting a viscous droplet of dormant spores known as the sorus (Raper, 1984). For about four days after spore formation, the 'young' spores do not germinate when washed and placed in phosphate-buffered solutions (Cotter & Glaves, 1989). They will germinate synchronously, however, if they are heat shocked at 45 °C for 30 min after being washed free of autoinhibitors such as discadenine, a zeatin-like molecule (Abe *et al.*, 1976; Cotter & Glaves, 1989). Young spores also respond and germinate when bacterial growth products called 'promoters' overcome the inhibition imposed by discadenine (Ihara *et al.*, 1990). When fruiting bodies are aged for 10–14 d at room temperature, the 'mature' spores gain the ability to germinate spontaneously (autoactivate) if washed and placed in phosphate-buffered solutions. During the initiation of spore swelling, autoactivating spores release an 'autoactivator' which may overcome discadenine and greatly increase the rate as well as the synchrony of spontaneous germination in the rest of the spore population (Cotter & Glaves, 1989). Mutant spores called 'premature-maturation mutants' or 'spontaneous germinators' have been isolated and named SG1 and SG2. These mutants gain the ability to autoactivate after only 1 d of ageing. They release autoactivator(s) with the same chromatographic characteristics as aged wild-type spores (Dahlberg & Cotter, 1978).

The interactions of autoactivators and autoinhibitors, as well as osmotic-pressure effects, suggested that dormancy and spore germination in *D. discoideum* were regulated by classical signal-transduction pathways (Cotter & Glaves, 1989; Glaves & Cotter, 1989; Cotter *et al.*, 1990). More recent work has indicated that spore dormancy in the fruiting body is maintained by at least two inhibitory cascades (Cotter *et al.*, 1992). The first involves low-molecular-mass autoinhibitors that stimulate tyrosine phosphorylation of actin, preventing cyto-

skeletal rearrangements and thus spore swelling (Gauthier *et al.*, 1997; Kishi *et al.*, 1998). This cascade may originate when discadenine activates the transmembrane two-component histidine kinase DHKB (Zinda & Singleton, 1998). The active DHKB may in turn, through a series of phosphoryl relays, inactivate the phosphodiesterase activity of RegA, allowing cAMP to rise and contributing to the high protein kinase A (PKA) activity of dormant spores. The second inhibitory cascade is regulated by the osmotic pressure of the extracellular material of the sorus. It is commonly observed that the sorus volume varies directly with humidity. Thus, low relative humidity results in drying of the sorus and an increase in osmotic pressure upon the spores. The sporulation-specific adenylyl cyclase (ACG) is a membrane-bound osmosensor and directly produces cAMP in response to osmotic pressure (van Es *et al.*, 1996; Virdy *et al.*, 1999). The pressures required *in vivo* to activate ACG and so maintain spore dormancy in the sorus may be simulated *in vitro* with glucose or sucrose solutions ranging from 100 to 250 mM (Cotter, 1977; van Es *et al.*, 1996; Virdy *et al.*, 1999). In osmotically inhibited spores, the increase in cAMP activates PKA to phosphorylate a number of 'dormancy proteins' (Lydan *et al.*, 1994a; van Es *et al.*, 1996; Virdy *et al.*, 1999). If the relative humidity is high, the sorus absorbs moisture from the air, and the lower osmotic pressure allows mature spores to produce auto-activators. The autoactivators override the auto-inhibitors and activate a phospholipase C pathway that mobilizes Ca^{2+} , which then interacts with calmodulin and calmodulin-binding proteins. These latter proteins appear to interact with the cytoskeleton, promoting rearrangement and thus spore swelling, followed by release of nascent amoebae (Lydan & Cotter, 1994, 1995; Lydan *et al.*, 1994a, b). Nomarski-differential interference microscopy and transmission electron microscopy demonstrate that actin in dormant spores is present in large rod-shaped structures in both the nucleus and the cytoplasm; correlations are noted between the dephosphorylation of actin, disappearance of the actin rods, initiation of cytoplasmic streaming and spore swelling (Sameshima *et al.*, 1994; Kishi *et al.*, 1994, 1998). Mutants such as *splA*⁻ produce spores without phosphorylated actin and are only transiently dormant (Kishi *et al.*, 1998).

The experiments described in this report show that the sori of fruiting bodies contain very high levels of ammonium phosphate that stimulate spore dormancy through the ACG osmosensor and its downstream effector PKA. This demonstrates that ammonia and ammonium ions interact with cAMP production in all stages of development. We suggest a model that explains most of the interactions between these two low-molecular-mass morphogens.

