Acidification of the Cytosol Inhibits Endocytosis from Coated Pits

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Abstract. Acidification of the cytosol of a number of different cell lines strongly reduced the endocytic uptake of transferrin and epidermal growth factor. The number of transferrin binding sites at the cell surface was not reduced in cells with acidified cytosol. Experiments with transferrin–horseradish peroxidase conjugates and a monoclonal anti-transferrin receptor antibody demonstrated that transferrin receptors were present in ~75% of the coated pits both in control cells and in cells with acidified cytosol. The cytosol efficiently blocks endocytosis of transferrin and EGF, whereas the uptake of the toxic plant protein ricin and of the membrane impermeant fluorescent dye, lucifer yellow, is only slightly reduced.

Materials and Methods

Materials

*[^14]C*dimethylloxazolidine 2, 4-dione (DMO) was obtained from New England Nuclear, Boston, MA. [*H]leucine, [*H]glucose, [*H]H2O, and Na [*H]I were from the Radiochemical Centre, Amersham, UK. Transferrin, horseradish peroxidase (HRP), pronase, EGF, 2-(N-morpholino)ethane sulfonic acid (MES), SPDP (5-[2-pyrrolidinyl]dimethylpentane-3,4-bisacy chloride), Hepes, Tris, nigericin, and valinomycin were obtained from Sigma Chemical Co., St. Louis, MO. Amiloride was a gift from Merck, Sharp & Dohme, Drammen, Norway. W7 and W5 were purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Transferrin was saturated with iron as described (9), and [*H]labeled ligands were prepared by the iodogen method (15).

Preparation of Conjugates for Electron Microscopy

Conjugates of ricin and transferrin with colloidal gold (particle size 5-10 nm, or 20 nm) were prepared by the method of Slot and Geuze (50), and the amount of protein necessary to stabilize the colloidal gold solution was determined by the method of Horisberger and Rønset (26).

Conjugates of ricin and transferrin with HRP were prepared by the SPDP method as previously described (56, 57). After the eluted fractions were filtered through a Sephacryl-200 column, they were analyzed by SDS-PAGE, and the monovalent conjugates (i.e., those containing one transferrin or ricin molecule and one HRP molecule) were selected for further experiments.

Cells. Hep 2-cells (human laryngeal carcinoma cells with HeLa markers) were obtained from Dr. P. Boquet, Institut Pasteur, Paris, France. A431 cells were obtained from the American Type Culture Collection, Rockville, MD.

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This solution were made in the same buffer and aliquots were taken and centrifuged in a Eppendorf centrifuge for 10 min. From the supernatant 200 μl was measured after incubation of the cells with 0.1 M lactose to test the specificity of conjugate binding. Similarly, in some experiments with the transferrin conjugates, preincubation at 0–4°C was performed with excess unlabeled transferrin (50 μg/ml). After the incubation with the conjugates, the cells were washed with buffer and further processed for electron microscopy as described below.

Ultrastuctural Immunocytochemical Detection of Transferrin Receptors

William 2 cells in monolayer cultures, with and without NH4Cl prewashing, were incubated in 0.14 M KCl, 20 mM Hepes, pH 7.2, for 60 min at room temperature. In experiments with HRP conjugates and peroxidase immunocytochemistry, the cells were then washed carefully with PBS and incubated with diaminobenzidine-H2O2 as previously described (56, 57). The cells were then scraped off the flask and centrifuged in buffer. Pellets were postfixed with Oso4, treated with 1% uranyl acetate in distilled water, embedded in Epon, cut at ~50 nm, and examined in a JEOL 100 CX electron microscope as previously described (56, 57). The frequency of coated pits at the cell surface was determined as reported elsewhere (31).

Results

To test if endocytosis of ligands that enter cells from coated pits is influenced by the pH in the cytosol, we studied the internalization of transferrin and EGF.

Both of these ligands have been shown to enter cells by endocytosis from coated pits (13, 14, 20, 21, 34). It should be noted, however, that a recent report suggests that EGF is also taken up from uncoated pits (24).

