Kinetics of endosomal pH evolution in *Dictyostelium discoideum* amoebae

Study by fluorescence spectroscopy

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**SUMMARY**

The evolution of endo-lysosomal pH in *Dictyostelium discoideum* amoebae was examined during fluid-phase endocytosis. Pulse-chase experiments were conducted in nutritive medium or in non-nutritive medium using fluorescein labelled dextran (FITC-dextran) as fluid-phase marker and pH probe. In both conditions, efflux kinetics were characterized by an extended lag phase lasting for 45-60 min and corresponding to intracellular transit of FITC-dextran cohort. During the chase period, endosomal pH decreased during ~20 min from extracellular pH down to pH 4.6-5.0, then, it increased within the next 20-40 min to reach pH 6.0-6.2. It was only at this stage that FITC-dextran was released back into the medium with pseudo first-order kinetics. A vacuolar H⁺-ATPase is involved in endosomal acidification as the acidification process was markedly reduced in mutant strain HGR8, partially defective in vacuolar H⁺-ATPase and in parent type strain AX2 by bafilomycin A1, a selective inhibitor of this enzyme. Our data suggest that endocytic cargo is channeled from endosomes to secondary lysosomes that are actively linked to the plasma membrane via recycling vesicles.

Key words: v-ATPase, endosomal pH, acidification, fluorescein-dextran, *Dictyostelium discoideum*

**INTRODUCTION**

The structural and functional organization of the receptor-mediated endosomal pathway of mammalian cells has been extensively studied and many of its steps have been characterized by both in vivo and in vitro studies (Courtoy, 1991; Griffiths and Gruenberg, 1991; Gruenberg and Howell, 1989; Mellman et al., 1986; Murphy, 1991; Watts and Marsh, 1992). Constitutive fluid-phase endocytosis (pinocytosis) has attracted relatively less attention although most cell types are capable of internalizing quite large volumes of medium by this pathway (Steinman et al., 1983). The interiors of endocytic vesicles and lysosomes are characterized by a low pH and H⁺-gradients that are considered to be critical for the normal functioning of endocytosis (Mellman et al., 1986; Yamashiro and Maxfield, 1988; Anderson and Orci, 1988).

Fluid-phase endocytosis is very active in the amoebae of the cellular slime mold *Dictyostelium discoideum* as it provides the major gateway for an efficient entry of nutrients in axenically growing strains (Loomis, 1975; North, 1983). Clathrin-mediated traffic operates in this eukaryotic microorganism. The average number of coated vesicles has been estimated to be ~340 per amoeba (Swanson et al., 1981). Clathrin heavy chain-deficient mutants had a 90% reduced fluid-phase endocytosis activity and this supports the idea that endocytosis in *Dictyostelium* proceeds only through the classical coated-vesicle pathway (O’Halloran and Anderson, 1992). Vesicular structures, called ‘acidosomes’ in reference to similar structures described in *Paramaecium* (Allen and Fok, 1983) and enriched in vacuolar-type ATPase have been proposed to be responsible for the acidification of the endosomal apparatus (Padh et al., 1991a,b; Nolta et al., 1991). Cell-free studies of endosome-acidosome association and of endosome-endosome fusion have been reported (Padh et al., 1991b; Lenhard et al., 1992).

In order to get a deeper insight into the kinetics of fluid-phase endocytosis and pH evolution along the endolysosomal apparatus in *Dictyostelium* amoebae, we studied the fate of internalized fluorescein isothiocyanate-labeled dextran (FITC-dextran) during constitutive fluid-phase endocytosis in *Dictyostelium* amoebae. This fluid-phase and pH marker is taken up by cells without being adsorbed to membranes and is not degraded inside lysosomes (Thilo and Vogel, 1980; De Chastellier et al., 1983; Klein and Satre,
Temporal details of pH changes showed a rapid acidification to pH 4.6-5.0 followed by an increase up to pH 6.0-6.2.