METHODS

Reagents. L-Glutamine, γ -glutamyl monohydroxamate, L-methionine sulfoximine (MSX) and Nessler's reagent were from Sigma. ADP was from Boehringer Mannheim. Media

components were obtained from Oxoid. All other reagents were of the highest purity available.

Culture conditions. *D. discoideum* strains NC4 and V12 used in the study were parents of the respective prematurely maturing mutants SG1 and SG2; the null mutant *acg*⁻ (Pitt *et al.*, 1992) was employed in a few experiments. All strains were grown on three types of agar media: standard medium (SM) (Sussman, 1987), SM/2 (SM diluted 1:1 but with the agar concentration kept at 1.5%), and glucose/salts agar, which requires that the food bacterium *Escherichia coli* B/r (ATCC 12407) be prototrophic. The glucose/salts medium is believed to leave fewer unused nutrients in the agar and to result in sori with less moisture (Cotter *et al.*, 1990). *D. discoideum* spores (10⁴ ml⁻¹) mixed with *E. coli* B/r were spread on the three types of solid media and incubated at 22 °C. The bacterial host was cleared by 72 h and fruiting bodies were initiated by 96 h.

Germination conditions. Fruiting bodies were allowed to age for at least 2 d before spores were removed for germination experiments. Spores 2–4 d old were obtained by shaving the sori with the edge of glass slides, which were then dipped in 10 ml water contained in a 50 ml beaker. The suspended spores were vortexed in a glass test tube to break up clumps, and centrifuged for 5 min at 400 g. The supernatant was used to determine ammonia and phosphate concentrations. The spores were resuspended in 10 mM potassium phosphate buffer at pH 6.5, washed twice by centrifugation in the phosphate buffer and resuspended in the buffer at a concentration of 1–7 × 10⁷ ml⁻¹ for autoactivation experiments. In heat-induced activation experiments, spores were heat shocked at 45 °C for 30 min and then the number was adjusted to 1–7 × 10⁷ ml⁻¹. Spores germinated in volumes less than 5 ml were kept in suspension in 10 ml test tubes by slowly spinning micro-stir bars. Spore germination in volumes greater than 5 ml utilized various sized flasks aerated on a temperature-controlled rotary shaker at 150 r.p.m. The progress of spore germination at room temperature was monitored by scoring at least 200 spores/amoebae every 60 min by phase-contrast microscopy as unswollen, swollen or emerged amoebae.

Sample preparation. During the germination experiments, samples were harvested by centrifugation at 400 g for 5 min. The supernatants and pellets were separated and both were quickly frozen at -20 °C. The spore number in most pellets for enzyme analysis was 4 × 10⁸; the spore number in pellets for RNA extraction was 1 × 10⁸.

Protein extraction. Nitric-acid-washed glass beads (300 µl, 212–300 µm diameter; Sigma) and 350 µl extraction buffer [25 mM Tris/HCl (pH 7.8), 10 mM L-glutamate, 5 mM β-mercaptoethanol, 1 mM MnCl₂] were added to spores or germinating spore pellets and the suspension was vortexed for 5 × 20 s periods with intervening cooling on ice for 30 s. Usually 60–70% of the cells were lysed by this procedure. The protein extract was decanted into a fresh tube, centrifuged at 100 000 g for 10 min at 4 °C and stored on ice until use.

RNA extraction. Fresh cell pellets harvested from spores at various stages of germination were mixed with 300 µl diethylpyrocatechol (DEPC) and nitric-acid-washed glass beads (212–300 µm diameter, Sigma) and 350 µl 50 mM Tris/HCl (pH 8.4) containing 1% DEPC and 2% SDS. The cells were disrupted as described above and the supernatant was mixed vigorously with an equal volume of phenol/chloroform (1:1). After centrifugation (12 000 g, 10 min) the aqueous phase was collected and re-extracted twice more with phenol/chloroform. Before the last extraction sodium acetate (pH 4.7) was added to a final concentration of 0.4 M. The aqueous

phase was then mixed with 2 vols 99% ethanol, and the RNA precipitated overnight and collected by centrifugation (12 000 g, 30 min, 4 °C) was washed once in 75% ethanol and dried under vacuum. The final pellet was resuspended in sterile water and stored at 4 °C (Dunbar & Wheldrake, 1997b).

Glutamine synthetase (GS) assay. GS activity was assayed by monitoring the formation of γ-glutamyl hydroxamate in the Mn²⁺-dependent transferase reaction as described by Dunbar & Wheldrake (1995).