EGF stimulates internalization of the hormone–receptor complex which is then degraded (8), while the receptors for transferrin seem to be internalized constitutively, i.e., internalization occurs whether or not ligand is bound, and the receptor is then recycled to the cell surface (3).

We have used three different methods to acidify the cytosol. In the first method, which is best tolerated by the cells, we preincubate the cells with NH4Cl to load them with NH3, and then transfer the cells to medium without NH4Cl (4). Ammonium ions are in equilibrium with NH3, which is membrane permeant. Therefore, upon removal of extracellular NH4Cl, NH3 will rapidly diffuse out of the cells, while the protons are left behind. After such treatment the Na+/H+ exchanger is normally activated, and the cytosolic pH is rapidly regulated back to neutrality (36, 46). However, the Na+/H+ exchanger can be inhibited by amiloride or blocked if the cells are transferred to Na+-free buffer. Under such conditions, low intracellular pH can be maintained for a considerable period of time.

We have also acidified the cytosol by a method based on the ability of certain weak acids, such as acetic acid, to rapidly penetrate the cell membrane in its undissociated form.
In a first set of experiments we lowered the pH in the cytosol by NH$_4$Cl preloading and subsequent removal, and measured the effect on the endocytosis of $^{125}$I-labeled transferrin and EGF. As shown in Fig. 1, $A$ and $B$, the internalization of both ligands was strongly reduced after preincubation with 25 mM NH$_4$Cl, whereas preincubation with 10 mM NH$_4$Cl had little or no effect. The reduction of endocytosis was not due to the incubation with NH$_4$Cl as such. Thus, there was no effect on the uptake of EGF and transferrin when NH$_4$Cl was present throughout the experiment (data not shown).

In experiments with the fluorescent probe, BCECF (2', 7'-bis-[2-carboxyethyl]-5-[and -6]carboxyfluorescein, acetoxymethyl ester), we have earlier found that when Vero cells are preincubated at pH 7.0 with 25 mM NH$_4$Cl and then transferred to medium without NH$_4$Cl, the pH in the cytosol is reduced to at least pH 6.0, which represents the limit of detection with this method (45). To measure the internal pH under the conditions here used, we measured the distribution of the weak acid $[^{14}$C]DMO (12) after incubation of cells with NH$_4$Cl and subsequent transfer to buffer without NH$_4$Cl. The data presented in Fig. 2 show that the internal pH decreased linearly with the concentration of NH$_4$Cl present during the preincubation, indicating that the internal buffering capacity is essentially constant between pH 6 and 7. This has also been found in other systems (4). By comparing the data in Figs. 1 and 2 it appears that to reduce the endocytosis, the internal pH must be reduced to values below pH 6.5.

It should be noted that the concentration of NH$_4$Cl required during the preincubation varied somewhat (25-40 mM) from experiment to experiment. The reason for this is not known. It is therefore necessary in each experiment involving electron microscopy (see below) to measure the endocytic uptake of transferrin or EGF in a parallel experiment.

Although acidification of the cytosol inhibited the endocytic uptake of transferrin, it increased the total amount of transferrin associated with the cells (Fig. 1 $C$). Scatchard analysis of the binding data indicated that the increased binding is due to an increased number of transferrin receptors on the cell surface (Fig. 3). Thus, acidification of the cytosol after preincubation with NH$_4$Cl increased the number of transferrin molecules bound per Vero cell from 100,000 to 175,000. Maximal increase in binding was obtained 5-10 min after the transfer to NH$_4$Cl-free buffer (data not shown). The increase in the number of transferrin receptors was dependent on Ca$^{2+}$ in the medium and could be inhibited by the calmodulin antagonists trifluoperazine and W7. W5, an inactive analogue of W7, had no effect (data not shown). The effect of acidification of the cytosol on the amount of EGF associated with the cells was much less, but also in this case a slight increase was observed (Fig. 1 $D$).

