Molecular Biology of the Cell
Vol. 10, 4419–4427, December 1999

Circulation of the Plasma Membrane in *Dictyostelium*

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Submitted March 29, 1999; Accepted October 1, 1999
Monitoring Editor: Guido Guidotti

We have developed a fluorimetric assay with the use of the dye FM1-43 to determine the rate at which *Dictyostelium* amoebae endocytose their surface membrane. Our results show that they do so about once each 4–10 min. A clathrin null mutant takes its surface up only ~30% more slowly, showing that this membrane uptake cannot be caused by clathrin-coated vesicles. Surprisingly, Ax2 and its parent, NC4, which differ in their rates of fluid-phase internalization by ~60-fold, take up their surfaces at the same rates. These results show that, in axenic cells, the uptake of fluid and of surface area are separate processes. The large activity of this new endocytic cycle in both Ax2 and NC4 amoebae appears capable of delivering sufficient new surface area to advance the cells' fronts during migration.

INTRODUCTION

Eukaryotic cells are capable of budding in segments of their plasma membranes and thereby internalizing some of the surrounding medium by a variety of different mechanisms (Mellman, 1996). In most mammalian cells, a principal route is via coated pits, which select a subset of plasma membrane proteins, including the receptors for transferrin, low-density lipoprotein, and some peptide hormones, to enter the cell (Pearse and Robinson, 1990). Other routes of internalization are less well characterized, including macropinocytosis (Haigler *et al.*, 1979; West *et al.*, 1989; Hacker *et al.*, 1997), transcytotic processes in epithelial cells (Peters *et al.*, 1985), and possibly caveolae in many cell types (Anderson, 1998). In each case, the internalized membrane is believed to be returned to the cell surface and complete an endocytic cycle. In a migrating fibroblast, the membrane internalized by coated pits is returned to the cell surface at the cell's leading edge; there, together with the actin cytoskeleton, it helps extend the front of the cell forward (Bretscher, 1996). In a slowly moving cell, such as a fibroblast, the membrane area taken up by coated pits each minute is approximately the same as that required to extend the front of the cell in 1 min. However, other cells move more rapidly than fibroblasts, and there is no evidence that endocytic processes could operate quickly enough to provide the surface area required to extend the fronts of these cells as they advance. We wished to determine whether, in a rapidly moving cell such as a *Dictyostelium* amoeba, surface membrane uptake could sustain the high speed at which these cells move.

Endocytosis has previously been monitored in a variety of ways in *Dictyostelium*. In an early report, the internalization of surface proteins labeled with radioactive galactose was followed; this suggested that an area equivalent to that of the plasma membrane is internalized once every 45 min (Thilo and Vogel, 1980; Thilo, 1985). A similar rate was subsequently found for a 120-kDa surface protein (Bacon *et al.*, 1994). By contrast, the cyclic AMP receptor cAR1 appears to be confined to the cell surface and does not enter the cell (Xiao *et al.*, 1997). Experience with mammalian cells, however, has shown that different surface proteins are endocytosed at different rates, making it difficult to obtain a rate of surface area uptake in this way. An alternative approach to the study of endocytosis in *Dictyostelium* has involved fluid-phase uptake; in this approach, the volume of fluid internalized is measured and—provided that the size of the primary endocytic vesicles is known—the rate of surface membrane uptake can be deduced. Unfortunately, the size of these vesicles is uncertain. However, the remarkable discovery was made that the wild-type strain NC4 takes up fluid ~100 times more slowly than does the axenic line Ax3 (Kayman and Clarke, 1983). More recently, mutants in which the clathrin heavy chain gene has been deleted were found to internalize fluid-phase markers at ~20% the rate of its parent Ax2 (Ruscetti *et al.*, 1994).

The only potential method for measuring surface area uptake in *Dictyostelium* uses the dye FM1-43 (Betz *et al.*, 1996; Haugland, 1996). This amphipathic molecule is virtually nonfluorescent in the aqueous phase, but it can partition into, and equilibrate with, a membrane in which it fluoresces intensely. Because its polar head group prevents it flipping across the bilayer, the only way in which it can enter a cell is by endocytosis when it becomes trapped inside a vesicle. This means that the rate of accumulation of this dye by a cell measures the rate of surface uptake. FM1-43 has been used to great effect to study membrane recycling in neural tissue, providing graphic evidence for the internalization and recycling of membrane in single neurons (Betz and Bewick, 1992;

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Meffert *et al.*, 1994; Lagnado *et al.*, 1996; Ryan *et al.*, 1996). We wondered whether it could also be used to measure the rate of surface uptake at a biochemical level. Another incentive to study *Dictyostelium* amoebae is that, unlike other rapidly migrating cells, they can easily be obtained in large numbers.

The reason for wishing to understand surface uptake in *Dictyostelium* amoebae may now be clear. Membrane flow models for cell locomotion (Abercrombie *et al.*, 1970; Bretscher, 1984), in which the advance of the leading edge is provided by membrane from internal pools, require rates of exocytosis, and hence of endocytosis, that can sustain this advance. For a cylindrical *Dictyostelium* amoeba, this means an area equivalent to its surface for each cell length it moves, which it can do once every few minutes. In other words, the deduced rate of surface uptake of once each 45 min (Thilo and Vogel, 1980; Thilo, 1985; Bacon *et al.*, 1994) implies that membrane flow models for how cells move could not apply to these cells. A similar conclusion comes from a comparison of NC4 and Ax3. With a 100-fold difference in fluid-phase endocytosis, and hence surface uptake, they could hardly be expected to move at the same rates; yet they do. All this might suggest that endocytosis and cell locomotion in *Dictyostelium* cannot be linked. We wished to examine this further.