Northern analysis. The amount of GS mRNA formed during spore germination was determined by the procedures of Dunbar & Wheldrake (1997b). To confirm that equal amounts of RNA were loaded in each lane, all RNA samples were diluted 1 in 500 and probed with a 3 kb *Eco*RI rRNA fragment labelled with [α-³²P]dCTP by the random-primed method.

Ammonia analysis. At the pH values used in these experiments, the term ammonium should be used. However, the biologically active component is most likely to be ammonia (Davies *et al.*, 1996) so the two terms will be used interchangeably. Samples (1 ml) of spore washes and germination supernatants were mixed with 0.25 ml Nessler's reagent (Sigma) and incubated at room temperature for 20 min before measuring A₄₈₀. Ammonia concentrations were determined by reference to a standard curve constructed with NH₄Cl. To confirm that the Nessler assay was specific, ammonia production was also measured by oxidation of NADH (A₃₄₀) at 30 °C as described by Pamula & Wheldrake (1990) using pure bovine liver glucose dehydrogenase, and with an ammonia electrode.

In some experiments, ammonia in sori was determined by using a dry glass slide to harvest sori; the spores and matrix material were quickly transferred to a 10 µl glass capillary tube. The contents of the capillary tube were rinsed into 10 ml distilled, deionized water. The spores were removed by centrifugation and ammonia in the supernatant was determined with Nessler's reagent. The spores were resuspended in phosphate buffer and counted.

Phosphate analysis. Inorganic phosphate in spore washes and germination supernatants was determined by adding to each 1 ml sample: 0.5 ml 3% HClO₄, 1.8 ml 0.8% FeSO₄ (in 0.1 M H₂SO₄) and 0.15 ml 6.6% ammonium molybdate (in 3.5 M H₂SO₄) and measuring A₆₂₀ after 20 min at room temperature. A standard curve was constructed with potassium phosphate (Fiske & Subbarow, 1925).

Development on Millipore filters and agar surfaces. To produce fruiting bodies as free of extraneous nutrients as possible, cells were washed in 10 mM potassium phosphate buffer and 5.5 × 10⁸ cells per filter plated on cellulose nitrate membrane filters (4.5 cm diameter, Millipore) resting on supporting filter pads saturated with 10 mM potassium phosphate buffer. The plates were incubated at room temperature until fruiting bodies formed. In other experiments, 10 mM MES buffer at pH 6.5, free of phosphate, was used in place of 10 mM potassium phosphate buffer.

Development of cells in micro-drops of less than 10 µl utilized 1.5% non-nutrient agar containing 10 mM potassium phosphate or 10 mM MES buffer. The cell number was adjusted to 10⁸ ml⁻¹ before the drops were plated on the agar surface with a micropipette (Cotter *et al.*, 1990).

Reproducibility of experiments. Each experiment was done three or more times, with the mean and standard error of the mean (SEM) calculated where appropriate. Enzyme assays,

ammonia determinations and phosphate determinations were done at least in triplicate for each experiment.

Much of the data are expressed in production or consumption by 10^8 spores; this number is not entirely arbitrary since several inhibitory properties of spores are evident at this concentration.

RESULTS

GS activity in spore extracts

Crude extracts of dormant spores prepared by glass-bead grinding 2 d after fruiting-body formation failed to reveal significant GS activity. To determine when and under what conditions GS returned to vegetative levels, spores of *D. discoideum* strain SG1 were washed in 10 mM potassium phosphate buffer at pH 6.5 and allowed to germinate by autoactivation (spontaneous germination). The germination process was highly reproducible in four separate experiments (Fig. 1). Similar germination kinetics were observed after spores were heat-activated and incubated in phosphate buffer (data not shown). Spore swelling began after 1 h incubation and the first amoebae were observed 2 h after the start of incubation. Germination was essentially complete by 5 h. Autoactivation and heat-induced germination of the opposite mating type (strain SG2) gave similar results (data not shown).

The specific activity of GS in 2–4-d-old dormant spores was extremely low (Fig. 2) when compared to that in newly formed fruiting bodies (Dunbar & Wheldrake,

Fig. 1. Autoactivation of SG1 spores in 10 mM potassium phosphate buffer at pH 6.5. Spores were incubated in 50–100 ml volumes in 250 ml flasks at $2\text{--}7 \times 10^7 \text{ ml}^{-1}$. Each hour the percentage of the spore population that was swollen (○) or present as nascent amoebae (●) was determined by phase-contrast microscopy; the mean $\pm$ SEM for four separate experiments are plotted. Samples of spores/amoebae were pelleted by centrifugation, and the supernatant and pelleted cells were frozen separately.

