IN VITRO FUSION OF *ACANTHAMOEBA* PHAGOLYSOSOMES

III. Evidence That Cyclic Nucleotides and Vacuole Subpopulations Respectively Control the Rate and the Extent of Vacuole Fusion in *Acanthamoeba* Homogenates

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ABSTRACT

Fusion of phagolysosomes has been previously demonstrated to occur during the incubation of phagolysosome-containing homogenates of *Acanthamoeba* (Oates and Touster, 1978, *J. Cell Biol.* **79**:217–234). Further studies on this system have shown that methylxanthines (0.2 mM) and/or cAMP (0.5–1 mM) markedly accelerate the average rate, but not the extent, of the in vitro phagolysosome fusion process. Adenosine, 5′-AMP, and ADP (0.5–1 mM) were without effect. ATP (0.5–1 mM) caused variable stimulation, whereas β,γ-methylene-ATP (1 mM) caused pronounced inhibition, as did GTP (1 mM) and cGMP (1 mM). Stimulation by 3-isobutyl-1-methylxanthine was blocked by GTP, but not by ATP or cAMP. These results indicate that the rate of phagolysosome fusion in *Acanthamoeba* homogenates may be regulated by cyclic nucleotides, with enhancement of the fusion rate by cAMP and inhibition of the rate by cGMP. The extent of the reaction increased spontaneously and markedly during the first few hours after preparation of the homogenates. This activation appears to be because of a slow conversion of a significant fraction of the vacuole population from a fusion-incompetent to a fusion-competent, cyclic nucleotide-sensitive state.

The phagolysosome fusion reaction in *Acanthamoeba* homogenates occurs relatively rapidly, in substantial yield, in the absence of exogenous fusion-inducing agents, and in the absence of detectable membrane damage (10). It shows a distinct temperature optimum at 30°–32°C, the growth temperature of the organism, and is arrested at 0°C (7). In this system, vacuole fusion is independent of the type of particulate material within the vacuole, retains the specificity of fusion between membrane types that is observed in vivo, and occurs by a mechanism morphologically similar or identical to that observed in vivo (9). The fusion reaction is unaffected by colchicine, cytochalasin B, 2,4-dinitrophenol, KCN, or low ($\leq$10 mM) concentrations of added KCl, but is inhibited by low concentrations of KF (I_{50} = 2.7 mM) (7, 10). Because these properties suggested that the *Acanthamoeba* homogenate system was a promising model of the fusion process in vivo, we have pursued our characterization of this system. We now report the identification of factors on which

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the rate of the fusion reaction is dependent, namely adenyl and guanyl nucleotides. Evidence is also presented that the extent of the fusion reaction is dependent on the relative size of a fusion-competent subpopulation. An abstract of part of this work has been previously published (8).

MATERIALS AND METHODS

Materials

3-Isobutyl-1-methylxanthine (MIX)[1] was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). β,γ-Methylene-adenosine triphosphate (β,γ-CH_2-ATP) was purchased from Miles Laboratories, Inc. (Elkart, Ind.). All other nucleotides were obtained as the sodium salts or the free acids in the purest grades available from Sigma Chemical Co. (St. Louis, Mo.). Enzyme grade Tris (base) and density gradient grade sucrose were obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.), and EM grade OsO_4 from Polysciences, Inc. (Warrington, Pa.). Certified Eosin Y was purchased from American Hospital Supply Corp., Harleco Div. (Gibbstown, N. J.). Other chemicals were reagent grade.

In Vitro Fusion Experiments

Acanthamoeba castellanii (Neff strain) were cultured axenically at 30°-32°C in 500-ml and 1-liter aerated bottles as described previously (9). Cells were harvested either during exponential growth phase (4-6 $\times$ 10^5/ml) or in stationary phase (1.8-2.5 $\times$ 10^6/ml). Generation time in exponential phase was 8-10 h. In vitro fusion experiments with vacuoles labeled with red blood cells (Rv) or with yeast (Yv) were carried out as previously described (10). Reaction rates were measured 2-3 h after homogenization.