MATERIALS AND METHODS

Cell Growth and Dye Uptake

Dictyostelium discoideum strains NC4 and Ax2 were maintained on nutrient agar plates at 22°C in association with *Klebsiella aerogenes*. Ax2 cells were also grown in a rotary shaker at 22°C in axenic medium (Watts and Ashworth, 1970). Myosin heavy chain null mutant cells (*mhcA*$^-$, strain HS2206 [Manstein *et al.*, 1989]) and clathrin heavy chain null mutant cells (*chc*$^-$, strain HO103 [Niswonger and O'Halloran, 1997]) were grown in association with *K. aerogenes*.

Cells were washed free of bacteria by differential centrifugation (100 × *g*, 3 min, four cycles) in KK_2 buffer (20 mM potassium phosphate, pH 6.1, 2 mM $MgSO_4$). Axenically grown cells were harvested during exponential growth (1–5 × 10^6 cells/ml) and washed twice in KK_2 buffer. Unless indicated otherwise, all procedures were carried out at ~22°C.

The lipophilic styryl dye *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM 1-43; Molecular Probes, Eugene, OR) was used as a surface membrane marker. Uptake of FM1-43 was studied quantitatively by fluorescence spectroscopy in KK_2 buffer containing 0.1 M sorbitol (KK_2-S) to repress vacuolar activity (Zhu and Clarke, 1992). Microscopic examination of Ax2 cells incubated in this buffer showed that the contractile vacuole was not labeled with FM1-43. In other preliminary experiments, we ascertained whether this dye might be toxic to the cells. Ax2 cells were incubated in KK_2-S for 2 h under the same conditions used for dye uptake. Cell viability was then determined and found to be >85%.

Dye uptake was carried out by two methods. The first of these requires fewer cells and is preferred for cells that grow poorly or are not robust (such as the mutants used here).

Direct Method Cells at 5 × 10^6 cells/ml were shaken in KK_2-S for 30 min and then diluted to 2 × 10^6 cells/ml, FM1-43 was added to a final concentration of 10 μM, and the sample was maintained in an orbital shaker. The change in fluorescence over time was monitored with the use of a Perkin Elmer-Cetus (Norwalk, CT) LS-5B fluorescence spectrophotometer (excitation wavelength, 470 nm; emission wavelength, 570 nm). To avoid phototoxicity, 300-μl aliquots were taken for each point (one-tenth of the sample volume), the fluorescence intensity was recorded, and the cells were returned to the flask. The best fit to the data was obtained with a two-exponent equation, and then the initial rate of FM1-43 uptake was calculated as the value of the derivative of the equation, expressed as fluorescence units per million cells per minute. To measure the cell surface membrane, different concentrations of cells were mixed with 10 μM FM1-43 and the initial fluorescence was recorded. A slope of ~12 fluorescence units per million cells was obtained for all of the strains. The rate of membrane internalization was obtained by referring the initial rate of FM1-43 uptake to the cell surface membrane. These results are expressed as the percentage of the cell surface taken up in 1 min or as the number of minutes in which a cell internalizes a surface membrane area equivalent to its plasma membrane.

Indirect Methods After the cells were shaken for 30 min in KK_2-S, they were centrifuged and resuspended at 2.5 × 10^7 cells/ml in KK_2-S. FM1-43 was added (10 μM), and its uptake was stopped at the appropriate time by diluting 200-μl aliquots in 10 ml of ice-cold KK_2-S. After two washes at 0°C, the cells were transferred to a different tube, washed once more at 0°C, and resuspended in 500 μl of 2% paraformaldehyde or 1% Triton X-100 in KK_2-S. The fluorescence intensity of the samples was monitored as described above. The rate of membrane internalization was calculated as in the direct method. In the case of detergent treatment, a conversion factor is needed to calculate the rate of membrane internalization because the cell surface membrane can be measured only in the absence of detergent and the spectral properties of FM1-43 in lipid membranes differ from those in detergent micelles. This conversion factor was determined with the use of lecithin vesicles. Egg lecithin was isolated (Pangborn, 1951), and unilamellar vesicles were prepared by the extrusion technique (Hope *et al.*, 1985) with the use of a stainless steel mini-extruder (Avanti Polar Lipids, Alabaster, AL) with polycarbonate filters of 100-nm pore size. A total of 1 mg of lecithin in 1 ml of KK_2 containing 5 μM FM1-43 was forced 10 times through the filters, and the vesicles were then separated from free dye by Sephadex G-25 chromatography. In this procedure, the dye is trapped in the inner monolayer of the vesicles. A standard curve was made by measuring the fluorescence of FM1-43 in lecithin vesicles diluted in KK_2-S. After this step, Triton X-100 was added to the samples to a final concentration of 1%, and the fluorescence was measured again. By comparing both curves, the fluorescence yield of FM1-43 was found to be reduced by 4.8-fold when the vesicles were dissolved in 1% Triton X-100.

We carried out preliminary experiments with the direct method to determine whether internalized dye reemerges from Ax2 when the cells are incubated in dye-free medium. Cells preloaded with FM1-43 for 75 min lost ~65% of the dye over the next 30 min, indicating that exit of the dye from the cells has a course similar to that of uptake.

It is worth noting that, despite the large volumes of fluid that Ax2 internalizes, the amount of FM1-43 that enters these cells by the aqueous phase is very small compared with that internalized by membrane association; this can easily be calculated from the number of dye molecules reported in synaptic vesicles (Henkel *et al.*, 1996).