The strong reduction in endocytosis occurring after incubation with 25-40 mM NH$_4$Cl was rapidly reversible when the internal pH was normalized. Thus, when cells that had been acidified by an NH$_4$Cl prepulse were incubated with transferrin in the presence of Na$^+$ to allow pH regulation by Na$^+$/H$^+$ exchange to occur, no reduction in endocytic uptake of transferrin was observed. In fact, the total amount of endocytosed transferrin was in that case higher than in con-
cated concentrations of acetic acid. ~25I-Transferrin (200 ng/ml, growing in 24-well disposable trays were incubated for 5 min at 37°C in Hepes medium (pH 7.0) with and without 25 mM NH4Cl. The medium was then removed and 0.14 M KCl, containing 2 mM CaCl2, 1 mM MgCl2, 1 mM amiloride, and 20 mM Hepes (pH 7.0) was added. After a 5-min incubation at 37°C, the cells were chilled to 0°C, increasing concentrations of ~25I-labeled transferrin (6,300 cpm/ng) were added in the absence and presence of 100 μg/ml unlabeled transferrin, and the cells were incubated for 2 h at 0°C. They were then washed three times in cold PBS, dissolved in 0.1 M KOH, and the radioactivity associated with the cells was measured. The amount of transferrin bound per cell was calculated, the amount of transferrin bound in the presence of excess unlabeled transferrin was subtracted, and the data were plotted according to Scatchard (47). r, number of transferrin molecules bound per cell; c, molar concentration of free transferrin.

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We also studied the effect of NH4Cl prepulsing on the endocytic uptake of ~25I-labeled ricin in Vero cells, Hep 2 cells, MCF7 cells, and A431 cells. In contrast to the results obtained with transferrin and EGF, endocytosis of ricin was only slightly reduced when the cytosol was acidified (Table I). This was confirmed by electron microscopic studies shown below. Also, there was no large effect on the endocytosis of the fluorescent fluid phase marker, lucifer yellow, when the cytosol was acidified by prepulsing of the cells with NH4Cl (Table I). In contrast, the uptake of lucifer yellow was strongly inhibited in ATP-depleted cells or when the temperature was reduced to 4°C (data not shown).

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Figure 3. Equilibrium binding of ~25I-labeled transferrin to Vero cells. Vero cells growing in 24-well disposable trays were incubated for 30 min at 37°C in Hepes medium (pH 7.0) with and without 25 mM NH4Cl. The medium was then removed and 0.14 M KCl, containing 2 mM CaCl2, 1 mM MgCl2, 1 mM amiloride, and 20 mM Hepes (pH 7.0) was added. After a 5-min incubation at 37°C, the cells were chilled to 0°C, increasing concentrations of ~25I-labeled transferrin (6,300 cpm/ng) were added in the absence and presence of 100 μg/ml unlabeled transferrin, and the cells were incubated for 2 h at 0°C. They were then washed three times in cold PBS, dissolved in 0.1 M KOH, and the radioactivity associated with the cells was measured. The amount of transferrin bound per cell was calculated, the amount of transferrin bound in the presence of excess unlabeled transferrin was subtracted, and the data were plotted according to Scatchard (47). r, number of transferrin molecules bound per cell; c, molar concentration of free transferrin.

Figure 4. Ability of acetic acid to decrease endocytosis of transferrin and to increase the total amount of bound transferrin. Vero cells growing in 24-well disposable trays were incubated for 5 min at 37°C in Hepes medium (0.2 ml, pH 5.0) with and without the indicated concentrations of acetic acid. ~25I-Transferrin (200 ng/ml, 38,600 cpm/ng) was then added and, after a 10-min incubation, surface and endocytosed transferrin was measured as described in Materials and Methods.

