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Dictyostelium discoideum mutants resistant to the toxic action of methylene diphosphonate are defective in endocytosis

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Summary

Methylene diphosphonate is taken up in *Dictyostelium discoideum* amoebae by fluid-phase pinocytosis, and it inhibits growth through the production of methylene analogs of adenosine triphosphate and diadenosine tetraphosphate. Methylene diphosphonate resistance was thus used as the basis of a screening strategy for the isolation of endocytosis mutants. Fifteen *Dictyostelium* mutants, whose growth was resistant to 7.5 mM methylene diphosphonate, were obtained and three of them were characterized in more detail. They were partially defective in fluid-phase pinocytosis (both the rate and extent of FITC-dextran entry were reduced to 40-50% of the parent type activity) and they had smaller amounts of several lysosomal enzymes, such as acid phosphatase, *N*-acetylhexosaminidase, α-mannosidase (20-60% of the parent type activities). In contrast to the lysosomal hydrolases, the mutants had unchanged activities for enzyme markers selective for other compartments. They appeared phenotypically similar to the *Dictyostelium* mutant HMW570, which is defective in fluid-phase pinocytosis and oversecretes lysosomal enzymes. The methylene diphosphonate-resistant mutants were found to be unable to acidify fully their endosomal compartments and they have an increased endosomal pH, as shown by the use of the pH-sensitive fluorescence of FITC-dextran. Furthermore, the hypothesis proposing a defective acidification of the endosomal pathway was supported by the measurement of ATP-dependent vesicular acidification with acridine orange, and by in vivo ^{31}P NMR spectroscopy with aminomethylphosphonate as a pH probe.

Key words: *Dictyostelium*, amoeba, methylene diphosphonate, lysosome, endocytosis, acidification.

Introduction

Endocytosis is highly active in the cellular slime mould *Dictyostelium discoideum* and it is the primary process by which amoebae take up nutrients (Loomis, 1975). The lysosomal system is a prominent feature of *Dictyostelium* ultrastructural organization and has been extensively studied at the biochemical level (Cardelli et al. 1990). A characteristic of the endo-lysosomal compartments is their acidity, which is crucial for the proper functioning of endocytosis (Mellman et al. 1986). Acidification of endosomal pathways in *Dictyostelium* has been studied and the role of the vacuolar ATPase-rich organelles, the acidosomes, has been emphasized (Padh et al. 1989, 1991).

In higher eucaryotes, mutants defective in endocytosis have been isolated on the basis of increased resistance to toxins or viruses and the majority of mutants are defective in endo-lysosomal acidification (Merion et al. 1983; Colbaugh et al. 1989; Robbins and Roff, 1987; Yamashiro and Maxfield, 1987).

Our screening strategy relied on the use of methylene diphosphonate (MDP), a toxic molecule for *Dictyostelium* amoebae, which enters by fluid-phase pinocytosis and kills the cells through the synthesis of methylene analogs of adenosine triphosphate and diadenosine tetraphosphate by aminoacyl-tRNA synthetases (Klein et al. 1988b). Considering the multiplicity of intracellular targets, which make it unlikely that MDP resistance could arise directly from mutations of the aminoacyl-tRNA synthetases, we reasoned that cell lines that have acquired resistance to MDP would be good candidates for endocytosis mutants. The experiments reported here show that three characterized MDP-resistant *Dictyostelium* mutants are partially defective in fluid-phase pinocytosis and have reduced amounts of several lysosomal enzymes. The mutants were found to be unable to acidify an early endosomal compartment.

Materials and methods

Isolation of MDP-resistant Dictyostelium *mutants*

Dictyostelium discoideum AX2 (ATCC 243974) and mutant

cell lines were grown in axenic medium (Sussman, 1987). Amoebae were washed with 17 mM Na, K-P_i buffer, pH 6.5, resuspended at 1×10^7 cells/ml and incubated for 30 min at 22°C with 3.4 mM *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Cells were washed in ice-cold 17 mM Na, K-P_i buffer, pH 6.5, and incubated for 48 hours at 22°C in axenic medium before addition of 7.5 mM MDP. After 5-7 days, MDP-resistant cells were cloned on lawns of *Klebsiella aerogenes* on nutrient agar.