Fluid-Phase Endocytosis

Fluid-phase endocytosis was measured with FITC-dextran (average molecular weight, 70,000) from Sigma Chemical (St. Louis, MO) as a fluid-phase marker (Thilo and Vogel, 1980). Cells were washed in KK_2 buffer and held at a concentration of 5 × 10^6 cells/ml in an orbital shaker in KK_2 containing 2 mg/ml FITC-dextran. Samples (1 ml) were taken at different times and diluted 10-fold into ice-cold KK_2. Cells were collected by centrifugation, washed twice in ice-cold KK_2, transferred to another tube, washed once, and resus-

pended in 500 μl of 100 mM Tris, pH 8.8, containing 0.2% Triton X-100. The fluorescence intensity of the samples was determined with the use of excitation and emission wavelengths of 490 and 520 nm, respectively. The pinocytosed volume was determined by comparison with a standard curve.

Microscopy

FM1-43 Ax2 or NC4 cells were allowed to attach to a coverslip for 20 min. The coverslip was inverted onto a chamber constructed with a 1-mm space between the coverslip and the slide. Cells were incubated for 5 min in KK_2-S containing 10 μM FM1-43 and then washed. Images were taken just after dye addition, after washing the dye away at 5 min of incubation, and then after leaving the cells for an additional 25 min without dye. Ax2 amoebae were also preincubated for 10 min in 10 mM sodium azide in KK_2-S and then incubated for 5 min in FM1-43 plus azide.

FITC-Dextran Ax2 or NC4 cells were allowed to attach to microscope slides for 20 min and incubated with 2 mg/ml FITC-dextran in KK_2 for 5 min at 22°C. After the dextran was washed away at 0°C, the cells were fixed in 2% formaldehyde for 30 min.

All samples were examined with differential interference contrast or fluorescence optics with a 40× objective. Images were recorded digitally with a cooled charge-coupled device camera and reproduced without changing the linearity of fluorescence response.

RESULTS

FM1-43 Uptake as Seen in the Microscope

The impermeant dye FM1-43 is essentially nonfluorescent in the aqueous phase but fluoresces when it partitions into the outer leaflet of a surface membrane (Betz *et al.*, 1996; Haugland, 1996). This binding is reversible, so when cells are washed, the dye is rapidly removed from the cell surface (Ryan *et al.*, 1996). However, if stained surface membrane is internalized, the dye becomes trapped within internal vesicles; therefore, its uptake can be used to measure endocytosis (Betz and Bewick, 1992; Betz and Wu, 1995). Because the dye interacts with the growth medium, all cells were first equilibrated in KK_2 buffer containing 0.1 M sorbitol; the sorbitol was included to repress vacuolar activity (Zhu and Clarke, 1992), because it has been reported that the related dye FM4-64 preferentially labels contractile vacuoles in newly germinated NC4 cells (Heuser *et al.*, 1993).

When Ax2 amoebae grown in axenic medium are placed in FM1-43 and viewed in a fluorescence microscope, they immediately fluoresce; this initial labeling appears limited to the plasma membrane, as expected (Figure 1A). After 5 min at room temperature, the cytoplasm has a distinctly uneven appearance that is more easily observed if the surface fluorescence is removed by washing the cells with buffer. After washing, there are bright dots (~0.5 μm in diameter) that represent dye internalized in vesicles (Figure 1C). If these cells are incubated for another 25 min in the absence of FM1-43, much of the remaining dye is seen to reside in larger (1–2 μm diameter) vesicles (Figure 1E). Dye internalization is inhibited by azide (Figure 1, G and H). When the wild-type strain NC4 is incubated for 5 min with FM1-43, the labeling pattern is indistinguishable from that of Ax2 (Figure 1, compare C and D). After another 25 min in the absence of FM1-43, the labeled vesicles inside NC4 cells appear smaller (still 0.5 μm in diameter) than those in Ax2 cells (Figure 1, compare E and F). We have investigated this difference and find that, when Ax2 cells are grown on bacteria and subsequently labeled with dye for 30 min (as in Figure 1E), only the smaller vesicles are seen (data not shown). This finding indicates that how Ax2 amoebae handle internalized membrane depends on how they are grown.

FM1-43 Uptake by Ax2 Grown Axenically

We have measured surface uptake with the use of FM1-43 in two different ways, which we call the "direct" and "indirect" methods.

In the direct method, cells are placed in the dye and the fluorescence intensity over time is monitored with a fluorimeter. Because the partitioning of dye into, and equilibration with, the plasma membrane is almost instantaneous (Ryan *et al.*, 1996), the increase in fluorescence over time measures unlabeled surface that has become exposed to the dye as a result of the combined processes of exocytosis and endocytosis. This direct route measures the zero-time fluorescence, which is due to the cells' plasma membranes; however, this same fluorescence provides a high baseline against which the subsequent uptake of FM1-43 is measured.

Alternatively, in the indirect methods, the cells are incubated in the dye for various times, chilled to 0°C, and then washed at 0°C to remove surface dye. The remaining FM1-43, which represents only internalized dye, can then be determined. The advantage of this approach is that the zero-time fluorescence is much lower, providing a lower baseline, and this improves the sensitivity of the assay, especially at short times of labeling.

In each case, the rate of surface membrane internalization has been calculated from the initial rate of FM1-43 uptake and from the cell plasma membrane fluorescence observed at zero time. The latter can be measured only when the cells are in the presence of the dye, i.e., by the direct method.

