Elevation of AMP Levels During Phagocytosis in *Acanthamoeba castellanii*

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Addition of particles to growing cultures of the amoeba, *Acanthamoeba castellanii* resulted in the production of large amounts of AMP, much of which was found extracellularly. At the time of maximal AMP production (13 h growth) adenylate energy charge values in conditioned and fresh medium were 0.58 and 0.1, respectively. Removal of particles from growth medium by filtration lowered the AMP level to 22% of the control value at the time of maximal AMP production; addition of particles to conditioned medium raised the level of AMP 3.6-fold.

Uptake of killed yeast was accompanied by relatively small changes in levels of ATP and ADP, but large (5.7-fold) changes in AMP levels. Successive cycles of phagocytosis were paralleled by cyclic changes in AMP levels.

INTRODUCTION

The soil amoeba *Acanthamoeba castellanii*, in common with other free-living amoebae (Chapman-Andresen, 1977), relies entirely on processes of endocytosis for uptake of nutrients. The plasma membrane of this organism has many similarities in its macromolecular composition to mammalian cells (Korn & Wright, 1973) and indeed the endocytic mechanisms appear similar to those occurring in many types of mammalian cells (Silverstein *et al.*, 1977; Klebanoff & Clark, 1978). Apart from feeding mechanisms, endocytosis is also important for the microbiocidal action of certain white blood cells (Klebanoff & Clark, 1978), membrane internalization and re-cycling (Schneider *et al.*, 1979; Bowers, 1980; Bowers *et al.*, 1981) and other important cellular functions (Silverstein *et al.*, 1977). While in *A. castellanii* pinocytosis (fluid uptake) appears to be a continuous feeding process (Bowers & Olszewski, 1972), phagocytosis (particle uptake) can be induced by the addition of latex beads (Weisman & Korn, 1967; Wetzel & Korn, 1969), yeasts (Ryter & Bowers, 1976) or horse red blood cells (Brown *et al.*, 1975).

During exponential growth of *A. castellanii* in batch culture in a glucose/proteose peptone/yeast extract medium, the initial 30 h are unusual in several respects (Edwards & Lloyd, 1977*a, b*). Respiration rates are low, but upon the addition of 1 mM-cyanide may be stimulated by up to 50% (Edwards & Lloyd, 1977*b*); glucose disappearance is negligible and adenylate energy charge values are low (less than 0.1) mainly due to the production of large amounts of AMP, much of which is found extracellularly (Edwards & Lloyd, 1977*a*). When grown in conditioned medium (i.e. having previously supported growth for 30 h), respiration is still cyanide-stimulated (Edwards & Lloyd, 1977*b*), but glucose disappearance is detectable from time zero, and low levels of extracellular AMP are found (Edwards & Lloyd, 1977*a*). Thus, it was suggested that the particles that are produced after autoclaving growth medium are preferentially ingested by the amoebae and that this phagocytic process is responsible for the elevated AMP levels.

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In the present study we have further examined this phenomenon using various modifications of the growth medium and by feeding the cells with heat-killed yeasts. We show that phagocytosis of particles of medium or yeast results in the production of large amounts of AMP, much of which is found extracellularly.

METHODS

Growth of the organism. *Acanthamoeba castellanii* was maintained and grown with shaking at 30 °C exactly as described previously (Edwards *et al.*, 1977) in a medium containing (% w/v): Difco proteose peptone, 0.75; Oxoid yeast extract, 0.75; glucose, 1.5. Organisms were counted in a Fuchs-Rosenthal haemocytometer slide (Baird & Tatlock, Chadwell Heath, Romford Essex) after suitable dilution with 50 mM-MgCl₂, pH 7.4.

Measurements of phagocytosis. Dried *Saccharomyces cerevisiae* (0.5 g) was heated to 100 °C for 1 h in 10 ml of 50 mM-phosphate buffer, pH 7.4 (Jenson & Bainton, 1973). The suspension was centrifuged at 1000 *g* for 5 min, the supernatant discarded and the pellet washed twice in 5 vol. of the above buffer. The heat-killed organisms were kept as a thick slurry at 4 °C for 1 week until required.