MATERIAL AND METHODS

Culture conditions

*Dictyostelium discoideum* parent strain AX2 (ATCC 24397) and methylene diphosphonate-resistant mutant HGR8 (Bof et al., 1992) were grown at 21°C in axenic medium (Watts and Ashworth, 1970). Cell numbers were determined with a Coulter model ZM counter.

Fluid-phase endocytosis assay and measurement of endosomal pH

Fluid-phase endocytosis and endosomal pH were measured as described previously (Klein and Satre, 1986; Bof et al., 1992) with FITC-dextran to provide a general fluid-phase and pH marker of the endosomal content (Ohkuma and Poole, 1978).

Pulse-chase experiments

Amoebae (1×10^7 cells/ml) were pulsed at 21°C with 20 mg/ml FITC-dextran or dextran either in axenic growth medium, pH 6.5, or in 40 mM MES-Na buffer, pH 6.5 and washed at the end of the pulse in ice-cold 40 mM MES-Na buffer, pH 6.5, 0.05% bovine serum albumin (BSA) (w/v). Cells were chased by incubation at 21°C in the medium used for the pulse but containing only dextran. Intracellular FITC-dextran and endosomal pH were measured on aliquot samples.

Chemicals

FITC-dextran was synthesized by reacting fluorescein isothiocyanate (isomer I) with dextran (M_r = 70,000). It was checked in particular that it contained no low molecular weight fluorescent contaminants (Klein et al., 1989). Identical results were obtained with various FITC-dextran batches in which labeling stoichiometries varied between 3 and 9 chromophores per dextran molecule. Bafilomycin A1 was obtained from K. Altendorf (University of Osnabrück, Germany).

RESULTS

Time course of endosomal pH evolution

*Dictyostelium* amoebae suspended in axenic growth medium that contained FITC-dextran accumulated the fluorescent probe (Fig. 1). The accumulation was initially rapid and linear for approximately 60 min at 21°C. After this time, the rate of uptake slowed, and at about 2 h it reached a quasi steady-state equilibrium value between uptake and efflux of FITC-dextran (Klein and Satre, 1986). To measure FITC-dextran efflux, amoebae were incubated for various times (10-160 min and 16 h) in FITC-dextran-containing axenic growth medium, washed at 0°C and reincubated at 21°C in FITC-dextran-free medium. Fluorescence of the lysate represented cell associated FITC-dextran. Efflux of FITC-dextran exhibited biphasic kinetics. There was an extended lag phase before egress and the longer the pulse, the less marked the lag time (Fig. 1). Similar results were obtained in non-nutritive medium (40 mM MES-Na buffer, pH 6.5). The kinetics of uptake and exocytosis in *Dictyostelium* amoebae were different from those obtained in cells such as macrophages (Besterman et al., 1981; Swanson et al., 1985) and endocytosed fluid was not recycled rapidly from an early compartment but passed entirely to later compartments throughout the cell before efflux.

FITC-dextran behaves as a pH probe and it has been used in various cells to measure endosomal pH (Yamashiro and Maxfield, 1988). In *Dictyostelium* amoebae from axenic strains AX2 or AX3, the average pH of endosomal compartments has been reported to be 5.4-5.8 (Cardelli et al., 1989; Bof et al., 1992; Brénot et al., 1992). To obtain a detailed picture of the time-course of acidification changes in various endosomal compartments of *Dictyostelium* amoebae, pulse-chase experiments were conducted and the fluorescence signal thus reflected the progression of the marker through endosomal compartments.