Fig. 2. GS activity in autoactivated SG1 spores. The mean $\pm$ SEM are plotted for three separate experiments with 2–4-d-old spores.

Fig. 3. Accumulation of GS mRNA during autoactivated SG1 germination. (a) RNA (20 μ g) isolated every hour throughout the 5 h of spore germination was electrophoresed, transferred to a nylon membrane and hybridized with a ^{32}P -labelled 177 bp PCR product of the GS gene. (b) RNA loading control; the same RNA samples as in (a) were electrophoresed on a separate gel and probed with an $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ -labelled 3 kb *EcoRI* rRNA probe.

1995). No increase was detectable during auto-activation until spores began to swell (Fig. 2). After 8 h incubation, activity had risen to near the vegetative-

Fig. 4. Ammonia production during autoactivation of SG1 spores. The supernatants from four germination experiments were tested with Nessler's reagent. The spore numbers ranged from 2 to 7×10^7 ml⁻¹; however, the data are plotted as the amount of ammonia produced by a theoretical spore population of 10^8 ml⁻¹. The means $\pm$ SEM are shown.

growth level. The increase occurred in the absence of bacteria or axenic media, and thus growth was not required to induce the vegetative level of GS. The results were not strain dependent since spores of the opposite mating type (strain SG2) yielded nascent amoebae with the same specific activity as above (data not shown). Similar results were found for spores activated by heat shock (data not shown).

MSX at 5 mM has been shown to completely inhibit GS activity *in vitro* (Dunbar & Wheldrake, 1995, 1997c). The inhibitor did not delay autoactivation, but the nascent amoebae did not clump in suspension as readily as uninhibited cells (data not shown). Plating of nascent amoebae, from MSX-treated spores on agar surfaces in micro-drops, resulted in rapid aggregation similar to that seen with uninhibited nascent cells.

Transcriptional control of GS

Northern blotting indicated that the return of GS activity in nascent amoebae may be transcriptionally controlled (Fig. 3). Again, there were no significant differences between the transcription patterns of nascent amoebae generated by autoactivation or heat-induced germination of spores (data not shown).

Ammonia production during spore germination

Earlier studies using ¹³C NMR suggested that the high concentrations of glutamine and trehalose, as well as of glutamate and asparagine, in dormant spores decreased during the 5 h of germination (Klein *et al.*, 1990). The amino acids are probably used primarily as energy sources rather than for the synthesis of spore-germi-

Table 1. Ammonium concentration in supernatants from germinating spores

All values are normalized to 10^8 spores ml⁻¹ in phosphate buffer. Each entry represents the mean $\pm$ SEM for three experiments.

Strain	Method of spore activation	Ammonium concn after 5 h germination (mM)
SG1	Autoactivated	2.47 ± 0.26
SG1	Heat activated	2.17 ± 0.10
SG2	Autoactivated	2.40 ± 0.33
SG2	Heat activated	2.76 ± 0.50

nation proteins (Ennis, 1981; Klein *et al.*, 1990; Cotter *et al.*, 1992) and would probably be catabolized to carbon dioxide and ammonia.

When ammonia was measured in autoactivating SG1 spores as described in Methods, dilution of the supernatants was usually necessary because of the large quantity produced. The amount produced in 5 h depended directly upon spore concentration in the range 2 – 7×10^7 ml⁻¹. The data for four experiments were plotted as the concentration of ammonium produced by a normalized spore population of 10^8 ml⁻¹ over 8 h (Fig. 4). Similar high levels of ammonia were released when SG1 spores were heat activated. Strain SG2 also released ammonia during germination (Table 1). Independent assays with a glutamate dehydrogenase coupled enzyme system (see Pamula & Wheldrake, 1990) confirmed that the amine released by germinating spores was ammonia. There was a 98 % agreement between the two methods (data not shown). Measurements with an ammonia electrode reconfirmed the above data.

Ammonia-induced reversible inhibition of spore germination

In several experiments, at very high spore densities, auto-induced spore germination was delayed and ammonia was detected in the time zero samples. This led us to examine (1) the effects of increasing concentrations of ammonia on spore germination, and (2) the concentration of ammonia in mature fruiting bodies. In initial experiments, the inhibitory effects of 5 mM ammonium chloride and ammonium sulfate were primarily due to chloride and sulfate anions (data not shown). Since germinating spores produced ammonia in 10 mM phosphate buffer in other experiments, phosphate was tested as a counterion. Low (14 mM) concentrations of ammonium in 10 mM phosphate buffer, pH 7.0, had very little effect on spore germination (data not shown). At a moderate concentration (28 mM) of ammonium, auto-induced spore germination proceeded rapidly after the short lag. Replacement of ammonium with potassium ions resulted in little or no lag (data not shown). The levels of GS in amoebae at 5 h were very similar in the