Assay Methods

In most experiments with homogenates prepared from exponential phase cells, fusion was monitored by measuring the postincubation levels of hybrid (red blood cell and yeast-containing) vacuoles (Hv), as described previously (10). In all experiments with homogenates prepared from stationary phase cells, and in some with exponential phase cell homogenates, the fusion reaction was studied in homogenates containing vacuoles labeled with red blood cells only. In these cases, the extent of fusion (%F) was defined as the number of fusion events which had occurred (equal to the decrease in the total number of vacuoles) relative to the maximum number of fusion events which could possibly have occurred (equal to the initial vacuole concentration, Vo, when $Vo \gg 1$), $\times$ 100. Under conditions where the particle-containing vacuoles are stable, %F is given by:

$$\%F = [1 - (No/N)] \times 100, \quad (1)$$

where No is the average number of particles per vacuole before the 30°C incubation, and N is the same quantity after the incubation. These values were obtained by examining pre- and postincubation samples under oil immersion bright field microscopy in the presence of 0.3% Eosin Y, as previously described (10); 600-800 vacuoles (800-1,500 particles) were counted per sample. The total number of particles in the vacuoles counted was divided by the total number of vacuoles counted to give N or No, from which %F was calculated by Eq. 1. Vacuole stability was monitored by the dye exclusion assay previously described (10).

In some experiments, the number of particles in various vacuole subgroups was determined at different time points during the fusion reaction. Results were expressed relative to the total number of membrane-bounded particles, a quantity which remains constant during the reaction (see Results). Vacuoles were classified under oil immersion as monomers, dimers, trimer, . . . etc., on the basis of the number of particles they contained (1, 2, 3, . . . etc.). The frequency of particles in each vacuole class was obtained by dividing the total number of particles found in a given class by the total number of particles counted in all classes (~1,600 particles [1,000 vacuoles] per sample).

Electron microscopy was performed as described before (10), except that to minimize handling of the rather fragile giant hybrid vacuoles (see Results), fixation of the undiluted reaction mixtures was done in these cases by carefully overlaying the relatively viscous homogenates with cold, unbuffered 1% OsO_4 which was 0.3 M in sucrose. The samples were allowed to fix for 4 h at 0°C before the processing plugs were inserted and the specimens were then embedded, sectioned, and stained as previously reported (10).

RESULTS

Rate of the Fusion Reaction

In homogenates prepared from stationary phase cells (1.8-2.5 $\times$ 10^6/ml), the rate of the in vitro fusion reaction slowed after the first 5 min and reached a plateau by 30 min (Fig. 1, open symbols). If MIX, a potent and specific inhibitor of cyclic nucleotide phosphodiesterase activity (1), was added to the homogenates before the incubation, the fusion reaction did not slow down after 5 min, but continued at the same rate and reached the same plateau value as reached by the control system in only ~7 min (Fig. 1, half-filled diamonds). Theophylline (1,3-dimethylxanthine) was observed to cause the same effect, but it was somewhat less potent than MIX, as has been reported in other systems (1) (data not shown). A result virtually identical to that found with 0.2 mM MIX was obtained in the absence of the phosphodiesterase inhibitor when the homogenates were supplemented with relatively high (nominally 0.5 or 1 mM) concentrations of cAMP (Fig. 1, half-filled circles and triangles).

The unlikely possibility that cAMP was causing adhesion, rather than fusion (see reference 10), of the vacuoles (to an extent which happened to coincide with the extent of fusion in the control

[1] *Abbreviations used in this paper*: β,γ-CH_2-ATP, β,γ-methylene-adenosine 5'-triphosphate; Hv, hybrid, i.e., red blood cell- and yeast-containing vacuole; MIX, 3-isobutyl-1-methylxanthine; Rv, red blood cell-containing vacuole; Yv, yeast-containing vacuole.