For measurements of phagocytosis, amoebae were grown for 36 h (cell density of approximately 10⁶ cells ml⁻¹) in duplicate flasks. The medium from one culture was collected by centrifugation at 1000 *g* for 10 min at 30 °C and the supernatant was termed 'conditioned medium'. This was used to dilute the contents of the second flask to a cell density of approximately 2 × 10⁵ cells ml⁻¹; the flask was equilibrated at 30 °C with shaking for 30 min prior to the addition of the heat-killed yeast. Initiation of phagocytosis was by the addition of the killed yeast suspension (usually about 10–35 µl to 50 ml culture) and samples were removed at frequent intervals for cell counts, or measurements of adenine nucleotides.

Experiments with conditioned medium. Conditioned medium was prepared as described above. Particles formed after autoclaving were removed by filtration through 0.22 µm pore-size filters. Where indicated, particles were introduced into conditioned media by washing the filter. Thus, four types of growth media could be produced: (1) freshly-autoclaved media; (2) freshly-autoclaved, filtered media; (3) conditioned media and (4) conditioned media with the particles from (2) added to it. All media were autoclaved before inoculation (10⁵ cells ml⁻¹) and amoebae were allowed to grow for about 24 h.

Analytical methods. Measurements of adenine nucleotides were as described previously (Edwards *et al.*, 1975) using luciferin-luciferase assays on 1 ml of culture prepared by extraction with 0.3 ml boiling chloroform. Rapid mixing using a 'Whirlimixer' ensured quenching within 0.2 s. ATP, ADP and AMP were measured following the procedure of Chapman *et al.* (1971) using ATP as calibration standard. After filtration of the culture (Whatman GF/A filters), adenine nucleotides in the growth medium were measured using 1 ml of the filtrate. The yeast suspension contained only AMP at a concentration of 0.26 nmol ml⁻¹ and this value was subtracted from all AMP measurements. Oxygen uptake was measured at 30 °C using the 'open' system of Degn & Wohlrab (1971). The system consisted of a stainless steel reaction vessel (working volume 4.5 ml) fitted with a membrane-covered Clark electrode (type D602; Radiometer, Copenhagen). With a constant partial pressure of oxygen in the gas phase, the respiration rate (V_r) was calculated after the attainment of a steady-state oxygen concentration in the liquid phase by the equation: $V_r = K(T_G - T_L)$, where K is the oxygen transfer constant, T_G is the oxygen concentration in the gas phase and T_L is the oxygen concentration in the liquid phase.

RESULTS

Changes in AMP concentration during growth

Previous work had suggested that the large quantities of AMP produced during the initial phase of growth in fresh medium may be the result of phagocytosis of particles that are produced after autoclaving (Edwards & Lloyd, 1977*a*). These high levels of AMP were not apparent during growth in conditioned medium which did not contain particles. Differing growth conditions, as described in Methods, were employed to further test this hypothesis. In culture 1 (freshly autoclaved medium) the AMP concentration was highest after about 15 h growth, at approximately 100 nmol (ml culture)⁻¹ (Fig. 1*a*). In culture 2 (freshly autoclaved but filtered) the AMP concentration was reduced to about 22 nmol (ml culture)⁻¹. In culture 3 (conditioned media containing no particles) AMP reached a maximum concentration of about 5 nmol (ml culture)⁻¹, while in culture 4 (conditioned media with added particles) the AMP concentration reached about 18 nmol (ml culture)⁻¹. Thus, removal of particles from fresh media reduced the AMP concentration to about 22% of the control value, whereas addition of particles to conditioned media elevated the AMP concentration 3.6-fold. Corresponding adenylate energy charge values for conditioned medium and fresh medium at 13 h growth were 0.58 and 0.1, respectively (Fig. 1*b*).

Fig. 1. Changes in AMP and adenylate energy charge values during growth of *A. castellanii*. Organisms were inoculated (10^5 cells ml^{-1}) at time zero into: freshly autoclaved medium (●); freshly autoclaved, filtered medium (○); conditioned medium (□) and conditioned medium with added particles (■). Samples were removed at intervals for cell counts (data not shown) and measurement of (a) AMP and (b) adenylate energy charge, defined by Atkinson & Walton (1967) as: adenylate energy charge = $(\text{ATP} + \frac{1}{2} \text{ADP})/(\text{ATP} + \text{ADP} + \text{AMP})$.