In the direct method, cell-associated fluorescence increases smoothly with time as soon as the FM1-43 is added to the amoebae, as shown in Figure 2A. The rate of increase decreases with time as the membranes of the endocytic compartment become labeled. This results in cellular fluorescence doubling within ~1 h, at which time the amount of membrane-associated dye inside the cells appears to be the same as that on the plasma membrane. Dye uptake measured fluorimetrically (Figure 2A), or as observed in the microscope (Figure 1, G and H), is energy dependent in that it is abolished by azide. The initial rate of increase in fluorescence indicates a surface uptake of ~10% of the plasma membrane per minute. Ax2 cells, then, appear to internalize a surface area equivalent to the plasma membrane once every 10 min.

In the indirect methods, amoebae were placed in FM1-43 for various times, chilled to 0°C, and washed to remove surface label. Cell-associated fluorescence was measured after cellular processes had been stopped by formaldehyde fixation. When dye uptake is followed in this way (Figure 2B, ▲), the kinetics are similar to those shown in Figure 2A, and this uptake does not occur at 0°C. The initial rate of membrane uptake by Ax2 cells is found to be ~14% of the plasma membrane per minute, indicating that a cell surface area equivalent is internalized once every 7.5 min.

There were three points that concerned us with the measurements described above.

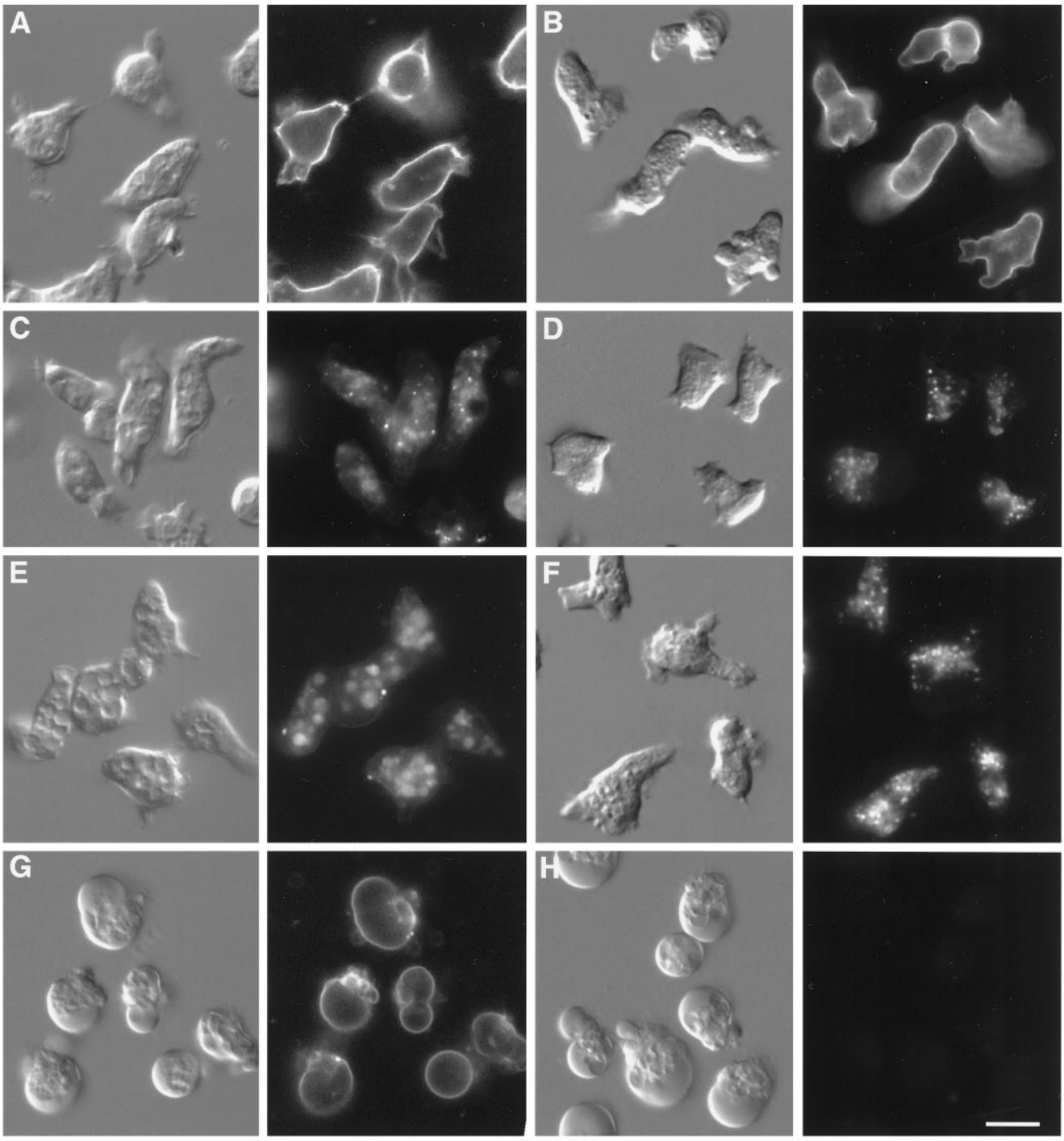

Figure 1. FM1-43 internalization by individual Ax2 and NC4 cells. (A–F) Dye associated with Ax2 (A, C, and E) or NC4 (B, D, and F). (A and B) Surface fluorescence at 0 min after FM1-43 addition. (C and D) Cells incubated for 5 min with dye and then washed. (E and F) Same as C and D, but with an additional 25 min without dye. (G and H) Ax2 cells pretreated with azide, observed at 0 min after addition of dye (G) or after 5 min of incubation in dye and then washed (H). Differential interference contrast (DIC) images are on the left of each pair, and fluorescence images are on the right. Minor differences between pairs of DIC and fluorescence images are due to movement of the live cells. Bar, 10 μm.