Endocytosis assay

Pinocytosis was measured as described previously (Klein et al. 1988a) with fluorescein-labeled dextran (FITC-dextran, 70,000 average molecular weight, Sigma) as a fluid-phase marker. Uptake data were analyzed on the basis of a kinetic model corresponding to a single compartment and were fitted to an equation of the form: $V(t)=V_{max}(1-\exp^{-kt})$; $V(t)$ being the volume of internalized fluid at time t and the plateau value (V_{max}) being the apparent volume of the endosomal compartment.

Endosomal pH and vesicular acidification assays

Endosomal pH was measured as described previously (Klein et al. 1988a) by the dual-excitation ratio method with FITC-dextran as a pH probe (Ohkuma and Poole, 1978). An in vivo calibration curve was generated by equilibrating intra- and extracellular pH values with weak acid and weak base. To this, FITC-dextran-loaded AX2 amoebae (1×10^7 cells/ml) were incubated for 45 min at 0-4°C in 10 mM Tris-HCl, 100 mM ammonium acetate, adjusted to the required pH between pH 4 and pH 9, before measuring the fluorescence excitation ratio R ($I_{490\ nm}/I_{450\ nm}$). The best-fit of the data corresponded to the following equation: $pH=6.1+\log[(R-0.88)/(5.7-R)]$ and pH values were calculated from the experimentally determined R data.

ATP-stimulated acridine orange (AO) accumulation was measured as described by Padh et al. (1991) in 0.1 M sucrose, 0.1 M KCl, 5 mM $MgCl_2$, 5 mM Hepes, 0.1 mM sodium vanadate, 0.1 mM sodium azide, 0.75 mM ethylene glycol bis(amino-ethyl ether)tetraacetate (EGTA), 1.5 μM AO, pH 7.0. The reaction was started by addition of 1 mM ATP. The initial rates of AO fluorescence quenching varied linearly with the amount of vesicles in the range 40-200 μg protein/ml. AO quenching was fully reversed by 3 μM nigericin.

^{31}P NMR spectra of amoebae suspensions were obtained with a Bruker AM400 spectrometer. Specific conditions are described in the legend to Fig. 3 (below). Aminomethylphosphonate (AMeP) was used as a pH probe as described previously for methylphosphonate (Satre et al. 1989). In vivo pH calibration curves, constructed as discussed above for FITC-dextran, showed that AMeP had a pK of 5.5 and a variation in chemical shift from +11.2 to +9.2 p.p.m. (parts per million) over the range pH 3.5-8.

Subcellular fractionation

All manipulations were done at 0-4°C. Cells (2×10^9) were suspended in 0.1 M sucrose, 5 mM glycine, pH 8.5 (final volume: 5 ml), and passed 10 times through a 8.02 mm precision bore in a metal block containing a 8.002 mm steel ball. The degree of breakage was 80-90%. The fractionation procedure was slightly modified after Padh et al. (1989). The homogenate was adjusted to 45% (w/w) sucrose by addition of 65% (w/w) sucrose, 5 mM glycine, pH 8.5, and centrifuged at 17,500 *g* for 30 min in a Beckman JA20 rotor. The supernatant was diluted to 5% (w/w) sucrose with 5 mM glycine, pH 8.5, and centrifuged at 25,000 *g* for 30 min. The pellet (about 15 mg protein) was resuspended in 1 ml in 0.1 M sucrose, 5 mM Hepes-Na, pH 7.0.

Enzyme assays

N-acetylglucosaminidase, α-mannosidase and acid phosphatase as lysosomal markers, aspartate alanine aminotransferase as cytosolic marker, alkaline phosphatase as plasma membrane marker, and oligomycin- or azide-sensitive ATPase as mitochondrial marker, were assayed following standard procedures (Wiener and Ashworth, 1970). Oligomycin and sodium azide were used at 10 μg/ml and 1 mM, respectively. Vacuolar ATPase was defined as ATPase activity measured at pH 7.0 and sensitive to 25 μM NBD-Cl (Padh et al. 1989). Protein content was measured with the Folin-Ciocalteu phenol reagent.

