Weak Bases and Ionophores Rapidly and Reversibly Raise the pH of Endocytic Vesicles in Cultured Mouse Fibroblasts

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ABSTRACT It has been shown that endocytic vesicles in BALB/c 3T3 cells have a pH of 5.0 (Tycko and Maxfield, Cell, 28:643-651). In this paper, a method for measuring the effect of various agents, including weak bases and ionophores, on the pH of endocytic vesicles is presented. The method is based on the increase in fluorescein fluorescence with 490-nm excitation as the pH is raised above 5.0. Intensities of cells were measured using a microscope spectrofluorometer after internalization of fluorescein-labeled α2-macroglobulin by receptor-mediated endocytosis. The increase in endocytic vesicle pH was determined from the increase in fluorescence after addition of various concentrations of the test agents. The following agents increased endocytic vesicle pH above 6.0 at the indicated concentrations: monensin (6 μM), FCCP (10 μM), chloroquine (140 μM), ammonia (5 mM), methyla- mine (10 mM). The ability of many of these agents to raise endocytic vesicle pH may account for many of their effects on receptor-mediated endocytosis. Dansylcadaverine caused no effect on vesicle pH at 1 mM. The observed increases in vesicle pH were rapid (1-2 min) and could be reversed by removal of the perturbant. This reversibility indicates that the vesicles themselves contain a mechanism for acidification. The increase in vesicle pH due to these treatments can be observed visually using an SIT video camera. Using this method, it is shown that endocytic vesicles become acidic at very early times (i.e., within 5-7 min of continuous uptake at 37°C).

A wide variety of ligands, including certain hormones, serum proteins, bacterial toxins, lysosomal enzymes, and enveloped viruses, enter cells via receptor-mediated endocytosis (reviewed in references 1 and 2). For many of these ligands, the pathway of entry into the cell has been shown to begin with the concentration of occupied receptors over clathrin-coated pits, followed by entry into endocytic vesicles (1, 2). In several cases, there is evidence that receptors enter endocytic vesicles along with the ligand (3-5).

Several workers have examined the effect of weak bases or ionophores on various steps in the receptor-mediated endocytosis pathway. A variety of effects have been reported, including reduced rates of endocytosis (6, 7), inhibition of receptor recycling (8-11), recycling of intact ligand-receptor complexes to the surface (4), inhibition of diphtheria toxin toxicity (12), and reduction of virus infectivity (13).

Since acidification is an early event in the endocytosis pathway (16), it is plausible that weak bases and ionophores could exert some of their effects by raising endocytic vesicle pH. To explore that possibility, I have directly measured the ability of several of these agents to raise endocytic vesicle pH. In general, the concentrations required to raise vesicle pH are similar to concentrations where various effects on the endocytosis pathway have been reported. The effects on vesicle pH are rapidly reversible. A notable exception is dansylcadaverine which does not raise vesicle (or lysosomal) pH at concentrations far above those which inhibit endocytosis (7), recycling (10), or viral infectivity (13).

The results presented in this paper indicate that vesicle acidification is a very early event after internalization and that endocytic vesicles contain a mechanism for regulating their internal pH. These results also indicate that acidification of endocytic vesicles may play a central role in the processing of many ligands and receptors.

MATERIALS AND METHODS

Materials

Monodansylcadaverine was obtained from Fluka, monensin from Calbiochem, and chloroquine diphosphate from Boehringer Mannheim. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), methyla- mine, propylamine, and hexylamine were obtained from Sigma Chemical Co., St. Louis, MO. α2-Macroglobulin (αM) and fluorescein-labeled αM (F-αM) were prepared as described previously (16).

Cell Culture

BALB/c 3T3 mouse fibroblasts were grown in Dulbecco-Vogt modified Eagle's medium (DME; Gibco, Grand Island Biological Co., Grand Island, NY) containing 10% (vol/vol) calf serum at 37°C in an atmosphere of 95% air and 5% CO2.
**Quantitative Fluorescence Microscopy**

Fluorescence intensities were measured using a Leitz MPV Compact microscope spectrophotometer mounted on a Diavert microscope. A 75 W xenon lamp was used for epifluorescence illumination. The microscope was modified by inserting a beam splitter to allow simultaneous intensity measurement and observation using an image intensifier television camera. A standard Leitz fluorescence filter cube was modified by removing the excitation filter and placing either a 490 or a 450-nm narrow bandpass filter in the incident light path. The incident beam was centered and stopped down to a diameter of ~50 μm, and the position of the beam was noted on the video monitor. The measuring diaphragm was adjusted to a diameter slightly smaller (in the image plane) than the illuminated area. pH measurements on endocytic vesicles and lysosomes were made as described previously (16) using the ratio of fluorescein fluorescence intensities with 450 and 490-nm excitation.