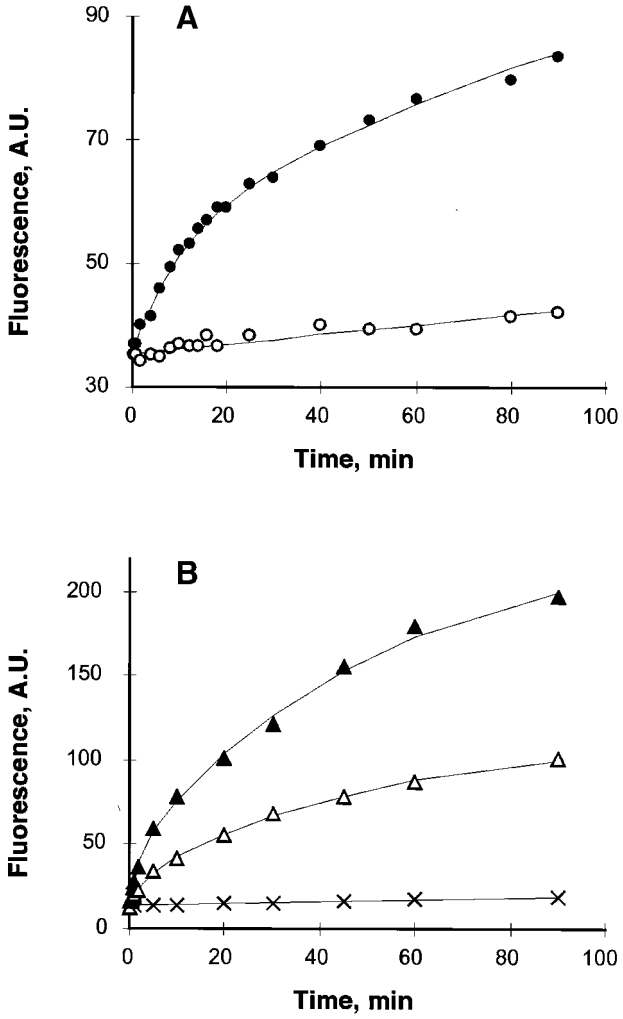

Figure 2. FM1-43 uptake by Ax2 amoebae grown axenically. (A) Direct method: uptake in the absence (●) or presence of 10 mM azide (○). (B) Indirect methods: uptake at 22°C (▲) or at 0°C (×), followed by formaldehyde fixation; and uptake at 22°C, followed by solubilization in 1% Triton X-100 (△). The lines represent the data fit to a two-exponent equation (●, ▲, △) or to a straight line (○, ×). In A, the uptake of FM1-43 was continued to 180 min; at this time, the level of fluorescence had nearly plateaued and the ratio of total-to-surface fluorescence was ~2.5. A.U., arbitrary units.

1. If the dye is taken up in very small vesicles and then added to larger (endosomal) structures that have a much larger volume-to-surface area ratio, the dye would equilibrate with this increased volume. If the partition coefficient of the FM1-43 favors the aqueous phase, the majority of the dye, taken up in the membrane of small vesicles, would move out into the aqueous phase in the endosomes and thereby lose its fluorescence. This would lead to an underestimate in the initial rate of surface uptake. This potential problem appears not to exist because the partition coefficient of FM1-43 lies too far in favor of the membrane phase (as can be deduced from the findings of Henkel *et al.* [1996]); although the aqueous phase to which the FM1-43 becomes exposed after entering the cell may increase substantially, dye loss from the membrane phase would be small.

2. Studies of mammalian cells have shown that the pH of endocytic vesicles decreases quickly after they have pinched off the plasma membrane, which may also be the case in *Dictyostelium* (Aubry *et al.*, 1993, 1997; Padh *et al.*, 1993). If so, this could affect the fluorescent properties of the endocytosed FM1-43. This dye has a conjugated electron system that resonates between a pyridinium nitrogen at the water/membrane interface and a tertiary amine near the hydrophobic end of the molecule; protonation of this system would remove its affinity for membranes and its fluorescence (Betz *et al.*, 1996). We have estimated the pKa of 1 μM FM1-43 in the presence of egg lecithin vesicles to be 4.1, which suggests that a substantial decrease of the pH inside vesicles could drive the FM1-43 into a nonfluorescent state. Furthermore, in cells prefixed in formaldehyde (Figure 2B), this problem might be exacerbated because formalin fixation of amino groups releases protons that may decrease the ambient pH. This could lead to an underestimate of the rate of endocytosis.

3. The spectral properties and partition coefficient of FM1-43 may be affected by the lipid composition of, or the potential across, the membrane in which it resides. These may contribute to the quite different spectral properties that this dye exhibits in different membranes (Betz *et al.*, 1996).

To counter the uncertainties raised in points 2 and 3 above, the labeled cells were washed at 0°C to remove surface dye and the remaining fluorescence was measured after dissolving them in Triton X-100 (Figure 2B). This should remove any effects of decreased vesicular pH, membrane potentials, or different lipid compositions of internal compartments. However, in doing this, we needed to find a way to relate this fluorescence, measured in detergent, to that caused by dye bound to the cell surface, which can be made only on intact cells. As described in MATERIALS AND METHODS, we determined that the fluorescence emission of FM1-43 is 4.8-fold lower when the dye is dissolved in 1% Triton X-100 than when it is dissolved in a lipid bilayer made from egg lecithin. We assume that the FM1-43 fluorescence emissions in lecithin and on the surface of *Dictyostelium* are similar; therefore, we used a conversion factor of 4.8 in calculating the rate of surface membrane uptake. This yields a faster rate at which Ax2 cells internalize their surfaces, being one plasma membrane equivalent every 4 min.