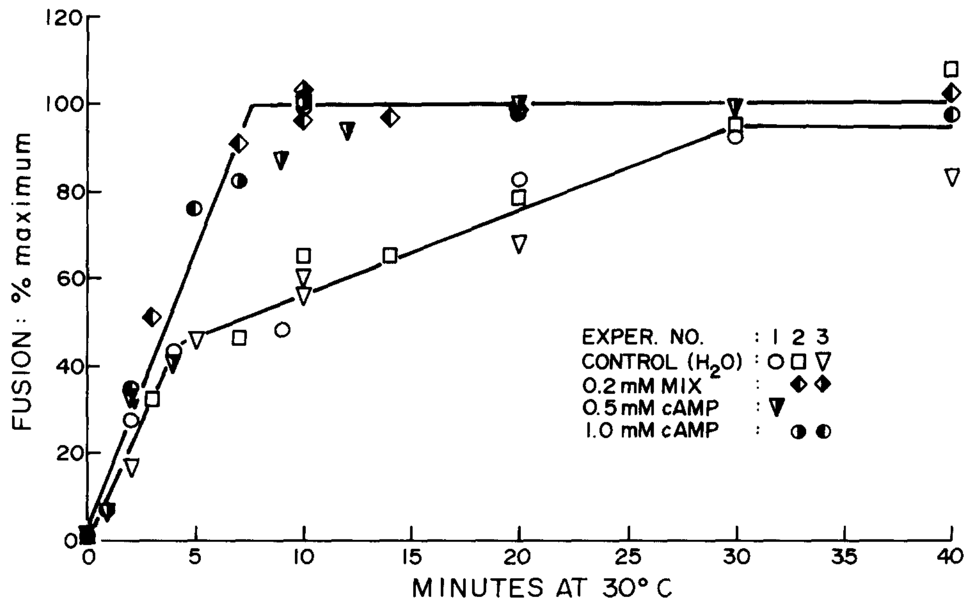

FIGURE 1 Effects of MIX and cAMP on the vacuole fusion reaction in Rv-containing homogenates prepared from stationary phase cells. The figure shows the pattern of fusion reaction in the controls (open symbols), and after the addition of 0.2 mM MIX (half-filled diamonds), 0.5 mM cAMP (half-filled triangles), and 1 mM cAMP (half-filled circles). Fusion (%F) was measured as described in Materials and Methods, and the results are plotted relative to the maximum amount of fusion obtained in each experiment (plateau level = 100%).

system) was ruled out by examination of 10-min samples from the above reaction mixtures by electron microscopy. No evidence of adhesion in any of the samples was found by EM, and a significantly higher amount of fusion, i.e., a higher average number of particle profiles/vacuole, was found in the samples treated with cAMP (data not shown). As measured by dye exclusion, the integrity of the phagolysosome membranes was unaffected throughout the course of the incubation in the homogenates prepared from stationary phase cells, as previously reported for the reaction in homogenates made from exponential-phase cells (10). In addition, at the concentrations employed in this study, none of the compounds used to perturb the reaction system had any noticeable effect on phagolysosome stability, as measured by dye exclusion (data not shown).

Stimulation of the above reaction rate, i.e., shortening of the time necessary to reach the reaction plateau, by MIX and/or cAMP was also seen in homogenates prepared from exponentially growing cells (4.0–5.0 × 10^5/ml). In this case, the control reached a plateau level by 10–15 min as previously reported (25), while the cAMP-supplemented reaction plateaued at the same level as the control by 4–7 min (data not shown).

The specificity of this rate-stimulating effect was then examined. As shown in Table I, MIX and cAMP accelerated the fusion reaction to the same extent, causing an average increase of 70% at 10 min relative to the control, while adenosine, 5'-AMP, and ADP had no significant effect at 1 mM concentration (Table I) or at 0.5 mM (data not shown). The effect of ATP was variable (note the relatively large standard deviation for this value in Table I): it either showed little effect or it stimulated as well as cAMP or MIX. In contrast, β,γ-CH_2-ATP inhibited the reaction ~50% in each of three experiments (Table I). In addition, GTP and cGMP were found to markedly inhibit the reaction (Table I). Finally, combination of MIX with cAMP or ATP did not cause significant additional fusion over that seen with MIX alone, but GTP was observed to block the stimulatory effect of MIX (Table I). These results indicate that the rate, but not the extent, of the in vitro fusion reaction depends on the endogenous concentrations of adenyl and guanyl nucleotides available.