Table I. Effect of Acidification of the Cytosol on the Ability of Vero, Hep 2, MCF 7, and A431 Cells to Endocytose ~25I-Ricin and Lucifer Yellow

| Treatment* | Endocytic uptake of ~25I-Ricin| Endocytic uptake of lucifer yellow|
|-----------|-----------------------------|----------------------------------|
| Prepulsing with 25 mM NH4Cl (Vero) | 82 ± 15 | 84 ± 12 |
| Prepulsing with 25 mM NH4Cl (Hep 2) | 69 ± 17 | |
| Prepulsing with 25 mM NH4Cl (MCF 7) | 65 ± 10 | |
| Prepulsing with 25 mM NH4Cl (A431) | 79 ± 10 | |
| 5 mM acetic acid in Hepes medium, pH 5.0 (Vero) | 78 ± 8 | |
| 0.14 M KCl, nigericin, valinomycin, pH 6.0 (Vero) | 30 ± 14 | 25 ± 9 |

* Cells growing in monolayer cultures were treated as described in the legends to Figs. 1, 4, and 5.
† The uptake of lucifer yellow was measured fluorometrically as described in Materials and Methods.
‡ The uptake of lucifer yellow was measured fluorometrically as described in Materials and Methods.

Ability of Acetic Acid to Inhibit Endocytosis of Transferrin

In experiments where we acidified the cytosol of Vero cells by incubation with acetic acid, we obtained essentially the same results as when the acidification was carried out by NH4Cl prepulsing. Thus, the endocytosis of transferrin was strongly reduced (Fig. 4 A), and the total amount of transferrin associated with cells was increased (Fig. 4 B). The extent of the increase in binding varied between different experiments. Furthermore, there was only a slight reduction in uptake of ~25I-labeled ricin (Table I).

Regulation of Cytosolic pH by Incubation of Cells with Nigericin in Isotonic KCI

When cells are incubated in isotonic KCl containing nigericin, the pH in the cytosol will be the same as that in the surrounding medium (53). Valinomycin was added to ensure a high K+ permeability. It is shown in Fig. 5, that also when the pH in the cytosol was lowered by this method, the endocytosis of transferrin and EGF was inhibited in all of the four cell lines tested. Consistent with the results described above, the pH must be reduced below pH 6.5 to inhibit the endocytosis efficiently.

It should be noted that after incubation with isotonic KCl in the presence of valinomycin and nigericin and then transfer to normal medium, the endocytosis was not restored as fast as when the cytosol had been acidified by the two other methods. This could be due to incomplete removal of the ionophores. Furthermore, this method also reduced the endocytic uptake of cell-bound ricin and of lucifer yellow (Table I) although not to the same extent as the uptake of transferrin and EGF (Fig. 5). Apparently, the method demonstrated in Fig. 5 has a general toxic effect on the cells (see below). Altogether it may be concluded that acidification of the cytosol by three different methods had essentially the same effect on the uptake of the markers here studied.

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To test if acidification of the cytosol has a general toxic effect on the cells, we transferred the cells back to normal medium to allow normalization of the internal pH to occur. In a first set of experiments, we added [3H]leucine to the cells 15 min later and measured their ability to incorporate the labeled amino acid during 10 min. The data in Table II show that in cells that had been treated with nigericin, valinomycin, and KC1, the rate of protein synthesis was strongly reduced, whereas in cells that had been acidified by the two other methods, the rate of protein synthesis was only reduced to approximately half the control value.

When we measured the rate of [3H]leucine incorporation 12 h later, the rate was close to that in the control cells. Clearly, acidification of the cytosol had only a transient effect on protein synthesis and did not affect cell survival, with the possible exception of acidification by treatment with nigericin, valinomycin, and KCl.

When the ATP level of cells is reduced to <10% of the control level, endocytosis in general is blocked (51) although exceptions may exist (19, 30). As shown in Table III, the ATP level was not strongly reduced by any of the three methods here used to acidify the cytosol. Altogether, it may be concluded that acidification of the cytosol by NH4Cl prepulsing or by treatment with acetic acid, does not induce any permanent damage to the cells, while the method of pH-clamping with KCI and ionophores may have a slight toxic effect.