Fig. 2 illustrates the evolution of both the amount of internalized FITC-dextran and endosomal pH during the chase period following a 5-min FITC-dextran pulse, either in non-nutritive MES-Na buffer or in axenic growth medium, both at pH 6.5. In MES-Na buffer (Fig. 2A), chase kinetics were characterized by an extended lag phase lasting about 40 min (T lag), followed by pseudo-first order exocytosis (k = 0.045 min^-1, t_1/2 ~15 min). Measurement of endosomal pH along the fluid-phase endocytosis pathway in *Dictyostelium* amoebae showed first a decrease in pH during a total time T_1, followed by an increase in pH during a total time T_2 and afterwards pH stabilization. Endosomal acidification was rapid, as endosomal pH at the end of the 5-min pulse was already much more acidic than pH of the loading medium (Fig. 2C). During the first 10 min of chase,
endosomal pH continued to decrease rapidly (the downward acidification rate was $-0.09$ pH unit/min for an extracellular pH of 6.5) to reach a minimal pH value close to 4.6 (pH$_{\text{min}}$). This acidification period (T$_1$) is likely to correspond to the interaction of incoming endocytic vesicles with acidosomes (Padh et al., 1991a,b). During the next 20-25 min of chase (T$_2$), pH steadily increased with a rate of +0.063 pH unit/min to stabilize at pH 6.0 (pH$_{\text{max}}$) at the end of the lag phase (T$_{\text{lat}}$) preceding the rapid FITC-dextran efflux. It should be pointed out that a close correspondence was observed between the total time required for endosomal pH evolution (T$_1$ + T$_2$ = 43.4 min) and the time required for efflux of FITC-dextran to occur (T$_{\text{lat}}$ = 46.3 min).

In Dictyostelium amoebae, fluid-phase endocytosis was shown to be more active in nutritive medium than in buffer and a comparison of FITC-dextran accumulation revealed that the initial rates of fluid influx were 5.3 and 7.9 fl/cell per min in MES-Na buffer and in axenic growth medium, respectively (see Table 1). In the presence of nutrients, an additional receptor-mediated component has been postulated to account for this increased activity (Maeda and Kawamoto, 1986). Nevertheless, pulse-chase experiments (Fig. 2B,D) showed that the kinetics of FITC-dextran efflux and pH evolution in axenic growth medium were largely similar to those in MES-buffer. Fluid-phase marker chase showed a ~60 min lag phase followed by a pseudo-first order efflux ($t_{1/2} = 24$ min). Endosomal pH decreased (~0.08 pH unit/min for an extracellular pH of 6.5) during T$_1$ = 20 min to reach a minimal value of pH 5.0. It then increased during T$_2$ = 40 min with a rate of +0.036 pH unit/min up to pH 6.2 at the end of the lag phase preceding FITC-dextran efflux (Fig. 2D). As observed in MES buffer, the total time of endosomal pH evolution in axenic growth medium (T$_1$ + T$_2$ = 56.5 min) corresponded to the time required for efflux of FITC-dextran to be effective (T$_{\text{lat}}$ = 59.3 min).

Very similar data were obtained when FITC-dextran pulse lengths varied between 3 and 10 min. Kinetic results are collected in Table 1. A comparison of parameters obtained in nutritive medium or in MES buffer shows that acidification rates were identical. In contrast, rate of endosomal pH increase and apparent fluid efflux were somewhat slower in nutrient medium.

Endosomal pH kinetics were also analyzed in strain HGR8, a methylene diphosphonate-resistant mutant with reduced vacuolar ATPase activity (Bof et al., 1992). Acid-

### Table 1. Fluid-phase endocytosis parameters and evolution of endosomal pH in Dictyostelium AX2 amoebae

(A) Endocytosis parameters

<table>
<thead>
<tr>
<th>Medium</th>
<th>Entry rate (fl/cell per min)</th>
<th>T$_{\text{lat}}$* (min)</th>
<th>Efflux constant $k$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutritive</td>
<td>7.9 ± 1.8 (32)</td>
<td>59.3 ± 3.7 (20)</td>
<td>0.029 ± 0.005 (44)</td>
</tr>
<tr>
<td>MES-Na</td>
<td>5.3 ± 1.1 (17)</td>
<td>46.3 ± 4.1 (10)</td>
<td>0.045 ± 0.009 (20)</td>
</tr>
</tbody>
</table>