Results

Isolation and growth characteristics of MDP-resistant mutants

After mutagenesis with MNNG, fifteen mutants able to grow in the presence of 7.5 mM MDP were isolated in three separate screenings. Resistance to MDP was found to be a stable property, since, if cells were grown for several generations without any added MDP, they still remained resistant. Three of these mutants (HGR5, HGR8 and HGR9) were selected for further characterization. Their growth parameters are summarized in Table 1. The generation times of the mutants were 1.5 to 1.7 times longer than that of AX2 and the maximal cell yields were decreased two- to fourfold. MDP was tested for its inhibitory effect. Whereas the growth of strain AX2 was markedly inhibited by 2 mM MDP in agreement with previous studies (Klein et al. 1988b), 7.5 mM MDP had only a slight inhibitory effect on the growth of HGR5, HGR8 or HGR9 (not shown). The mutants were found to be about fourfold more resistant to MDP than AX2. Fruiting body formation on filters and spore germination took place with the mutants in the presence of 7.5 mM MDP. In contrast, morphogenesis in strain AX2 was prevented by 2.5 mM MDP (Satre et al. 1989).

To ensure that the MDP-resistant phenotype was not the result of a general perturbation of cellular permeability, we checked whether the mutants were cross-resistant towards other inhibitors. The concentrations required to inhibit growth were identical for AX2 and for the mutants with respect to several drugs, such as

Table 1. *Growth characteristics of* Dictyostelium *strain AX2 and of the MDP-resistant mutants*

Strain	Generation time (hours)	Cell density at the plateau ($\times10^7$/ml)
AX2	8.2 ± 0.2	2.7 ± 0.2
HGR5	13.3 ± 1.3	0.7 ± 0.2
HGR8	12.2 ± 1.0	1.2 ± 0.1
HGR9	14.2 ± 2.2	0.8 ± 0.1

Growth parameters in axenic medium were determined at 22 (±1)°C in the absence of added MDP (mean ± s.d. from three separate experiments).

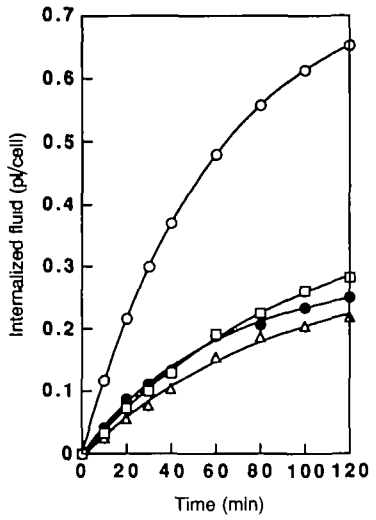

Fig. 1. Fluid-phase pinocytosis in *Dictyostelium* AX2 and MDP-resistant mutants. Amoebae (1×10^7/ml) were incubated at 22°C in axenic growth medium containing 2 mg/ml FITC-dextran. The amount of internalized FITC-dextran was determined at the indicated times. The lines drawn through the data points represent best fits of function $V(t) = V_{max} \times (1-\exp^{-kt})$ as described under Materials and methods. The following symbols were used: (○) AX2, (●) HGR5, (△) HGR8 and (□) HGR9.

inhibitors of microtubules (5 μg/ml nocodazole), protein synthesis (0.25 mg/ml cycloheximide) or DNA replication (1 mM hydroxyurea). MDP-resistant mutants were still sensitive to inhibition by compounds affecting endocytosis, such as vanadate or caffeine (Klein et al. 1989; Gonzalez et al. 1990).

Fluid-phase pinocytosis is decreased in the MDP-resistant mutants

The kinetic course of entry of FITC-dextran into AX2 and into the MDP-resistant mutants, is illustrated in Fig. 1. Fluid-phase pinocytosis was remarkably reduced in all three MDP-resistant mutants. The initial velocities of entry of FITC-dextran into HGR5, HGR8 and HGR9 were reduced, respectively, to 49, 40 and 46% of the value for AX2. Similarly, the pinocytosis plateau was decreased in the mutants (Table 2). The data suggest a diminished endosomal volume in the MDP-resistant mutants or alternatively a less efficient concentrating mechanism occurring during fluid-phase pinocytosis in *Dictyostelium* (Klein and Satre, 1986).