Electron Microscopical Observations

To visualize the effect of low pH in the cytosol on endocytosis, we carried out ultrastructural studies. In the following experiments we used coated pits as structural markers ranging from a coated portion of the plasma membrane to a coated vesicular profile located less than three profile diameters away from the plasma membrane (see also reference 31). Both in Vero and Hep-2 cells coated pits were present at the cell surface at approximately the same frequency in control incubations and in experiments where the cytosol was acidified (see Figs. 6 and 7, and Table IV). Also smooth pits or invaginations were observed under both control and experimental conditions. Their size varied considerably, from ~50-70 nm (being the most frequent diameter) and up to ~250 nm (Fig. 6). Serial section analysis revealed that many endosome-like vacuolar structures in the peripheral cytoplasm were in fact surface connected, thus representing pits (Fig. 6). The frequency of the various smooth pits was, however, difficult to determine. In the first place, the number (particularly that of the small ones) varied considerably from region to region of the cells. Furthermore, it is meaningless to discriminate shallow pits from simple invaginations due to irregular cell surface geometry. However, the general impression obtained was that there are from one to eight times as many smooth pits (all sizes included) than coated pits (see also reference 31). The acidified cells showed a somewhat altered morphology, most notably in the Golgi complexes, which exhibited a marked swelling and vesiculation (not shown).

We have earlier shown that when Ri-Au or Ri-HRP conjugates are added to cells at 0-4°C, ricin binding sites are...
Figure 6. Electron micrographs of Vero cells incubated with various ricin conjugates. a shows a portion of a cell incubated with monovalent RI-HRP conjugate at 4°C. The conjugate binds evenly to the cell surface and also labels coated pits (Cp) and smooth pits (Sp). b represents
evenly distributed all over the cell surface (55, 57). Control experiments showed that the binding of these markers was specific. Thus, preincubation with excess of unlabeled ricin or with 0.1 M lactose prevented the binding of these conjugates to the cells (Fig. 6 b, and data not shown).

The data in Fig. 6 show that both conjugates were found in practically all coated pits as well as in smooth pits of various sizes, and no differences could be established between control and acidified cells.

At 37°C the amount of surface labeling decreased due to internalization of the conjugates, but it could still be observed in coated and smooth pits (Fig. 6). The conjugates were also present in endosomal elements (Fig. 6), approximately to the same extent both in control and acidified cells. The ultrastructural observations are therefore in agreement with the biochemical data.

In our first attempts to visualize binding and uptake of transferrin, a conjugate of transferrin and gold (Tf-Au) was used. The conjugate was found at the cell surface both as single particles and as aggregates of various sizes. The conjugate showed a preferential localization to coated pits and their surroundings, while smooth pits were labeled only on occasions. This pattern was found in both control and acidified cells. However, since preincubation with unlabeled transferrin did not completely prevent surface labeling, particularly by the larger aggregates, we considered the Tf-Au conjugate as an unreliable marker of the surface distribution of transferrin receptors.

To circumvent the problem of unspecific binding we carried out further experiments with a monovalent Tf-HRP conjugate. The binding of this conjugate could be prevented by preincubation with unlabeled transferrin (data not shown). In agreement with previous reports on the transferrin receptor distribution (23, 25) the HRP reaction product was mainly found close to or within coated pits, leaving the smaller smooth pits largely unlabeled (Fig. 7). The largest smooth pits were never labeled. The frequency of Tf-HRP labeled coated pits in nonacidified and acidified Hep-2 cells was approximately the same (Table V).

At 37°C TF-HRP was frequently observed in endosomes in control cells (Fig. 7). In contrast, almost no endosomal structures were labeled in the acidified cells. This was as expected since the parallel Hep-2 culture flasks (same series of experiments) revealed a decrease in endocytosed [125I]transferrin from 60% of the total bound transferrin (10 min after addition of transferrin) in control cells to ~5% in acidified cells (not shown).

In another set of experiments we visualized transferrin receptors by using immunoperoxidase cytochemistry and a monoclonal mouse anti-human transferrin receptor antibody. Because of the specificity of the antibody in these experiments we used exclusively Hep 2 cells, which are of human origin. This approach gave approximately the same results as described above for the Tf-HRP conjugate (Fig. 7 and Table V).