(Bi) Endosomal pH evolution: early acidification phase

<table>
<thead>
<tr>
<th>Medium</th>
<th>Acidification rate (A pH/min)</th>
<th>T$_1$* (min)</th>
<th>pH$_{\text{min}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutritive</td>
<td>$-0.083 ± 0.018$ (17)</td>
<td>18.9 ± 2.8 (17)</td>
<td>5.02 ± 0.17 (17)</td>
</tr>
<tr>
<td>MES-Na</td>
<td>$-0.090 ± 0.025$ (10)</td>
<td>20.5 ± 3.9 (10)</td>
<td>4.64 ± 0.30 (10)</td>
</tr>
</tbody>
</table>

(Bii) Endosomal pH evolution: endosomal pH increase and stabilization phases

<table>
<thead>
<tr>
<th>Medium</th>
<th>Alkalisation rate (A pH/min)</th>
<th>T$_2$ (min)</th>
<th>pH$_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutritive</td>
<td>$+0.036 ± 0.011$ (18)</td>
<td>37.6 ± 7.2 (18)</td>
<td>6.21 ± 0.13 (21)</td>
</tr>
<tr>
<td>MES-Na</td>
<td>$+0.063 ± 0.026$ (8)</td>
<td>22.9 ± 5.0 (8)</td>
<td>5.96 ± 0.07 (10)</td>
</tr>
</tbody>
</table>

Data correspond to pulse-chase data with pulse lengths between 3 and 10 min. Mean ± s.d.; the number of independent experiments is indicated in parentheses.

* T$_{\text{lat}}$ and T$_1$, the duration of the lag phase and the acidification phase, respectively, are measured from the start of the pulse (see Fig. 2).
ification rate during phase $T_1$ and pH increase during phase $T_2$ were reduced, respectively, to 30% and 40% of their values in parent strain AX2 (see Table 1). Characteristic endosomal pH values measured during the chase were $pH_{min} = 6.0$ and $pH_{max} = 6.5$ (Fig. 2D).

**Effect of weak bases, nigericin and bafilomycin on endosomal pH and fluid-phase endocytosis**

Protonated forms of permeant weak bases concentrate inside acidic endocytic compartments and the H⁺-gradient is eventually lost when active acidification can no longer compensate for weak base accumulation (Poole and Ohkuma, 1981). Incubation of pulsed cells with imidazole or ammonium chloride at different times during the chase (0, 15 and 60 min) effectively induced the expected rise in endosomal pH. An identical reduction of pH gradient between endosomal compartments and cytosol at pH 7.4 (Martin et al., 1987) was obtained if amoebae were resuspended in the presence of a K⁺/H⁺ ionophore, nigericin (not shown).

Endocytosis kinetics and endosomal pH were next examined in cells that were continuously exposed to 40 mM imidazole-Cl, pH 6.7. Internalization of FITC-dextran in Dictyostelium amoebae was only moderately inhibited and initial influx rates and plateau values were both reduced by ~20-25% (not shown). Results of a pulse-chase experiment are shown in Fig. 2A and C. Efflux parameters, $T_{lat} = 67$ min and $k = 0.038$ min⁻¹ were similar to control values (see Table 1) but endosomal pH remained fixed at pH 6.7 during the whole course of the chase.

Fig. 3 shows the effect of increasing concentrations of bafilomycin A1, a selective inhibitor of vacuolar type H⁺-ATPase (Bowman et al., 1988), on fluid-phase endocytosis rate and on the average endosomal pH measured after 30 min loading with FITC-dextran. A clear effect of bafilomycin A1 was observed on both processes. Influx rate was depressed by up to 60% and endosomal pH was raised by 0.6 units to a pH of 6.1. The amount of inhibitor, IC₅₀ ≈ 7 nmoles/mg protein, was only slightly higher than needed in subcellular Dictyostelium preparations (Rooney and Gross, 1992).