Surface Uptake by Wild-Type and Mutant Cells

Wild-type *Dictyostelium* cells (NC4) are known to take up the fluid phase, measured with fluorescent dextran, much less effectively than Ax3 when grown in axenic medium (Kayman and Clarke, 1983). Surface membrane uptake with the use of FM1-43, therefore, was measured in NC4 and axenic (Ax2) cells grown either axenically or in association with bacteria. The results, obtained by the direct method of FM1-43 uptake described above, are presented in Figure 3A. There is little difference in the initial rates of uptake of the dye by NC4 or Ax2 cells grown on bacteria or in axenic medium. This reflects the uptake of FM1-43 as seen by fluorescence microscopy (Figure 1), but it is surprising given the difference in fluid-phase uptake reported between NC4 and Ax3 strains.

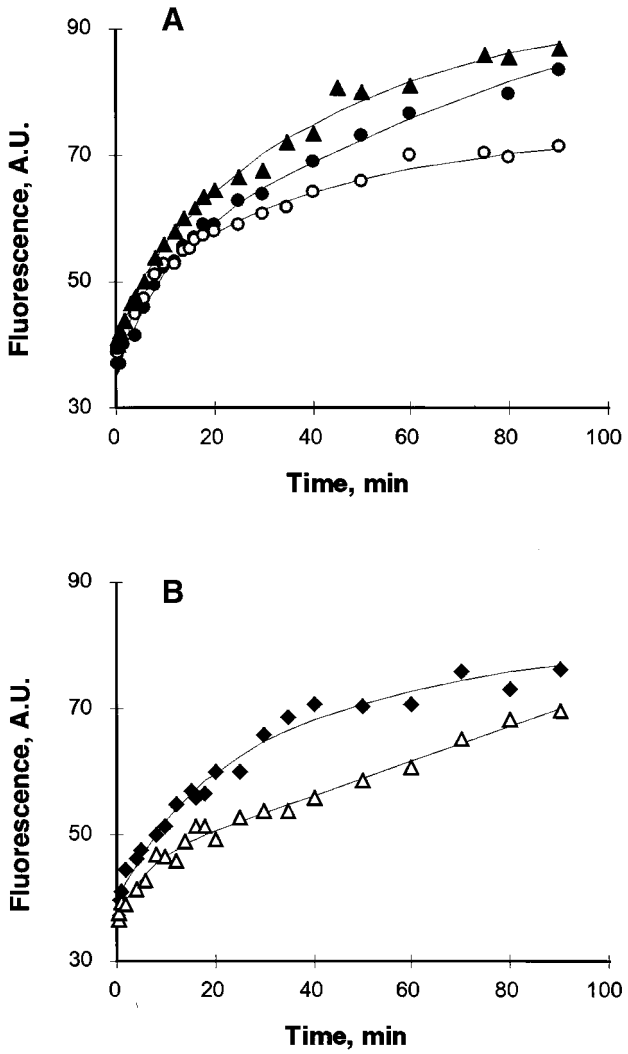

Figure 3. FM1-43 uptake by *Dictyostelium* strains grown with bacteria and measured by the direct method. (A) NC4 (▲) and Ax2 (○). The data from Figure 2A for Ax2 cells grown axenically have been included for comparison (●). (B) *mhcA*$^-$ (◆) and *chc*$^-$ (△). The lines represent the data fit to a two-exponent equation. A.U., arbitrary units.

The rate of surface uptake by amoebae that had no clathrin heavy chain (*chc*$^-$) was also examined, because these amoebae have greatly reduced rates of pinocytosis (O'Halloran and Anderson, 1992; Ruscetti *et al.*, 1994). When placed in medium containing FM1-43, *chc*$^-$ cells internalize the dye at a slightly slower rate than Ax2 (Figure 3B), appearing to take up a surface membrane equivalent about once every 13 min. FM1-43 uptake was also studied in a myosin II heavy chain null mutant, which gave a rate of surface internalization similar to that shown by the *chc*$^-$ cells.

A summary of the rates of membrane uptake is presented in Table 1, in which additional data obtained for NC4 with the indirect methods are included.

Table 1. Rate of membrane internalization

	Initial rate of FM1-43 uptake (fluorescence units · 10^6 cells^{-1} · min^{-1})	Time to internalize a plasma membrane area equivalent (min)
Direct method		
Ax2 axenic	1.18	10.2
Ax2	1.22	9.8
NC4	1.29	9.3
mhcA$^-$	0.86	14.0
chc$^-$	0.92	13.1
Indirect methods		
Fixation		
Ax2 axenic	1.64	7.3
NC4	1.60	7.5
Triton X-100		
Ax2 axenic	2.97[a]	4.0
NC4	2.93[a]	4.1

The initial rate of FM1-43 uptake was calculated as the value of the derivative of a two-exponent equation and is expressed as fluorescence units per million cells per minute. The fluorescence associated with the cell surface is 12 fluorescence units per million cells. This number has been used to calculate the time in which one cell surface area (plasma membrane) equivalent is internalized.

[a] These numbers have been corrected to account for the lower fluorescence yield of the dye in detergent, as described in MATERIALS AND METHODS.