Extent of the Fusion Reaction

An analysis of the changes in vacuole species

TABLE I

Specificity of Nucleotide Effects on Vacuole Fusion

Additions	% Fusion
Control (H_2O)	20.4 ± 3.6 (11)*
0.2 mM MIX	37.2 ± 5.2 (7)
1 mM cAMP	32.8 ± 6.0 (9)
1 mM adenosine	22.7 ± 4.5 (3)
1 mM 5'-AMP	19.7 ± 0.2 (2)
1 mM ADP	20.9 ± 4.9 (2)
1 mM ATP	27.9 ± 10.0 (6)
1 mM β,γ-CH_2-ATP	9.9 ± 2.9 (3)
1 mM cGMP	5.5 ± 1.9 (3)
1 mM GTP	4.8 ± 3.3 (2)
0.2 mM MIX + 1 mM cAMP	42.7 ± 3.3 (2)
0.2 mM MIX + 1 mM ATP	42.1 ± 0.7 (2)
0.2 mM MIX + 1 mM GTP	23.8 ± 5.1 (2)

* Mean ± SD. The number of determinations is shown in parenthesis. All samples were incubated for 10 min at 30°C.

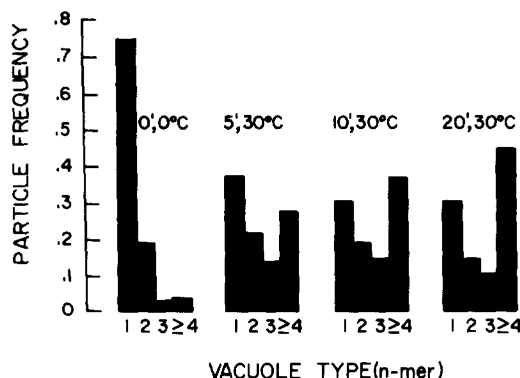

FIGURE 2 Distribution of membrane-bounded particles among various vacuole types during the course of a typical fusion reaction in homogenates prepared from exponential phase cells. Incubations were carried out 90 min after homogenate preparation. Vacuole types were defined in terms of the number of particles they contained (single-particle vacuole = monomer, two-particle vacuole = dimer, etc.). Note the constancy of the monomer population between 10 and 20 min, while the $n \geqslant 4$ population has increased at the expense of the dimer and trimer populations. See text for further discussion.

which occur during the course of a typical fusion reaction carried out 90 min after homogenate preparation is shown in Fig. 2. As can be seen, the percentage of total membrane-bounded particles present as single-particle vacuoles ("monomers") decreased to 40% of the initial level by 10 min and remained constant thereafter. However, while the monomer population ceased fusion activity at 10 min, an increase in the larger-sized $n \geq 4$ vacuole class was seen to proceed between 10 and 20 min at the expense of the dimer and trimer populations (Fig. 2, 10 vs. 20 min). In parallel experiments, cAMP and MIX each were found not to affect the final (20 min) particle distribution, but only the rate at which the final distributions was achieved (data not shown). These results indicate that a fixed and substantial fraction of the initial vacuole population is incapable of fusing at relatively early times after homogenate preparation.[2]

In mixtures of Rv- and Yv-containing homogenates prepared from exponentially growing *Acanthamoeba*, hybrid vacuole formation is virtually complete by 10 min of incubation at 30°–32°C (10). Somewhat surprisingly, however, the plateau level reached at 10 min was found to increase during the first few hours after preparation of the homogenates, after which it declined (Fig. 3). While the exact time and extent of maximum fusion varied from experiment to experiment, peak fusion values were typically obtained between 3 and 5 h posthomogenization, and the extent of the reaction typically increased severalfold[3] during the first few hours, reaching as high as 80% hybrids on some occasions. Transmission electron microscope observations on specimens taken at peak fusion times showed large groups of particles surrounded by single, continuous, morphologically undamaged unit membrane profiles, such as shown in Fig. 7 in reference 9. No evidence of aggregation or of fusion of other types of organelles was observed.

This "activation" (time-dependent increase in the plateau level) was also observed when the two

[2] The same conclusion is suggested by the previously published kinetics of the reaction (Fig. 5 in reference 10), where in a mixture of reacting Rv and Yv, the same fractions of both populations ultimately react, but the Rv, initially present in roughly twice the concentration as Yv, are observed to continue fusing for several minutes after hybrid formation and Yv fusion have ceased.