Lysosomal enzyme content is selectively reduced in MDP-resistant strains

To characterize further the MDP-resistant mutants, several enzymic activities were measured and a significant decrease in the lysosomal enzyme activities was noticed (Table 3). The extent of the deficiencies was variable according both to the measured enzymic activities and to the mutants themselves. In contrast to lysosomal hydrolases, no difference was observed between AX2 and the MDP-resistant mutants in the total protein content and the activities of the enzyme markers of the other subcellular fractions.

Secretion of lysosomal enzymes

Dictyostelium amoebae secrete lysosomal enzymes during growth (Dimond et al. 1981). Experiments were thus conducted to determine whether an increased secretion could account for a decreased lysosomal hydrolase content. Amoebae were collected and suspended at various densities in fresh axenic medium at 22°C. They were allowed to grow for 24 hours, after which the intracellular and extracellular activities of two lysosomal hydrolases: acid phosphatase and *N*-acetylhexosaminidase, were determined (Fig. 2). These two enzymes were shown previously to belong to different classes with respect to secretion (Dimond et al. 1981). In the case of acid phosphatase, the behavior of the MDP-resistant mutants was drastically different from that of AX2. In parent strain AX2, 75-80% of the total acid phosphatase activity was found associated with the amoebae. In the three MDP-resistant mutants examined, and as shown for HGR9 in Fig. 2, the intracellular acid phosphatase activity decreased from 50% to 20% of the total (amoebae + medium) activity as the cell density increased. In the case of hexosaminidase, the difference between AX2 and the MDP-resistant mutants followed a similar trend, but was less prominent. The data suggest that an increased secretion of lysosomal enzymes could account, at least partially, for the decreased intracellular content of lysosomal hydrolases.

Table 2. *Fluid-phase pinocytosis characteristics, endosomal pH, in vitro acidification and vacuolar ATPase activity of* Dictyostelium *amoebae from strain AX2 and MDP-resistant mutants*

Strain	Initial velocity*	Endosomal apparent volume*	Endosomal pH†	AO fluorescence quenching†	Vacuolar ATPase†
AX2	0.0093 ± 0.0012	0.90 ± 0.10	5.73 ± 0.19 (16)	42.8 ± 15.3 (13)	49.4 ± 9.9 (4)
HGR5	0.0046 ± 0.0023	0.28 ± 0.05	6.08 ± 0.24 (6)	12.2 ± 2.6 (5)	18.2
HGR8	0.0037 ± 0.0014	0.32 ± 0.06	6.10 ± 0.17 (13)	4.3 ± 1.6 (6)	16.5 – 22.3
HGR9	0.0043 ± 0.0010	0.38 ± 0.08	5.99 ± 0.10 (7)	23.2 ± 3.6 (4)	30.9 – 37

*Initial velocities are given in pl fluid internalized/min/cell and plateau values (endosomal apparent volume) in pl fluid internalized/cell. Results are the mean ± S.D. from four separate experiments.

†The number (n) of independent experiments is indicated in parenthesis. Values obtained for the endosomal pH in MDP-resistant mutants were significantly different from AX2 ($P<0.01$). The rate of AO fluorescence quenching is given in arbitrary units/min/mg protein and vacuolar ATPase in nmoles ATP hydrolysed/min/mg vesicular protein.