**Endocytic compartments in Dictyostelium amoebae**

The simplest hypothesis to explain the temporal evolution of endosomal pH in Dictyostelium amoebae is to consider the sequential involvement of kinetically distinct compartments, corresponding, respectively, to endosomal, lysosomal (or vacuolar) and recycling compartments. The evolution from early endocytic vesicles to the lysosomal compartment corresponds to $T_1$ (see Fig. 2C). During this phase, acidosomes acidify the interior of incoming vesicles down to pH 4.5-5.0. Period $T_2$ corresponds to various lysosomal stages. Perhaps the acidosomes dissociate during this period and the pH gradient may be used for transport functions. Intravesicular pH thus progressively increased to pH 6.0-6.2. The time period after $T_{lat}$ (equal to $T_1 + T_2$) demonstrates the transit of recycling vesicles at pH 6.0-6.2 back to the plasma membrane. The parameters derived from pulse-chase experiments were compared to fluid-phase endocytosis kinetics (see Fig. 1) to estimate apparent volumes of physically distinct compartments. Volumes given in Table 2 may only be taken as apparent volumes, since efficient concentration of fluid-phase probe has been shown to occur in endosomal compartments in Dictyostelium amoebae (De Chastellier et al., 1983; Klein and Satre, 1986), as in other cells (Steinman et al., 1983).

The endo-lysosomal compartments of Dictyostelium amoebae were observed by fluorescence microscopy after fixation of cells with glutaraldehyde. Amoebae that had been pulsed for 5 min with FITC-dextran displayed numerous fluorescent vesicles of heterogeneous sizes and distributed throughout the cytoplasm (Fig. 4A). The largest vesicles were up to ~2 µm in diameter. After 15 min chase, fluorescent vesicles in fixed cells exhibited a more fragmented structure and a brighter appearance, suggesting concentration of FITC-dextran in smaller vesicles (Fig. 4B). After 60 min chase, a few large vesicles remained together with a profusion of small and very small vesicles (<0.25 µm) (Fig. 4C). However, in unfixed cells, the general appearance and distribution of fluorescent vesicles were not stable.

**Table 2. Apparent volumes of compartments in the fluid-phase endocytic pathway of Dictyostelium AX2 amoebae**

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Medium</th>
<th>Endosomal</th>
<th>Lysosomal (vacuolar)</th>
<th>Recycling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutritive</td>
<td>150 ± 14</td>
<td>297 ± 57</td>
<td>160 ± 56</td>
<td></td>
</tr>
<tr>
<td>MES-Na</td>
<td>109 ± 6</td>
<td>121 ± 25</td>
<td>77 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>106 ± 32*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Volume obtained from in vivo 31P-NMR data with 2-aminooxyethylphosphonate probe (Breton et al., 1992).
obviously changed over the duration of the chase (Fig. 4D,E,F). The reasons for the differences between fixed and unfixed cells are not clear. Possibly, the glutaraldehyde fixed cells reflects the in vivo situation. Alternatively, perturbation of the endo-lysosomal system by glutaraldehyde (Swanson et al., 1987) reveals subtle differences in organisation.

DISCUSSION

In this study, pH within endo-lysosomal compartments of *Dictyostelium discoideum* amoebae was measured in pulse-chase kinetic experiments conducted with FITC-dextran during fluid-phase endocytosis. We found that efflux kinetics measured after short pulse loading were characterized by an extended lag phase. This result is in contrast to those reported in macrophages and fibroblasts (Besterman et al., 1981; Swanson et al., 1985), which showed a rapid efflux of fluid-phase marker by recycling from early endosomal compartments. Nevertheless, the situation in *Dictyostelium* amoebae is similar to that obtained in macrophages stimulated by phorbol esters where a major proportion of endocytosed material is directed to lysosomes (Swanson et al., 1985). The long latency observed in *Dictyostelium* before any FITC-dextran efflux suggested that recycling from early endosomal compartments was reduced and that all endocytosed material passed into late endosomes and lysosomes. This latter hypothesis, i.e. that all ingested elements were to face digestive conditions, would be fully consistent with the primary nutritive function of endocytosis in these amoebae.