Fig. 5. Reversible inhibition of SG2 spore autoactivation by 69 mM ammonium. Spores were harvested and washed three times with ammonium (69 mM)/phosphate (50 mM) buffer at pH 7.0. Samples in glass tubes were resuspended in the above buffer (5 ml) (▼) with the aid of small stirring bars for 30 h at room temperature. At 0 h, portions were centrifuged and resuspended in potassium (14 mM)/phosphate (10 mM) buffer at pH 7.0 (○), and in potassium (69 mM)/phosphate (50 mM) buffer at pH 7.0 (●). The spores resuspended in potassium solutions were incubated in glass tubes as above. At 5 (■) and 24 h (△) additional portions were centrifuged, resuspended in the potassium (69 mM)/phosphate (50 mM) buffer at pH 7.0, and incubated as above. The experiment was repeated three times and the means $\pm$ SEM are plotted.

two cultures [control value, $38 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$; ammonium value, $37 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$]. Thus 28 mM ammonium ion had no effect on the activity of GS in emerging amoebae.

Spores of strains SG1 and SG2 did not germinate in 100 mM ammonium phosphate buffer at pH 6.5–7.0 (data not shown). When spores of strain SG2 were harvested in an ammonium phosphate solution (69 mM ammonium and 50 mM phosphate ion, pH 7.0), auto-induced spore germination was inhibited by 99% (Fig. 5). The few amoebae that emerged were normal in appearance and viable. Inhibition imposed by 69 mM ammonium ion was relieved when spores were centrifuged and resuspended in phosphate buffer solutions containing either 14 or 69 mM potassium ion (see Fig. 5). Relief was also observed when spores incubated for 5, or even 24, h with 69 mM ammonium were pelleted and resuspended in 50 mM phosphate containing 69 mM potassium ion (see Fig. 5). These experiments were repeated three times and were remarkably reproducible.

In other experiments we extended the incubation of spores in 69 mM ammonium for up to 7 d without loss in viability or ability to germinate upon removal of the inhibitory ion (data not shown). Similar germination kinetics are observed when spores are directly harvested from fruiting bodies and incubated in non-inhibitory phosphate buffers (Cotter & Glaves, 1989).

Table 2. Ammonium concentrations in sori containing young dormant spores

All values are normalized to 10^8 spores in $10 \mu\text{l}$; each numerical entry represents the mean $\pm$ SEM for three experiments.

Strain	Method of harvesting	Ammonium concn (mM)	Phosphate concn (mM)
SG1	Wet slide	124 ± 24	117 ± 10
SG1	Dry capillary	115 ± 15	132 ± 31
SG2	Wet slide	227 ± 27	116 ± 10
SG2	Dry capillary	223 ± 30	178 ± 30
NC4	Dry capillary	275 ± 32	123 ± 15
V12	Dry capillary	246 ± 28	238 ± 20

Ammonium and phosphate levels in sori of *D. discoideum*

The above experiments led us to examine ammonium levels in young fruiting bodies. The concentrations of ammonium and phosphate were surprisingly high (over 100 mM) whether the matrix and spores were harvested by a wet slide as in the experiments described above, or a dry slide with transfer to a capillary tube, followed by dispersion in distilled water (Table 2). The results in Table 2 are normalized to 10^8 spores per $10 \mu\text{l}$ sori but approximately 5×10^7 spores were usually harvested from relatively dry fruiting bodies into a $10 \mu\text{l}$ capillary tube and hence the concentrations of ammonium and phosphate would be half of those recorded in Table 2; nevertheless these ammonium concentrations exceeded the 69 mM sufficient to block spore germination (see Fig. 5). When the spore number was closer to 10^8 , loading of capillaries was very difficult. Even 5×10^7 spores occupy a substantial portion of the $10 \mu\text{l}$ volume shared with the soluble matrix components and the stalk tissue. Thus, the values in Table 2 may be minimal estimates that rise rapidly when fruiting bodies dried slightly. The volume of the sorus is proportional to the cube of the radius, so a decrease in radius by half during ageing/drying would increase the ammonium concentration eightfold, keeping the spores dormant.