[3] The observed increase in the extent of fusion was actually more dramatic than the data in Fig. 3 might indicate, because the hybrids formed at peak fusion times were often of relatively enormous size (>50 μm in diameter and >75 particles per vacuole), representing many more fusion events than the smaller hybrids formed at earlier times. Under such circumstances, accurate determination of the average number of particles per vacuole was not possible with the method employed.

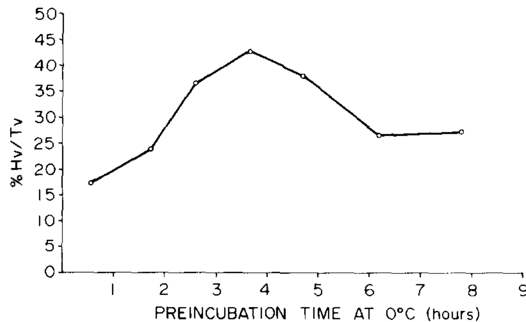

FIGURE 3 A typical plot of the extent of hybrid vacuole formation in vitro vs. the time after preparation of the homogenates at which the incubation was performed. Aliquots from a mixture of Rv-containing and Yv-containing homogenates (prepared from exponential phase cells) were preincubated for the times indicated and then incubated for 10 min at 31°C. The number of hybrid vacuoles resulting from each incubation was measured by light microscopy, and plotted relative to the total number of yeast-containing (Yv + hybrid) vacuoles. Hv, number of hybrid vacuoles; Tv, total number of yeast-containing vacuoles.

vacuole populations were maintained in separate test tubes on ice and mixed just before the incubation, indicating that the observed increase in the extent of fusion is not caused by facilitated vacuole contact (e.g., adhesion) occurring during the preincubation time. Activation was also seen when vacuoles from exponential phase cells were incubated for 10 min at 30°C, cooled on ice, and reincubated 1–2 h later for 10 min at 30°C (data not shown). Similar results were obtained in homogenates prepared from stationary phase cells.

DISCUSSION

Extent of the Fusion Reaction

At any given time, the in vitro system shows a fixed potential for the total number of vacuole fusion events that it will support; while the fusion rate may vary, the plateau level remains constant (e.g., Fig. 1). Analysis of the changes in vacuole species during the course of the reaction (Fig. 2) indicates that there are at least two vacuole subpopulations present: those that are competent to fuse, and those that are not. The size of the fusion-incompetent subpopulation relative to the total vacuole population is substantial (e.g., 40% of the monomers, the predominant vacuole type initially present, are fusion-incompetent in Fig. 2). In addition, the final distribution of vacuole species is not altered by cAMP, only the rate at which the vacuole population reaches this distribution (see Results). These observations suggest that the fixed potential for fusion in this system at any given time is a function of the relative size of the fusion-competent, cAMP-sensitive vacuole subpopulation.

Exhaustion of the pool of fusion-competent monomers coincides with a sharp drop in the overall reaction rate, followed shortly thereafter by cessation of the reaction (Fig. 2; cf., e.g., Fig. 5 in reference 10). While depletion of fusion-competent vacuoles explains why fusion ceases in the monomer subpopulation, it does not explain why fusion ceases shortly thereafter in the other subpopulations. However, although further experiments will be necessary to definitively answer this question, it seems clear that as fusion between fusion-competent vacuoles proceeds, the ratio of nonreactive to reactive species will continuously increase, which will cause the opportunities for contact between fusion-competent vacuoles to decrease continuously. The extent of the reaction will therefore be self-limiting in a manner that is independent of the rate of fusion, as is observed (e.g., Fig. 1).