Phagocytosis of yeast

While the above evidence showed that the presence or absence of particles in the growth media greatly altered AMP levels, a more direct, controllable system of phagocytosis was tested. Heat-killed yeasts were added to a suspension of amoebae and the rate of phagocytosis was measured by counting the number of yeasts free in the medium (Fig. 2). With an initial ratio of 10 yeasts to 1 amoeba phagocytosis was completed by 30 min, i.e. virtually no yeast were detected free in the medium. When the initial ratio of yeast to amoebae was increased to 20:1, the rate of phagocytosis was biphasic. By 4 min the amoebae had engulfed approximately 16 yeasts each; no further phagocytosis took place for a further 6 min and then another burst of phagocytosis was observed. Presumably this lag phase represented a period of digestion of phagocytosed yeasts which must be completed before further uptake could occur. When the initial ratio of yeast to amoeba was increased further to 40:1, each amoeba took up approximately 30 yeast during the initial burst of phagocytosis. This increased uptake presumably results from the increased collision of yeasts with amoebae, which must occur before ingestion; however, it must be pointed out that we have not distinguished between yeasts which were engulfed and those merely attached to the cell surface. Also, at such high yeast to amoeba ratios extensive clumping of amoebae occurs, probably trapping yeasts. Thus, these numbers of yeast ingested per cell must be considered to be maximal.

Changes in oxygen uptake rates during phagocytosis of yeasts

A suspension of amoebae (2×10^5 cells ml^{-1}) in conditioned medium was placed in the open oxygen electrode vessel and allowed to reach a steady-state with air in the gas phase. The steady-state respiration was calculated to be $1.54 \text{ nmol O}_2 \text{ min}^{-1} (\text{ml culture})^{-1}$ (Fig. 3). As indicated, yeasts were added to give a yeast to amoeba ratio of 10:1 and, after a lag phase of about 15 min, the steady-state level of oxygen dropped indicating an increase in respiration rate to $1.88 \text{ nmol O}_2 \text{ min}^{-1} (\text{ml culture})^{-1}$. Thus, a 22% increase in respiration rate occurred during phagocytosis.

Fig. 2. Phagocytosis of yeast by *A. castellanii*. *Saccharomyces cerevisiae* was heat-killed as described in Methods. Cultures of *A. castellanii* were grown to approximately 10^6 cells ml^{-1} and diluted to 4×10^5 cells ml^{-1} (■) or 5×10^5 cells ml^{-1} (●, ○) with conditioned medium. At time zero yeast were added to give initial yeast to amoeba ratios of 40:1 (○); 20:1 (●) and 10:1 (■). Phagocytosis was estimated by microscopic counts of the number of yeast free in the medium.

Fig. 3. Changes in oxygen uptake rates during phagocytosis of yeasts. A culture of *A. castellanii* was grown to 10^6 cells ml^{-1} and diluted to 2×10^5 cells ml^{-1} with conditioned medium. Diluted culture (4.5 ml) was introduced into the open oxygen electrode system until the steady-state concentration of oxygen in the liquid phase was attained. Heat-killed yeasts were added (arrow) to give an initial ratio of yeast to amoeba of 10:1. The bar marker represents 10 min.

Changes in adenine nucleotide pool levels during phagocytosis of yeast

Yeast were added to a suspension of amoebae to give an initial ratio of 30 yeast to 1 amoeba. Phagocytosis was initially rapid and proceeded in three distinct phases (Fig. 4a). The first phase was completed by about 20 min and the second by about 45 min. Concentrations of ATP (Fig. 4b), ADP (Fig. 4c) and AMP (Fig. 4d) were constant before the addition of yeast and were about 0.6, 1.55 and 6 nmol (ml culture) $^{-1}$, respectively, where the culture included cells plus medium. Upon the addition of yeast, levels of all three adenine nucleotides dropped initially; ATP levels dropped by 50%, ADP by 26% and AMP by 81%. Levels of all three then increased as the initial burst of phagocytosis declined, decreased again (AMP remained constant) at the onset of the second burst of phagocytosis and then declined as the second burst of phagocytosis slowed down. The third phase of phagocytosis did not show such dramatic changes in adenine nucleotide levels. The maximal increases in levels (about 45 min after the addition of yeast)