Table 3. *Protein content and enzymic activities of amoebae from* Dictyostelium *strain AX2 and MDP-resistant mutants*

Strain	AX2	HGR5	HGR8	HGR9
Protein	0.75 ± 0.15	0.77 ± 0.08	0.88 ± 0.14	0.80 ± 0.20
Acid phosphatase	106 ± 23	53 ± 12	49 ± 6	69 ± 15
Hexosaminidase	78 ± 22	19 ± 2	14 ± 1	36 ± 5
Mannosidase	4.2 ± 1.2	2.6 ± 0.2	1.6 ± 0.3	3.0 ± 0.3
Alkaline phosphatase	16.5 ± 4.0	13.5 ± 2.0	11.6 ± 1.0	14.1 ± 3.6
ATPase (oligomycin^s)	162 ± 34	143 ± 8	149 ± 28	139 ± 18
ATPase (azide^s)	174 ± 32	161 ± 17	166 ± 24	155 ± 15
Ala aminotransferase	35.7 ± 7.0	34.8 ± 5.5	50.7 ± 8.7	35.3 ± 6.6

Values are means ± S.D. from five separate experiments; except for oligomycin-sensitive ATPase and alanine aminotransferase; $n = 3$. Protein data are given in mg protein/10^7 cells. All enzymic activities are expressed in nanomoles substrate converted/min/mg protein.

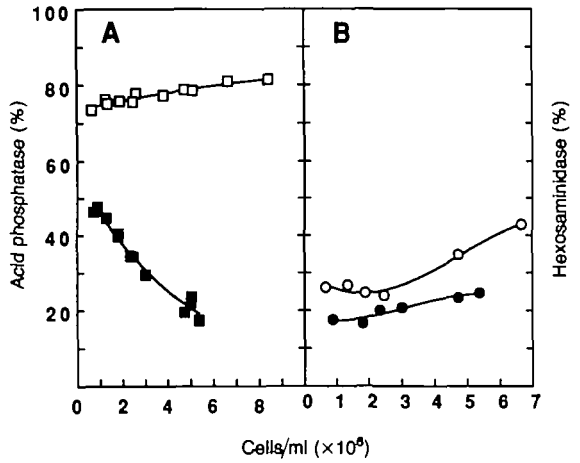

Fig. 2. Secretion of lysosomal hydrolases during axenic growth. Washed *Dictyostelium* amoebae were suspended in axenic growth medium at various cell concentrations (from 1×10^5/ml to 3×10^6/ml) and allowed to grow at 22°C. After 24 hours, the cell titer was determined and acid phosphatase (A (□,■)) and hexosaminidase (B (○,●)) activities were measured both in the amoebae and in the extracellular cell-free medium. The percentage of intracellular activity was plotted as a function of cell density. The open symbols correspond to the parent strain AX2 and the filled symbols to the MDP-resistant mutant HGR9.

Evidence for an altered acidification of the endosomal compartment in the MDP-resistant Dictyostelium *mutants*

Dictyostelium amoebae were loaded with FITC-dextran by a 3-hour incubation in axenic medium. The average endosomal pH was close to pH 5.7 for AX2 amoebae (Table 2), a value slightly higher than endosomal pH (pH 5.4) in *Dictyostelium* AX3 (Ebert et al. 1989). This may be due to a strain difference or to the use of an in vivo standard curve in this work. In MDP-resistant strains, the average endosomal pH values were significantly higher than in AX2, especially in HGR5 and HGR8, with endosomal pH reaching values of 6.1 (Table 2).

To analyze further the acidification capacity, the ATP-induced generation of a proton gradient was assayed in acidosome-rich fractions (Padh et al. 1989). In the MDP-resistant mutants, both the rate of acidication and the fractional loss of fluorescence were markedly deficient. The maximal rates of decrease in AO fluorescence resulting from acidification were reduced to 29%, 10% and 54% of AX2 activity in HGR5, HGR8 and HGR9, respectively (Table 2). Vacuolar ATPase followed a similar trend and activities were markedly deficient, especially in HGR5 and HGR8 fractions (Table 2). These findings further substantiate the endosomal pH data and suggest that the MDP-resistant mutants were affected in the acidification of their endosomal pathways, possibly at the level of acidosomes.