The initial concentration of ammonium and phosphate in sori of young fruiting bodies was independent of the type of growth medium, but as fruiting bodies aged, the sori on SM agar had a tendency to swell with a concomitant decrease in ammonium and phosphate concentration (data not shown). This may account for the observation that spores of strains SG1 and SG2 are unstable in fruiting bodies produced on SM agar and may germinate in the sori (Cotter *et al.*, 1990). The stalk tissue of a fruiting body does not appear to act as a straw transferring ammonium and phosphate from the spent growth medium into the sorus. Rather, the compounds are produced endogenously, for when cells were washed in MES buffer and allowed to fruit on filters containing MES buffer, the resulting sori contained levels of ammonium and phosphate similar to those of Table 2 (data not shown).

Fig. 6. Germination inhibition of SG2 autoactivation by ammonia. Spores were washed three times by centrifugation with 10 mM potassium phosphate buffer at pH 6.5. The pellets were resuspended at a density of 2.5×10^7 spores ml^{-1} in one of four solutions: 50 mM potassium phosphate buffer at pH 6.2 (▲), 50 mM ammonium phosphate buffer at pH 6.2 (▼), 50 mM potassium phosphate buffer at pH 7.2 (●), and 50 mM ammonium phosphate buffer at pH 7.2 (■). All of the solutions were brought to the appropriate pH using KOH. The means $\pm$ SEM are plotted for three separate experiments.

The effects of pH on the ratio of ammonia/ammonium and the dormancy of spores

Ammonia affects other parts of the developmental life cycle including aggregation density, period of slug migration and culmination. Davies *et al.* (1993) suggested that the active species is the unprotonated form, ammonia, as a result of its greater membrane mobility. Whether ammonia exerts a greater effect than ammonium ions in blocking spore germination was tested by incubating spores of strain SG2 in 50 mM ammonium phosphate buffer brought to pH 6.2 and 7.2 with KOH (Fig. 6). At pH 7.2 the spores were fully inhibited whereas at pH 6.2 spores germinated but with a long lag. In osmotic controls using 50 mM potassium phosphate brought to pH 6.2 and 7.2 using KOH, germination began after a lag of only 1 h. The Henderson-Hasselbach equation predicts that the concentration of ammonia decreases by 90% as the pH decreases from 7.2 to 6.2, whereas the ammonium ion concentration increases by only 0.8%. Clearly, it is ammonia in the ammonia/ammonium pair that blocks germination when potassium phosphate does not stimulate the osmosensor ACG to maintain spore dormancy.

Spores of the *acg*⁻ null mutant are not inhibited by ammonia

The availability of an *acg*⁻ mutant allowed us to test one possible target of ammonia action. This mutant produces normal-looking fruiting bodies (Pitt *et al.*, 1992) in which spores remain dormant through the activity of

Table 3. Autoactivation of strains SG1 and *acg*⁻ in 50 mM ammonium or potassium phosphate buffers

Spores were incubated in the buffers at 10^7 ml^{-1} for 12 h.

Strain	Ammonium phosphate*	Potassium phosphate*	Percentage germination†
SG1	+	-	2.0 ± 1.3
SG1	-	+	97.1 ± 3.2
<i>acg</i> ⁻	+	-	85.3 ± 6.0
<i>acg</i> ⁻	-	+	92.4 ± 5.2

* 50 mM ammonium or potassium dihydrogen phosphate was titrated to pH 7.2 with KOH.

† Shown as mean germination $\pm$ SEM of three experiments after 12 h incubation

discadenine (van Es *et al.*, 1996). However, such spores lack the dormancy response to high osmotic pressure that can be generated by sucrose concentrations near 250 mM (van Es *et al.*, 1996). Washed spores of *acg*⁻ were not inhibited from germinating in 50 mM ammonium phosphate solutions titrated to pH 7.2, while spores of strain SG1 were inhibited (Table 3). Both strain SG1 and *acg*⁻ spores germinated in the osmotic control solutions containing potassium phosphate buffer at pH 7.2. In other experiments, *acg*⁻ spores germinated in 100 mM ammonium phosphate solutions at pH 7.2 (data not shown).

DISCUSSION

Ammonia and ammonium ions are lethal to both vertebrates and invertebrates at concentrations between 0.5 and 5 mM (see Withers, 1992). Thus, the social amoebae may be unique in using very high concentrations of ammonia as a morphogen controlling development. However, the reaction of social amoebae is usually avoidance. The organism either moves away from the source of the ammonia, or attempts to consume the morphogen.