Immunocytochemical reaction product clearly was most distinct within or close to coated pits (Fig. 7). Smaller smooth pits were sometimes weakly labeled, but no reaction product was observed in distinct, large pits. The labeling was almost completely prevented when the primary antibody was omitted from the incubation protocol. It should also be noted that similar experiments carried out with other human cell types (39) gave identical results (data not shown).

From the ultrastructural studies the following conclusions can be made. (a) Molecules binding ricin are distributed with a high frequency all over the cell surface including coated pits and smooth pits and invaginations of various sizes, whereas transferrin receptors are preferentially localized to coated pit regions. (b) Coated pits are present in acidified cells with approximately the same or at a slightly higher frequency than in control cells. (c) Also in acidified cells the coated pits contain transferrin receptors.

**Toxic Effect of Ricin Endocytosed at Normal and Acidic Internal pH**

Ricin is a toxic protein that exerts its action on the ribosomes (38). We have earlier shown that endocytic uptake of ricin is involved in the mechanism of entry into the cytosol (43, 45). Since the endocytic uptake of ricin is not strongly reduced by acidification of the cytosol, we decided to study if ricin endocytosed under such conditions can intoxicate cells. In these experiments we used the NH4Cl prepulse method to acidify the cytosol. Toxin was added and endocytosis was allowed to proceed for 15 min. Then the cells were transferred to normal medium containing neutralizing amounts of antitoxin to inactivate any toxin present at the cell surface. After incubation overnight the ability of the cells to incorporate [3H]leucine was measured. The data in Fig. 8 show that protein synthesis in cells treated in this way was inhibited to the same extent as in control cells, where the endocytic uptake of the toxin occurred at normal internal pH.

**Discussion**

The major new observation in the present study is that acidification of the cytosol to values below pH 6.5, selectively inhibits endocytosis of transferrin and EGF, while it only slightly reduces the endocytic uptake of ricin and lucifer yellow. This conclusion is based on three different methods of acidification and the endocytosis is evaluated both by biochemical and morphological methods.

To explain why acidification inhibits endocytosis at least three possibilities must be considered: (a) At low intracellular...
Figure 7. Electron micrographs of Vero and Hep-2 cells incubated with Tf-HRP or anti-transferrin receptor antibody. a shows a Vero cell incubated at 4°C with monovalent Tf-HRP. b represents a Hep-2 cell incubated with Tf-HRP for 10 min at 37°C. c and d are Hep-2 cells acidified by prepulsing with 40 mM NH₄Cl and then incubated with Tf-HRP for 10 min at 37°C. In all cases Tf-HRP binds preferentially to coated pits (Cp). Smooth pits (Sp) are unlabeled. In b is also shown a Tf-HRP containing endosome (En). e–l shows Hep-2 cells incubated at 4°C with a mouse monoclonal anti-human transferrin receptor antibody, and thereafter with peroxidase-conjugated goat anti-mouse IgG. e–h represent nonacidified cells, and i–l represent cells acidified by prepulsing with 40 mM NH₄Cl. Transferrin receptors are in both cases localized close to or within coated pits (Cp). Smooth pits (Sp) are unlabeled. Bars, 0.25 μm.
lar pH, certain receptors (e.g., transferrin and EGF receptors) could be selectively prevented from gaining access to coated pits and subsequent internalization. (b) At low internal pH the formation of coated pits at the cell surface could be inhibited as in experiments with K⁺-depletion after hypotonic shock (28–31). (c) Low internal pH may inhibit coated pits from pinching off from the cell surface.

The first two of these possibilities are contradicted by our ultrastructural observations. Thus, coated pits were present at the cell surface of acidified cells in approximately the same number as in control cells, and in both cases the majority of the coated pits contained transferrin receptors. Therefore, our results are in favor of the third possibility, that acidification prevents pinching off of coated pits.