In vivo ^{31}P NMR spectroscopy was used for intracellular pH measurement in conjunction with AMeP as pH prove. Aminophosphonates enter *Dictyostelium* amoebae by following the fluid-phase endocytic pathways. In the ^{31}P NMR spectrum of AX2 amoebae incubated in the presence of 6.5 mM AMeP (Fig. 3A), the AMeP spectral region consisted of a major line at +9.3 p.p.m., corresponding to extracellular AMeP at pH 7.1, flanked by a satellite resonance at +11.0 p.p.m. This resonance was detected at early times (Fig. 3, inset) and its appearance was prevented by 2 mM vanadate or 7.5 mM caffeine, two inhibitors of endocytosis in *Dictyostelium* (Klein et al. 1989; Gonzalez et al. 1990). The new resonance corresponded to intracellular AMeP in a very acidic compartment. From the in vivo calibration curve, the pH of this compartment was determined to be 4.3 ± 0.2. Similar experiments were performed with the three MDP-resistant mutants. Fig. 3B corresponds to ^{31}P NMR data accumulated with HGR5 after addition of 6.5 mM AMeP. Identical results were obtained with HGR8 and HGR9 (not shown). The satellite resonance at +11.0 p.p.m. was not detected even for long incubation times. This indicated the absence of a pH 4.3 compartment in the MDP-resistant mutants.

Discussion

Previous results have shown that MDP enters *Dictyostelium discoideum* amoebae by the fluid-phase pinocy-