The pH reported during the chase by the FITC-dextran cohort decreased, in 20 minutes, from that of the external medium to pH 4.6-5.0. Previous studies had shown that the dextran marker was localized at this stage in intracellular compartments operationally defined as endosomes (Padh et al., 1991a,b). However, it fully co-localized with lysosomal hydrolase activities and thus it also defined compartments with the properties of prelysosomes (or late endosomes) and secondary lysosomes (Ebert et al., 1989; Padh et al., 1991a,b). The early stages of the *Dictyostelium* endosomal pathway are thus analogous to those of higher eukaryotes where endosomal pH drops with similar kinetics from pH 6.2-6.3 in early- and sorting-endosome, down to pH 5.0-5.5 in late-endosome (prelysosome) and to pH 4.8-5.4 in lysosomes (Yamashiro and Maxfield, 1988). In later stages of *Dictyostelium* endocytic pathway, however, we found that endosomal pH steadily increased up to pH 6.0-6.2. It remained stable at pH 6.0-6.2 in those vesicles able eventually to recycle the FITC-dextran marker, most likely from lysosomes back to the external medium. Recycling vesicles at pH 6.4-6.5 have been characterized only in the early recycling endocytic pathway of higher eukaryotes (Yamashiro and Maxfield, 1988).

Fluorescence pH measurements were in agreement with previous data from in vivo $^{31}$P-NMR experiments. The NMR technique discriminates between compartments at discrete pH values and it has allowed the detection of two kinetically distinct endocytic compartments in *Dictyostelium* amoebae, a first one at pH 4.3-4.5, followed by a second compartment at pH 5.8-6.0 (Brénot et al., 1992). Temporal evolution of endosomal pH in *Dictyostelium* was found to be independent of the presence of nutrients suggesting that fluid-phase endocytic activity in growth medium and in non-nutritive buffer follows identical or similarly controlled pathways.

Two sets of evidence favour the involvement of a vacuolar type H$^+$-ATPase in the acidification phase during fluid-phase endocytosis in *Dictyostelium* amoebae. First, the

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**Fig. 4.** Intracellular fluorescence after fluid-phase endocytosis of FITC-dextran. Amoebae were pulsed in axenic growth medium for 5 min with 10 mg/ml FITC-dextran, washed and chased in the same medium for 0 (A,D), 15 (B,E) and 60 min (C,F). (A,B,C) cells fixed with 1.25% glutaraldehyde. (D,E,F) unfixed cells. Bar, 10 µm.
trough in endosomal pH evolution was no longer observed when whole cells were treated with bafilomycin A1, an inhibitor highly specific for vacuolar-type ATPases. Second, in the membrane-diphosphonate-resistant mutant HGR8, partially-deficient in its vacuolar ATPase activity (Bof et al., 1992), average endosomal pH was much higher than in parent type amoebae and the acidification process was almost obliterated.

In conclusion, our data suggest that during fluid-phase endocytosis in *Dictyostelium* amoebae internalized material is channeled from endosomes to secondary lysosomes and acidification to pH 4.6–5.0 occurs. Lysosomal compartments are efficiently linked to the plasma membrane via recycling vesicles at a less acidic pH (pH 6.0–6.2).

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REFERENCES


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Note added in proof

Recently, Padh, H., Ha, J., Lavasa, M. and Steck, T. L. (*J. Biol. Chem.* (1993) 268, 6742-6747) have published a study of endocytosis in *Dictyostelium* amoebae using FITC-dextran, the results of which also demonstrate the presence of a nearly neutral post-lysosomal recycling compartment.