The finding that the extent of the fusion reaction (plateau level) increased during the initial hours after preparation of the homogenates (e.g., Fig. 3) was unexpected. The results presented strongly suggest that the fraction of vacuoles that are fusion-competent increases during this time. The mechanism by which this occurs is not known. However, while the observed activation process could represent an artifact of the in vitro state (e.g., "recovery" of the vacuole membranes after the trauma of homogenization), the findings that the dye exclusion values remained unchanged during the activation process, and that the specificity of the fusion reaction was maintained, suggest that nonspecific modification of the vacuole membranes is not occurring. In addition, the observations of Ryter and Bowers (14) provide evidence that, for a number of hours after phagocytosis, the phagocytic vacuoles of *Acanthamoeba* consist of at least two subpopulations in vivo: those that have fused with lysosomes and other phagocytic vacuoles, and those that have not. It will be noted that the vacuole subpopulations histochemically detected by Ryter and Bowers are of similar size to the subpopulations observed in this study, and that the relative size of the histochemical subpopulation that has fused with lysosomes increases slowly over a period of hours in vivo. It does not

seem unreasonable, therefore, to suggest that the slow acquisition of fusion competency and cyclic nucleotide sensitivity by the phagocytic vacuoles observed here in vitro, may reflect a similar membrane processing step which occurs in vivo.

Rate of the Fusion Reaction

The methylxanthines are specific inhibitors of cyclic nucleotide phosphodiesterase activity in a wide variety of systems, and have been reported to cause the elevation of both cAMP and cGMP levels (1). The present observations that theophylline and MIX each cause stimulation of the average rate of in vitro vacuole fusion (e.g., Fig. 1) therefore suggest that the reaction rate is dependent on the concentration of cyclic nucleotide(s). The results that the stimulatory effect of MIX was reproduced by the addition of 1 mM cAMP (Fig. 1 and Table I), but not by addition of 1 mM cGMP (Table I), indicate that the effect seen with the methylxanthines is due primarily to an elevation of the endogenous cAMP concentration. The relatively high (nominally 1 mM) concentration of exogenous cAMP required for full stimulation presumably reflects the high level of phosphodiesterase activity present in *Acanthamoeba* homogenates.[4] The lack of effect of adenosine and 5'-AMP (Table I) indicates that the effect is not caused by a metabolite of cAMP. In addition, the strongly antagonistic effect of cGMP (Table I) suggests that besides regulating a wide variety of other cellular processes (2), cAMP and cGMP may also regulate the phagolysosomal membrane fusion process.

Indirect observations on other types of systems (e.g., reference 4) have suggested that vesicle fusion in vivo is an ATP-requiring process. The present observations that exogenous ATP can maintain the initial fusion rate, that the effect is highly specific, and that it is apparently dependent on splitting of the terminal pyrophosphate linkage of ATP (Table I), indicate that the rate of vacuole fusion is linked to the ATP concentration in these homogenates. The reason for the lack of effect of exogenous ATP in some preparations is not known, but it presumably indicates that the endogenous ATP concentration was not always rate-limiting. It should be emphasized, however, that it is presently unclear whether the effect of ATP in this system is distinct from that of cAMP, as ATP may simply provide the substrate necessary for adenyl cyclase to generate cAMP. On the other hand, if cAMP exerts its action by activating a protein kinase, as is thought to occur in virtually all cases (5), then ATP would also probably be necessary as a phosphoryl donor at one or more subsequent phosphorylation steps.

The stimulation of the fusion reaction by cAMP was particularly noteworthy in view of the fact that F^-, which typically stimulates adenyl cyclase in broken cell preparations from mammalian systems (11), inhibits fusion (10). This apparent paradox was resolved by the recent observation by Achar and Weisman that adenyl cyclase of *Acanthamoeba* is inhibited by fluoride in concentrations similar to those found to inhibit the fusion reaction.[4] The previously reported inhibition by fluoride therefore appears to be attributable to at least two actions: (*a*) inhibition of glycolysis, which would lower the endogenous ATP concentration, and (*b*) inhibition of adenyl cyclase, which would lower the endogenous cAMP concentration. Interestingly, cGMP and GTP were found in the present study to be more than three times as potent as fluoride in inhibiting the fusion reaction (50% inhibition by KF occurs at ~3 mM (10), whereas 1 mM cGMP or GTP inhibits 75% [Table I]). The mechanism of inhibition by the guanyl nucleotides is presently unknown, as is the mechanism of stimulation by the adenyl nucleotides. Studies of the effects of these nucleotides in this system on membrane phosphorylation (13), endogenous calcium levels (12), and/or endogenous phospholipase A activity (3, 6), are likely to shed further light on their role in the vacuole fusion process.

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[4] S. B. Achar and R. A. Weisman, personal communication.

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