Fluid-Phase Endocytosis

Because of the apparent paradox—that NC4 and axenically grown cells internalize their surface membranes at the same rates measured with FM1-43 yet might behave quite differently toward fluorescent dextran—we also examined how our Ax2 cells take up fluid phase markers from the medium. NC4 and axenically grown Ax2 cells were labeled for 5 min in medium containing fluorescent dextran, washed, and fixed. As shown in Figure 4, Ax2 cells contain many labeled vesicles that are absent from the NC4 cells. The rates of uptake of fluid labeled with fluorescent dextran were also measured. NC4 cells (grown on bacteria) endocytose ~0.07 nl·10^6 cells^{-1}·min^{-1}, which is close to the rate reported previously (Kayman and Clarke, 1983); Ax2 cells grown on bacterial plates or axenically take up 0.5 and 4.2 nl·10^6 cells^{-1}·min^{-1}, respectively. Although the difference between NC4 and axenically grown Ax2 is not quite as large as that reported for NC4 and Ax3 (Kayman and Clarke, 1983), our results show that, despite a difference of ~60-fold in the rates of fluid-phase endocytosis between these cells, there is little difference in the rates of surface membrane internalization as measured by FM1-43 uptake.

DISCUSSION

Rate of Surface Uptake

When FM1-43 is added to amoebae, fluorescence microscopy shows that it first labels the cell surface, but after a brief incubation (5 min) it marks a population of intracellular vesicles ~0.5 μm in diameter. This uptake is blocked by

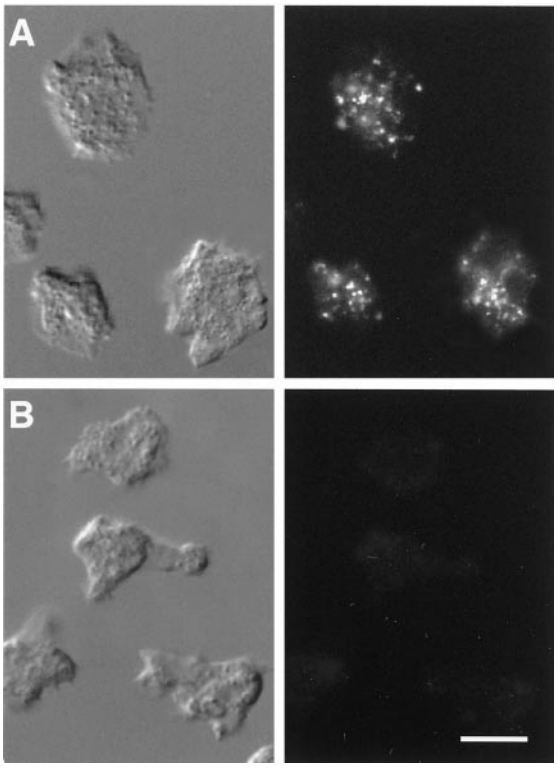

Figure 4. FITC-dextran uptake by Ax2 and NC4 cells. Ax2 (A) and NC4 (B) cells were incubated with 2 mg/ml FITC-dextran for 5 min, washed, and fixed. Differential interference contrast images are on the left of each pair, and fluorescence images are on the right. Bar, 10 μm.

azide, showing it to be an energy-dependent process (Figure 1). When dye uptake is measured fluorimetrically, the amount taken up increases smoothly with time, the uptake is energy dependent, and uptake does not occur at 0°C (Figure 2). All of these features indicate that, as in neuronal tissue (Betz and Bewick, 1992; Meffert *et al.*, 1994; Betz *et al.*, 1996; Lagnado *et al.*, 1996; Ryan *et al.*, 1996), FM1-43 provides a measure of endocytic activity in these cells.

The measured initial rates of endocytosis in Ax2 vary between 4 and 10 min for one cell surface area to be endocytosed (Table 1). Because styryl dyes have not been used in this way before, we measured the amount of internalized dye by a direct method and two indirect methods. The direct method follows total cell-associated fluorescence (plasma membrane plus internalized membrane). It has a high baseline because the plasma membrane contribution is always present, but it is best used with fragile cells. In the indirect methods, the cell surface label is washed away at 0°C and the fluorescence remaining inside the cells as a result of endocytosis is quantitated either after formaldehyde fixation or after the cells are dissolved in detergent.

These two alternatives are not equivalent and give different rates of surface internalization. The principal difference is that dissolving the cells in detergent ensures that the total dye accumulated is monitored, removing certain potential problems that are discussed in RESULTS. However, it does require a conversion factor, which we have determined, to account for the change in fluorescence properties of FM1-43 when it is moved from a membrane to a detergent micelle. We think that the indirect methods are more reliable for determining internalized dye at short times of incubation: they yielded a rate of endocytosis of one cell surface equivalent each 4–7.5 min. This is an average for a large population of cells, and it seems probable that at any moment some individuals are more active and others less so.

The rate of endocytosis in the wild type, NC4, has also been measured; regardless of which method is used, the rate obtained is indistinguishable from that of Ax2. A somewhat slower rate was measured (by the direct method) for the *mhcA*$^-$ and *chc*$^-$ mutants; these internalize a plasma membrane equivalent once every 13–14 min. It may be worth noting that even the fastest rates of cell surface uptake described here are slow compared with what exists in the terminals of goldfish bipolar cells. When these terminals, which are about the size of a small amoeba (having a diameter of ~10 μm), are stimulated, the ensuing endocytosis leads to the equivalent of 2.7 entire terminal surfaces being internalized in 1 min (Lagnado *et al.*, 1996).

Fluid-Phase and Surface Uptake

That NC4 and axenically grown Ax2 internalize their surfaces at the same rates is remarkable, given the 60-fold difference in the rates at which they accumulate fluorescent dextran in the fluid phase. This surprising result might be explained in various ways.