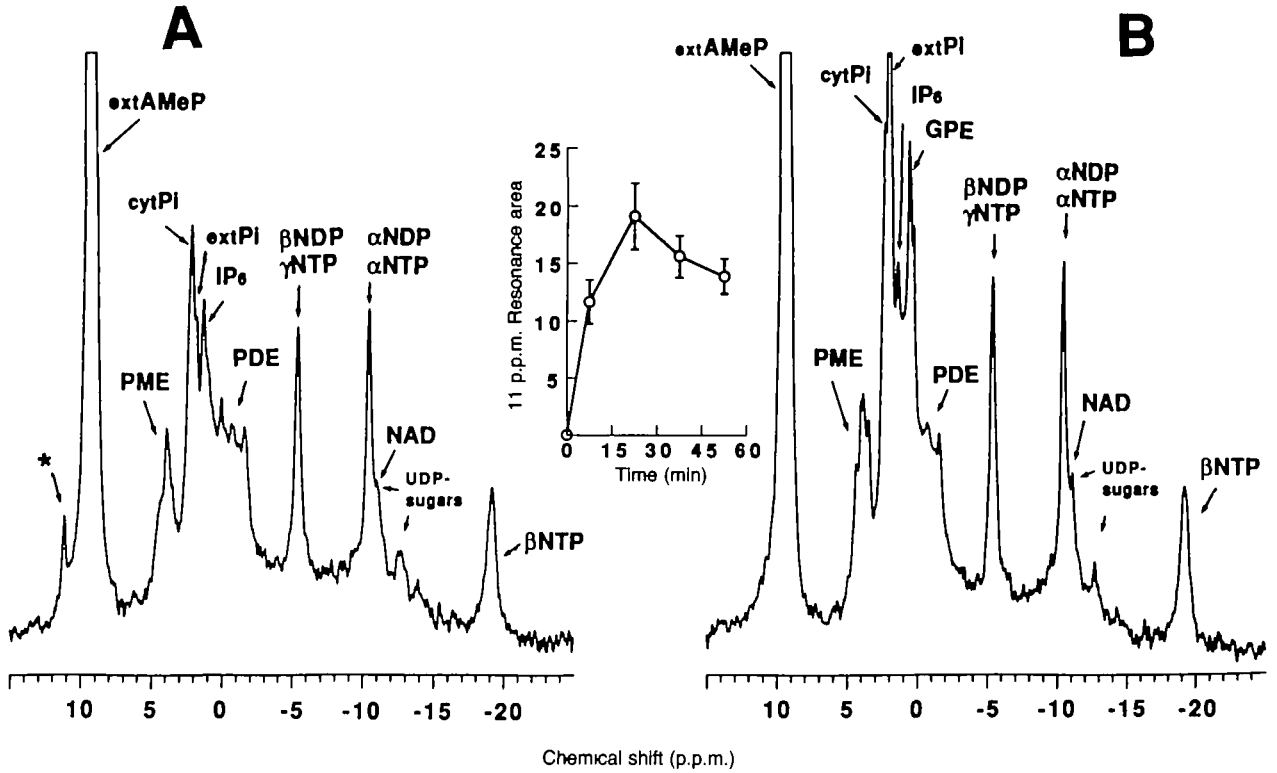

Fig. 3. ^{31}P NMR spectra of AMeP entry in *Dictyostelium* amoebae. Cells were suspended at a concentration of 3.5×10^8/ml in 40 mM Hepes-Na buffer, 6% (v/v) D_2O, pH 7.1, in a final volume of 6.5 ml and bubbled with oxygen. After equilibration at 22°C in the NMR spectrometer, AMeP (6.5 mM, final concentration) was added to the suspension. ^{31}P NMR spectra shown are the sum of 1500 free induction decays with 1.2-s interpulse delays and correspond to a 30-min accumulation between incubation times 15 and 45 min. Spectrum A, AX2. Spectrum B, HGR5. Peak assignments: PME, phosphomonoesters; cyt- and extP_i, cytoplasmic- and extracellular P_i; IP_6, inositolhexa(kis)phosphate; NDP, NTP, nucleoside di- and triphosphate; PDE, phosphodiesters; extAMeP, extracellular AMeP. Resonance at +11.0 p.p.m. labelled (*) in spectrum A corresponds to intracellular AMeP in an acidic compartment (see Results for details). Resonance at +0.5 p.p.m. in spectrum B was tentatively assigned to glycerophosphoethanolamine (GPE). The inset graph shows the variation of the area of the satellite resonance at +11.0 p.p.m. as a function of time.

tic pathway and inhibits growth (Klein et al. 1988b). This observation was used to screen for endocytosis mutants by isolating cell lines that were resistant to 7.5 mM MDP. Fifteen mutants were isolated and three of these were examined in more detail. They were partially defective in fluid-phase pinocytosis and both the rate and extent of FITC-dextran entry were reduced by more than 50%. Another finding was that the MDP-resistant mutants had a greatly decreased lysosomal enzyme content and a higher secretion rate for lysosomal hydrolases.

Evidence from several experiments presented here indicates that the mutants are impaired in the acidification of their endo-lysosomal compartment. First, they showed an increased average endosomal pH. Second, AO accumulation by their acidosome fractions was reduced. Third, the pH 4.3 compartment detected in *Dictyostelium* AX2 by ^{31}P NMR was absent in the mutants.

Various parameters of acidification in *Dictyostelium* may be affected: (1) the activity of the proton-translocating ATPase itself; (2) the permeability of the vesicle membrane to protons, either directly as a leaky membrane or by way of defective functioning of ionic carriers driven by the proton gradient; (3) the association of endocytic vesicles with acidosomes (Padh et al. 1991). The fractionation data suggest that vacuolar ATPase activity could be partially affected in HGR5 and HGR8, but that the lesion in HGR9 may be different. The resistance of *Dictyostelium* mutants to the toxic action of MDP could arise in several ways and the defective endosomal acidification could provide a simple explanation. The mechanisms of MDP transport from the endo-lysosomal compartments into the cytosol are not known; however, they could involve a co-transport with protons. A decreased pH gradient would diminish the cytosolic entry of MDP and thus reduce its toxic effect.

The MDP-resistant mutants are phenotypically analogous to *Dictyostelium* mutant HMW570, a member of class IV secretion mutants (Cardelli et al. 1990; Ebert et al. 1990), that oversecretes several lysosomal enzyme activities during growth and has a decreased fluid-phase pinocytosis. It has been suggested that the mutation in

HMW570 prevents formation of secondary lysosomes and is independent of the acidification of lysosomal and endosomal compartments (Ebert et al. 1989).

In conclusion, we have described here *Dictyostelium* MDP-resistant mutants that have defective fluid-phase pinocytosis and lower levels of lysosomal enzymes. The phenotype of the mutants appears to result from a defective endosomal acidification. It is anticipated that further characterization of these mutants should provide information on the mechanisms of endocytic pH regulation and lysosomal function.

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