GS activity is very high in sporulating cells of *D. discoideum*, allowing regulation of ammonia concentrations during culmination (Dunbar & Wheldrake, 1995, 1997a). However GS activity was virtually absent in dormant spores 2–4 d after culmination, suggesting that ammonia cannot be assimilated and instead accumulates in the sori of mature fruiting bodies. The GS activity began to rise 1 h after spores were induced to germinate by autoactivation or heat-induction. It continued to increase even after 5 h when spore germination was complete: the full vegetative level was not attained until 8 h. Since spore germination uses endogenous reserves and bacteria were not present in the culture, cell growth is not required to return the enzyme to vegetative levels. The transcriptional control of GS activity shown by mRNA blotting is similar to that found during culmination (Dunbar & Wheldrake, 1997b). At the

Fig. 7. Modulation of ACA and ectopically expressed ACG activities by sodium chloride and ammonium chloride. The data are replotted from Figs 1c and 3b of Schaap *et al.* (1995). ACA relay activities were examined by incubating aggregation-competent cells at 10^8 ml^{-1} in 20 mM HEPES buffer at pH 7.4 for 15 min with various concentrations of sodium chloride (Δ) and ammonium chloride ($\circ$). The cells were then stimulated with 2'HcAMP in 50 mM DTT for 5 min; the reaction was terminated by addition of 3.5% perchloric acid. A similar protocol was used to measure *in vivo* cAMP accumulation by *aca*⁻/ACG cells under the influence of sodium chloride ($\blacktriangle$) and ammonium chloride ($\bullet$) for 15 min, except that cells were starved for 1–2 h and only 50 mM DTT was added at the time of harvest. Cell lysates in both assays were neutralized with potassium bicarbonate and cAMP was measured by isotope dilution (see Schaap *et al.*, 1995 for additional details).

same time that GS activity is increasing in germinating spores, ammonia is being released in what appears to be a futile ammonia cycle. Previous work suggests that a substantial amount of ammonia released during germination is derived from the degradation of endogenous glutamine, glutamate, asparagine and other amino acids (Ennis, 1981; Jackson *et al.*, 1982; Klein *et al.*, 1990). Since exogenous ammonium at 28 mM did not modulate GS activity, the enzyme appears to be an integral part of the germination programme; i.e. it is not regulated by the environment, but is synthesized once the commitment to germination occurs. There is evidence that GS has a major function other than the synthesis of glutamine; i.e. it may be required to regulate free ammonia in various compartments of cells (Cotter *et al.*, 1992; Dunbar & Wheldrake, 1995, 1997a). Thus the enzyme may return as part of the preparation for normal vegetative growth, but it also may modulate the level of ammonia in the germination buffer.

The effects of various concentrations of ammonia during aggregation, slug migration and culmination were confusing until the work of Schaap *et al.* (1995), who studied the activity of ACA (adenylyl cyclase for aggregation) and ACG (expressed ectopically) in vegetative cells exposed to various osmotic agents. A portion of their data is replotted in Fig. 7 to aid the following discussion. At 100–250 mM, substances such

as sucrose, glucose and polyalcohols effectively maintain spore dormancy (Cotter, 1977) by stimulating ACG to its maximum activity (van Es *et al.*, 1996; Viridy *et al.*, 1999). At concentrations near 100 mM, any exogenous, non-penetrating substance such as potassium phosphate, sodium chloride, ammonium chloride or ammonium phosphate can activate ACG through its osmosensing function (see Fig. 7). At concentrations of 300 mM or more, ACG is inhibited and heat-activated spores are unable to return to dormancy because of partial dehydration (Cotter, 1977). At lower concentrations such as between 50 and 69 mM, ammonium phosphate (depending upon the pH) inhibits spores from germinating in a fully reversible manner whereas potassium phosphate does not. This suggests that ammonium phosphate is not acting via the osmosensing function of ACG. Ammonium chloride is a more effective activator of ACG than sodium chloride at concentrations around 10–50 mM (see Fig. 7). The pH of the cytosol of amoebae is normally 7.2–7.4 (Satre *et al.*, 1989), and it can be decreased to 6.4 with weak acids such as propionic acid (Satre *et al.*, 1989), or alternatively raised to 7.9 with weak bases (Van Lookeren Campagne *et al.*, 1989). The activity of ACG is highly pH dependent and peaks around pH 7.8 with little activity below pH 7.0 (Schaap *et al.*, 1995). We suggest that prevention of spore germination and enhanced activation of ACG by ammonium salts over potassium or sodium salts at 50–69 mM is a result of slight alkalization of the spores since the active species of the ammonia/ammonium ion pair is ammonia. Any increase in pH in the cortical layers of the spore would stimulate the plasma-membrane-bound ACG without greatly changing the overall cytosolic pH. Thus, both high (100 mM) and moderate (50–69 mM) concentrations of ammonium phosphate may maintain spore dormancy in sori by activating ACG through an osmotic as well as a pH effect. The evidence that *acg*⁻ spores are not inhibited by either 250 mM sucrose (van Es *et al.*, 1996) or 50 mM ammonium phosphate at pH 7.2 (Table 3) is consistent with the above hypothesis.