To determine the effect of various agents on endocytic vesicle pH, cells were incubated with F-α2M (100 μg/ml) in DME for 14 min at 37°C. The cells were rinsed four times with warm DME and placed in 1 ml of measuring buffer (155 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 10 mM glucose, 20 mM HEPES, pH 7.4). The cells were immediately placed on the microscope stage and observed with a 63 power, N.A. 1.3 objective, and a single cell, or a group of 2-4 cells, was centered (under bright field illumination) in the measuring area.

An initial intensity measurement was made using 490-nm excitation and an illumination period of 0.1 s. Immediately after this measurement, 1 ml of measuring buffer containing the test agent was added to the dish. The medium was gently mixed with a Pasteur pipet, taking care not to move the dish. Intensity measurements were made 1, 2, and 3 min after addition of the test agent. After the third measurement, an additional 1 ml of measuring buffer was added containing the agent and the final intensity was measured after another 3 min. The total time from the addition of F-α2M to the final measurement did not exceed 22 min. The total fluorescence illumination time was 0.7 s. The video system was used to check for movement of the cells after the initial measurement and to check for F-α2M binding to extracellular debris in the measurement field. Video images were recorded, displayed, and photographed as described previously (16).

**Binding Studies**

α2M was iodinated by the chloramine-T procedure (17) to a specific activity of 2 x 10⁶ cpm/μg. [125I]-α2M was separated from Na[125I] by chromatography on a Sephadex G-25 column. Epidermal growth factor (EGF) was prepared by the method of Savage and Cohen (18). [125I]-EGF was prepared by the method of Carpenter and Cohen (19) with a specific activity of 4.1 x 10⁹ cpm/ng. Measuring buffer was adjusted to a range of pH values from 6.4 to 7.8 using HCl or NaOH. Confluent monolayers were rinsed with ice-cold measuring buffer and incubated on ice with [125I]-α2M (3 nM) or [125I]-EGF (2 nM) in measuring buffer with 1% bovine serum albumin. Incubations were continued on ice, with constant shaking, for 1 h (EGF) or 4 h (α2M). At the end of the incubation, cells were rinsed four times with ice-cold measuring buffer and dissolved in 1 N NaOH. Radioactive counts were measured on a Beckman 4000 gamma counter (Beckman Instruments, Inc., Fullerton, CA). Nonspecific binding was determined by competition with excess α2M (2 mg/ml) or EGF (2 μg/ml). All values were determined in triplicate.

**RESULTS**

The pH-dependence of the excitation profile of fluorescein makes it a very useful probe for measuring intracellular pH (14). Fig. 1 shows the pH dependence of fluorescein labeled α2M (F-α2M) fluorescein using 450 or 490-nm excitation in the instrument used for these studies. Between pH values of 4.5 and 7.5, the fluorescence excited at 450 nm is relatively independent of pH, but the 490-nm fluorescence increases sharply with increasing pH. In a previous study (16), the ratio I450/I490 was used to estimate the pH of endocytic vesicles, and we obtained a value of 5.0 ± 0.2 (SE) after a 15-min continuous uptake and 5-min chase with F-α2M. In that study, light and electron microscopy were used to demonstrate that α2M is in nonlysosomal structures at the time of measurement.

To measure the effect of various agents on endocytic vesicle pH, cells were incubated with F-α2M for 14 min, and then the effect of the test agent on the intensity of fluorescein emission with 490-nm excitation was measured (see Materials and Methods). Agents which raise endocytic vesicle pH should increase the fluorescence intensity, with the extent of the increase dependent on the change in pH. We measured the fluorescence intensity (0.1-s illumination) on cells before and after the addition of various agents (listed in Table I). Within 1-2 min after addition, the fluorescence reached a stable value. We then added a high concentration of methylamine (50 mM) to the cells to elicit a ‘maximal’ increase in the vesicle pH and again measured the intensity. The increase in intensity caused by the test agent was then compared to the increase caused in the same cells by the high concentration of methylamine. The relative increase, ΔI, was defined as:

$$\Delta I = (I_{\text{test}} - I_{\text{init}})/(I_{\text{max}} - I_{\text{init}})$$

where $I_{\text{test}}$ is the stable intensity value after addition of the test agent, $I_{\text{init}}$ is the intensity before addition of the agent, and $I_{\text{max}}$ is the intensity after addition of 50 mM methylamine.