First, an NC4 cell may have a denser polysaccharide matrix on its surface than Ax2, such that fluorescent dextran is filtered out of the fluid entering forming endocytic vesicles. We consider this possibility unlikely because it does not explain why fluorescent dextran uptake by Ax2 grown on bacteria is much lower than that in the same cells grown on medium, yet surface uptake is the same.

Second, axenically grown cells take in large gulps of medium, which provide the nutrients needed for growth (Hacker *et al.*, 1997), whereas the NC4 cells may take in many tiny vesicles (or very narrow tubules). In this way, the two cell types could take in the same surface areas but quite different fluid volumes. Thus, if the 0.5-μm vesicles observed in Ax2 labeled after 5 min in FM1-43 were primary vesicles, the primary vesicles in NC4 would have to have diameters of ~0.01 μm and then coalesce to form the observed 0.5-μm vesicles. This seems unlikely, because vesicles with a diameter as small as 0.01 μm are unknown.

A third view, which we favor, suggests that the sizes of the primary vesicles that are responsible for the bulk of the membrane uptake are the same in NC4 and Ax2. These vesicles are probably small, so that they bring into the cell only a small volume of fluid. However, superimposed on this in Ax2 (but not in NC4) is a large fluid uptake that enables these cells to grow in a synthetic medium. In other words, the fluid and membrane uptake in Ax2 may reflect largely different processes.

A similar, but less dramatic, difference is also found when comparing Ax2 and the clathrinless mutant. This has a fivefold reduced ability to endocytose the fluid phase (O'Halloran and Anderson, 1992), yet the rate of surface uptake is reduced by only ~25%. This result also argues that

the processes we measure as surface and fluid uptake reflect different cellular mechanisms.

It may be helpful to compare endocytosis in amoebae and mammalian tissue culture cells. In unstimulated mammalian cells, the most widely used device to take up surface and fluid seems to be the clathrin-coated pit, although other endocytic processes do occur (Mellman, 1996). Some cell lines, upon hormonal stimulation, actively ruffle and in so doing encapsulate large droplets of fluid, a process called macropinocytosis (Haigler *et al.*, 1979; West *et al.*, 1989). In Ax2, much of the fluid phase is taken up by a mechanism that appears to be analogous to macropinocytosis. These cells form membrane structures on their surfaces that resemble circular ruffles called "crowns," which can fuse at their distal tips and thereby enclose a droplet of fluid for ingestion (Hacker *et al.*, 1997). This mechanism for fluid-phase uptake by Ax2, in which the internalized fluid is not introduced into the cell by coated vesicles, fits well with the paucity of coated pits seen along the plasma membrane of amoebae in thin sections (O'Halloran and Anderson, 1992; Hacker *et al.*, 1997). It suggests that a difference between Ax2 and NC4 is that Ax2, when grown in synthetic medium, is constitutively activated to ruffle. The reason why the clathrinless mutant is severely impaired in fluid-phase uptake (O'Halloran and Anderson, 1992) may be that ruffle production in Ax2 depends on membrane emerging on the cell surface from a coated pit–driven cycle, as appears to be the case with hormonally generated ruffles in higher cells (Bretscher and Aguado-Velasco, 1998).

We believe that our results show that superimposed on the processes that are responsible for fluid uptake in Ax2 is a new cycle detected by FM1-43. This cycle is more important in terms of surface uptake and occurs to almost the same extent in Ax2, NC4, and mutants lacking clathrin and myosin II.

Membrane Flow and Locomotion

Membrane flow models for locomotion require that the frontal extension of a cell as it advances is provided by the insertion of fresh membrane from intracellular sources, as has been shown to occur in migrating *Physarum* (Sesaki and Ogihara, 1997). This requirement implies that a close quantitative relationship exists between the rate of endocytosis and the maximum speed of a cell, a relationship that depends on the shape of the cell (Bretscher, 1996). For a (roughly) cylindrical cell such as a streaming *Dictyostelium* amoeba, one surface equivalent would have to be exocytosed at the front of the cell to advance it by its own length. This implies that a cell could not move its own length in less time than that required to endocytose its surface. In the present work, two major objections to membrane flow as a mechanism for locomotion in *Dictyostelium* amoebae appear to have been eliminated.

First, the earlier rates of membrane uptake determined in Ax2 suggested that one cell surface equivalent is taken up about every 45 min (Thilo and Vogel, 1980; Thilo, 1985; Bacon *et al.*, 1994). Because Ax2 cells move one cell length in a few minutes, the earlier rates were incompatible with a membrane flow model for locomotion. However, the rate measured here is about one cell surface equivalent every 4–10 min, and the lower end of this range is now compatible with a membrane flow model for cell locomotion.

Second, it could be inferred that NC4 and axenically grown Ax2 cells take up their surfaces at vastly different rates because of the great difference in the rates at which they drink. From this perspective, membrane flow models for locomotion would have predicted that Ax2 should move much more rapidly than NC4. Yet, the two cell lines move indistinguishably, which implied that membrane flow schemes for locomotion could not apply to *Dictyostelium* amoebae. In fact, these cells take up their surfaces at about the same rates and move at about the same rates, which fits nicely with the model. Indeed, one may ask, if the wild-type NC4 does not require fluid-phase endocytosis and membrane uptake to obtain nutrients, for what purpose could this surface uptake be used other than locomotion?

ACKNOWLEDGMENTS

We thank Manuel Sánchez del Pino, Leon Lagnado, Rob Kay, and Barbara Pearse for many helpful discussions. C.A.-V. was supported by a European Molecular Biology Organization long-term fellowship.

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