Concentrations of ammonium phosphate below 50 mM in sori, resulting from dew formation at high relative humidities, do not maintain spore dormancy without the aid of other autoinhibitors such as discadenine (Abe *et al.*, 1976). When unwashed dormant spores are diluted to 10^8 ml^{-1} , they often do not germinate spontaneously in phosphate buffer (unpublished), nor in response to nutrient media (Abe *et al.*, 1976; Zinda & Singleton, 1998). Since the concentration of ammonium phosphate is then only 1–3 mM, dormancy must be maintained by other factors such as discadenine (Abe *et al.*, 1976; Gauthier *et al.*, 1997). Discadenine may function through activating the histidine kinase DHKB, which then inhibits RegA hydrolysis of cAMP produced by ACG (Zinda & Singleton, 1998). At low relative humidities when fruiting bodies are relatively dry, wild-type spores would be subjected to high concentrations of ammonium phosphate and discadenine, both conditions favouring the maximum production of cAMP by ACG

and its minimal hydrolysis by the phosphodiesterase activity of RegA. The regulation of spore dormancy through maximal stimulation of cAMP and therefore maximum stimulation of PKA may be one of several times in the life cycle that development must be driven by at least two independent signals precisely controlled both spatially and temporally (Thomason *et al.*, 1998). That precise control of PKA activity is at the centre of regulation is indicated by studies with null mutants of ACA expressing just 1.6 times the catalytic subunit activity of PKA in wild-type cells (Wang & Kuspa, 1997). Such mutants rapidly form complete fruiting bodies with viable spores. It may be significant that an optimal concentration of ammonium (10–20 mM) increases ACA activity up to 1.7 times that given by the same concentration of sodium. At 50 mM and above, however, both sodium chloride and ammonium chloride are highly inhibitory to ACA activity (Fig. 7; Schaap *et al.*, 1995). The very large shift in ACA activity from stimulation to inhibition over a narrow range of ammonia concentrations may account for the confusion in the literature on the effects of ammonia upon cAMP accumulation (Riley & Barclay, 1990; Williams *et al.*, 1984).

The work reported here in conjunction with that of others suggests that the functions of ACA, GS and ACG are coupled in a sequential way during development. As much as 70 mM exogenously added ammonium chloride is required to block culmination (Davies *et al.*, 1993), which is probably triggered by a decrease in the ammonia concentration of the slug as originally suggested by Schindler & Sussman (1977, 1979) and shown by Wilson & Rutherford (1978). The decrease in ammonia to 10–20 mM by an increase in GS activity (Dunbar & Wheldrake, 1995, 1997a, b, c) would stimulate ACA to produce cAMP, in turn activating PKA almost twofold in the prespore region of the slug, and resulting in culmination. A marked rise in cAMP has been noted around the time of culmination (Abe & Yanagisawa, 1983). After fruiting-body formation is nearly complete, a decrease in GS activity in sporulating cells (Dunbar & Wheldrake, 1995, 1977a) without a corresponding decrease in ammonia production in dying stalk cells, would result in ammonia accumulation and severe inhibition of ACA activity (see Fig. 7). This could potentially decrease cAMP production and PKA activity, triggering spore germination (van Es *et al.*, 1996; Viridy *et al.*, 1999). However, it is at this time in development that *acg* is transcribed and translated in spores (Pitt *et al.*, 1992). Replacing ACA with ACG allows increasing concentrations of ammonia to act as osmotic and pH modifying agents stimulating ACG to produce cAMP, and thus promoting high PKA activity to maintain spore dormancy. Various histidine kinases may regulate RegA to fine tune the level of cAMP, both during culmination and post-sporulation (Shaulsky *et al.*, 1998; Thomason *et al.*, 1998; Zinda & Singleton, 1998). The above model may work in conjunction with a decrease in the acidification of vesicles by ammonia (Davies *et al.*, 1996) since lysosomal cysteine proteinases remain in a non-

acidified, unactivated state in dormant spores (North *et al.*, 1990, 1996; Cotter *et al.*, 1997).

NOTE ADDED IN PROOF

Recently, a third adenylyl cyclase activity (ACB), inhibited by high osmolarity, has been discovered by Meima & Schaap (1999). The enzyme may play a role similar to that postulated for ACA in the initiation and completion of culmination.

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