Fig. 4. Changes in adenine nucleotide pool levels during phagocytosis of yeasts. A culture of *A. castellanii* was grown to 10^6 cells ml^{-1} and diluted to 1.8×10^5 cells ml^{-1} with conditioned medium. The culture was returned to the growth vessel for 30 min. Five samples were removed from the culture and rapidly extracted with boiling chloroform, prior to the addition of yeasts. At time zero (arrow) heat-killed yeasts were added to give an initial yeast to amoeba ratio of 32:1. At frequent time intervals, samples were removed for measurement of (a) yeast cell numbers, (b) ATP, (c) ADP and (d) AMP. Levels of adenine nucleotides are expressed as $\text{nmol (ml culture)}^{-1}$, where culture included cells plus medium. Results are typical of five separate experiments.

above those before phagocytosis was initiated were: ATP 50%; ADP 16%; AMP 567%. At this time no ATP could be detected in the culture filtrate, while 4% of the ADP and over 40% of the AMP were detected extracellularly.

DISCUSSION

The results presented here confirm the previous suggestion (Edwards & Lloyd, 1977*a*) that phagocytosis of particles by *A. castellanii* results in elevation of AMP levels, thus lowering values of adenylate energy charge. This phenomenon therefore provides another example which, together with some cell cycle-dependent variations (Lloyd *et al.*, 1982), argues against the concept of stabilized adenylate energy charge values in growing organisms (Chapman & Atkinson, 1977). Phagocytosis has been observed under two conditions in the present study using particles of growth medium that are produced upon autoclaving the medium and a more controllable experimental system using heat-killed yeast. Removal of particles from the growth

medium reduced AMP levels to 22% of the maximal value, while addition of these particles to conditioned medium elevated AMP levels 3.6-fold. Phagocytosis of yeasts showed that elevation of AMP levels occurred within 3 min of the initiation of uptake. Repeating cycles of AMP production corresponding to successive cycles of uptake of yeasts add further strength to these conclusions.

Whether AMP production and subsequent extracellular export is a primary or secondary response to phagocytosis is not known at present. While all three adenine nucleotides showed responses similar to that of AMP during yeast uptake, the magnitude of the changes in ATP and ADP were less dramatic. Final levels of ATP were only about 15% higher than the level before the initiation of phagocytosis, whereas AMP levels were increased by a maximum of 5.7-fold. Since ATP is believed to be necessary for the phagocytic response (Silverstein *et al.*, 1977), a drop in ATP levels at the onset of yeast uptake does not seem surprising. ATP turnover in growing micro-organisms is very rapid (Chapman & Atkinson, 1977) and, since *A. castellanii* were not nutrient-limited, regeneration of ATP, with perhaps an 'overshoot' in production, rapidly occurred. Changes in AMP levels may be correlated with this phenomenon, since ADP produced by ATP hydrolysis may be further dephosphorylated to AMP and exported from the cells. In this way favourable ATP : ADP or ATP : AMP ratios may be maintained intracellularly (Knowles, 1977). Alternatively, there may also be some contribution to elevated AMP levels by increased RNA degradation (Klebanoff & Clark, 1978).

The increased oxygen uptake rate which was observed during phagocytosis resembles the 'metabolic burst' of leucocytes (see Silverstein *et al.*, 1977; Klebanoff & Clark, 1978 for references), where phagocytosing cells may show a two- to threefold increase in oxygen consumption rate. It is doubtful, however, in some cases if this cyanide-insensitive respiration is directly concerned with the increased energy demands. The response in *A. castellanii*, apart from being of much lower magnitude, was also different in that the increase in oxygen uptake rate occurred much later after the initiation of phagocytosis than can be considered typical for a 'metabolic burst.'

Further work is necessary to ascertain the origin and fate of the extracellular AMP and to determine any similarities and/or differences in endocytic properties to those in mammalian cells.

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