This procedure was chosen for several reasons. A microscope spectrophotometer was used because it allows one to observe
the cells as they are measured. Because of the pH dependence of fluorescein fluorescence, even a small amount of F-α2M bound extracellularly to debris or dead cells would make a large contribution to the signal compared with F-α2M at pH 5. Any measurement based on small numbers of cells must be internally calibrated to avoid artifacts arising from cell-to-cell variability in α2M uptake (20). In our previous study, internal calibration was provided by using the ratio I450/I490 which is independent of total uptake. In the present study, internal calibration was provided by comparing the effect of the test agent to the effect of 50 mM methylamine on the same cells. The value of ΔI should be independent of total F-α2M uptake. The average value of IMax/Ini was 1.7. Addition of the agents listed in Table 1 to cells which had not been incubated with F-α2M caused no change (±3%) in the cellular autofluorescence.

Also, these agents do not, by themselves, change the fluorescence of F-α2M. An advantage of the present method is that cellular autofluorescence, scattered light, and extracellular F-α2M should all contribute equally in each intensity measurement. Therefore, these sources all cancel out in the evaluation of ΔI. A disadvantage is that pH values cannot be determined directly from ΔI. However, by using Fig. 1 and measuring the pH in the presence or absence of 50 mM methylamine, one can estimate the pH from ΔI. As previously described, the pH in endocytic vesicles is 5.0 (16). Using the same method (i.e., measuring I450/I490), a pH of 7.0 ± 0.2 was obtained in the presence of 50 mM methylamine.

Ohkuma and Poole have measured intralysosomal pH in macrophages by placing a monolayer of cells in a modified fluorescence cuvette (14, 21). When measuring stable pH values, they used the shape of the fluorescein excitation profile, but for rapid time-dependent processes they measured changes in Iα55. They reported essentially equivalent results with the two methods. They proposed that weak bases, such as chloroquine or ammonia, raise lysosomal pH by diffusing across membranes as the uncharged species and binding protons within the acidic organelles.

Time and Concentration Dependence

Fig. 2 shows the effect of 10 μM monensin on the intensity of the fluorescence from F-α2M in endocytic vesicles. The intensity rapidly increases to a new stable value, indicating a rise in the intravesicle pH. Removal of monensin from the medium by extensive rinsing returns the fluorescence intensity to approximately the initial value. For all of the agents tested, stable intensity values were obtained within 1–2 min after addition. The increase in fluorescence caused by addition of amines was also reversible (not shown). The rapid rise in pH and the rapid reversibility are in general agreement with results obtained in measurements of changes in lysosomal pH (14, 21).

The concentration dependence of ΔI for hexylamine and the proton ionophore FCCP are shown in Fig. 3. Curves similar to that shown for hexylamine were obtained for all of the agents listed in Table 1 except FCCP and dansylcadaverine, and the concentrations which gave ΔI = 0.5 are listed. The electrogenic proton ionophore, FCCP, produced only a partial increase in ΔI even at high concentrations (Fig. 3 B). Monensin, an electroneutral ionophore, gave ΔI values near 1.0 at concentrations >15 μM. For monodansylcadaverine, I found ΔI = 0 for concentrations as high as 1 mM. This concentration is substab-
tially higher than the concentrations required to affect the behavior of several ligand-receptor systems (1, 7, 10, 13). A substantial rise in fluorescence excited at 490 nm was observed when 50 mM methylamine was added to cells in the presence of 1 mM monodansylcadaverine, indicating that dansyl fluorescence would not interfere with the detection of ΔI. Mono-dansylcadaverine (500 μM) also failed to raise lysosomal pH significantly in human fibroblasts (22) or in BALB/c 3T3 cells (not shown).

In the presence of 50 mM methylamine, the pH of endocytic vesicles was 7.0 ± 0.2 (3 experiments, 18 cells). If one takes this as the pH value when ΔI = 1.0 and used pH 5.0 for ΔI = 0 (16), then using Fig. 1, one can estimate that ΔI = 0.5 corresponds to a pH value near 6.2.

The increase in fluorescence intensity can be observed visually as shown in Fig. 4. Fig. 4A and B show the increased emission from endocytic vesicles upon addition of 50 mM methylamine after a 15-min incubation in F-α2M. Fig. 4C and D show that a similar increase can be observed after a 5-min incubation, indicating that the endocytic vesicles are acidic within 5 min or less. Almost no bright spots could be observed on these cells before addition of methylamine.

**pH Dependence of α2M and EGF Binding**

The pH dependence of α2M and EGF binding is shown in Fig. 5. The pH dependence of EGF binding has been described previously (7). As seen in Fig. 5, both ligands bind poorly...
We have developed a microspectrofluorometric method to measure the pH of endocytic vesicles which contain F-a2M. By observing the increase in F-a2M fluorescence almost immediately after formation, we found that the vesicles are capable of reaching pH 5 within 1 min or less. This rapid reacidification also indicates that the vesicles themselves contain a mechanism for regulating their internal pH. The measurements reported here can serve as a basis for determining whether it is reasonable to consider an increase in endocytic vesicle pH as the cause of the observed effects.

The agents listed in Table I have all been reported to affect one or more steps in the receptor-mediated endocytosis pathway. The results reported in the literature often seem contradictory, and it is difficult to make generalizations about the interpretations of the observations. Since entry into acidic vesicles is an early step, it is possible that raising the vesicle pH could be responsible for some or many of the observed effects. The measurements reported here can serve as a basis for determining whether it is reasonable to consider an increase in endocytic vesicle pH as the cause of the observed effects.

Many enveloped viruses require an acidic pH for fusion with cell membranes and entry of the nuclear capsid (23). Several weak bases have been reported to block infectivity, and it has been suggested that this might occur by raising lysosomal pH (13). The concentrations of weak base required are generally consistent with the concentrations required to raise endocytic vesicle or lysosomal pH. However, monodansylcadaverine (500 \( \mu \)M) blocks influenza virus infectivity at concentrations which do not raise vesicle pH. It has also been reported that monodansylcadaverine blocks endocytosis of vesicular stomatitis virus, and this inhibition was reported to occur by reducing the entry of virus into coated pits (23). This would be consistent with a mode of action for monodansylcadaverine other than raising pH.

"Nicked" diphtheria toxin can penetrate plasma membranes at pH 5.0 (25), and it has been shown that diphtheria toxin enters the same endocytic vesicles as a2M (26). Weak bases and monensin (12) are known to inhibit the toxicity of diphtheria toxin at approximately the concentrations which raise endocytic vesicle or lysosomal pH.

In the case of the enveloped viruses and diphtheria toxin, either the endocytic vesicles or lysosomes could be the site of cytoplasmic penetration. Since the endocytic vesicles are the first step on the pathway, they would seem a likely candidate for the site of penetration. Virus particles or toxin chains which did not escape from the endocytic vesicles would be delivered to lysosomes where they might penetrate into the cytoplasm before degradation.

Many of the ligands which enter cells by receptor-mediated endocytosis are delivered to lysosomes, and many of the receptors are recycled to the plasma membrane. Several investigators have described the effects of the agents listed in Table I on various steps in the endocytosis pathway. The goal of these previous studies has been to identify the steps in the pathway which are sensitive to these agents and, eventually, to understand the underlying basis for the sensitivity.

It is likely that at least some of the effects on endocytosis produced by the agents listed in Table I (except monodansylcadaverine) are due to an increase in endocytic vesicle pH. For example, it has been reported that ammonia blocks delivery of mannose-containing ligands to lysosomes in macrophages (4). Both monensin and chloroquine cause a loss of low density lipoprotein receptors from the cell surface, and this has been attributed to an inhibition of receptor recycling (9).

Monodansylcadaverine has been reported to block the endocytosis of a number of ligands, including epidermal growth factor (7), a2-M (24), 3,3',5-triiodo-2-thyronine (27), and vesicular stomatitis virus (24). It has also been reported to reduce the number of surface a2-M receptors (10). From our results, it is clear that at the concentrations used (20-500 \( \mu \)M), monodansylcadaverine is not acting as a weak base to raise endocytic vesicle or lysosomal pH. In several cases (1, 10, 13, 28), it has been suggested that dansylcadaverine produces effects similar to other weak bases. Either monodansylcadaverine and the weak bases produce similar effects by different mechanisms, or the effects of both are not related to the ability to raise the pH of acidic organelles.

There is growing evidence that exposure to an acidic environment is important for the processing of many ligands and receptors. Much of this evidence has been based on treatment of cells with agents which would be expected to raise the pH of acidic organelles. We have recently shown that the endocytic vesicles which are involved in uptake of many ligands are acidic. In this paper it has been shown that many of the agents which affect receptor-mediated endocytosis rapidly and reversibly raise endocytic vesicle pH at concentrations which affect endocytosis. These results are consistent with acidification of vesicles being an important processing step. It is important to realize, however, that these agents also may elicit effects at other sites, some of which are independent of their effects on pH.
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