It remains uncertain how ricin and lucifer yellow were internalized in acidified cells. One possibility is that these molecules were taken up by a population of newly formed coated pits and vesicles that were not immobilized at the cell surface during acidification in contrast to those containing transferrin receptors. However, our findings are also compatible with the possibility that an alternative (uncoated) endocytic pathway is responsible for the uptake of most ricin and lucifer yellow. A similar conclusion was recently reached by Moya et al. (37) and Madshus et al. (31) in studies of internalization of transferrin and ricin in K⁺-depleted cells.

The crucial and still unanswered question now is: what is the structural equivalent to such an alternative endocytic pathway? Obviously, one must look for surface pits containing binding sites for ricin, but not for transferrin. We found that the smooth pits, in particular the larger ones, represent such structures. At least four recent electron microscopic studies using ferritin or gold probes have suggested that smooth surface pits are involved in endocytosis.

Thus, Huet et al. (27) found that IgG-ferritin binding to the major histocompatibility surface antigens of cultured human fibroblasts was localized to smooth pits rather than coated pits and that the ligand was subsequently internalized. Furthermore, Montesano et al. (35) found that tetanus toxin and cholera toxin bound to smooth pits rather than to coated pits and were subsequently internalized by smooth vesicles in cultured liver cells. Hopkins et al. (24) found that while transferrin was taken up via coated pits and vesicles in cultured epithelial A431 cells, EGF induced formation of smooth pits which then internalized the ligand. Furthermore, Ghitescu et al. (18) found that smooth pits and vesicles were involved in receptor-mediated transcytosis of albumin-Au in capillary endothelial cells.

However, criticisms against an endocytic role of the small, smooth surface pits have been raised in studies analyzing thin serial sections of endothelial cells (5, 16). These studies showed that practically all structures believed to be endothelial vesicles were in fact surface connected and therefore probably not involved in endocytosis. Also, that a given small, smooth surface pit contains a certain ligand does not necessarily imply that it represents a forming endocytic vesicle. Previous studies have shown that recycling vesicles being in the process of exocytosis may have the same size and appearance (54). It should also be noted that the appearance of secretory vesicles (the constitutive pathway) coming from the Golgi complex is largely unknown, and some small, smooth pits at the cell surface could therefore represent secretory vesicles. Altogether, it appears that the small smooth pits at the cell surface may very well represent a heterogeneous population of structures with various, largely non-endocytic, but hitherto unknown functions.

The larger smooth pits (Fig. 6, f–l, and n) at the cell surface represent an alternative to the small, smooth pits as can-

| Table IV. Quantitative Data on the Number of Coated Pits at the Cell Surface |
|------------------|------------------|------------------|
| Cell type | Experiment | Coated pits | Amount of surface analyzed |
| | | No./mm | mm |
| Vero | Control | 36 | 2.6 |
| | Acidified (NH₄Cl prepulsing) | 58 | 3.1 |
| Hep-2 | Control | 52 | 1.7 |
| | Acidified (NH₄Cl prepulsing) | 59 | 1.5 |

Table V. Frequency of Coated Pits in Hep-2 Cells Binding Monovalent Tf-HRP and Mouse Monoclonal Anti-Human Transferrin Receptor Antibody (Anti-Tf-R) |

| | Nonacidified cells | Acidified cells (NH₄Cl prepulsing) |
|---|------------------|------------------|
| Tf-HRP | 62% (n = 65) | 70% (n = 33) |
| Anti-Tf-R | 81% (n = 68) | 75% (n = 32) |
didates for structures involved in endocytosis of ricin (see also reference 55). Although it is impossible at present to prove their role in endocytosis, it is obvious that even a relatively few such structures would engulf sufficient material to quantitatively be of great importance in endocytosis. However, further studies are still necessary to prove or disprove this concept.

Schlossman et al. (49) found that both spontaneous and ATP-dependent release of clathrin from coated vesicles were inhibited at low pH. However, as shown here, inhibition of endocytosis at low pH is not due to accumulation of coated vesicles in the cytosol. Rather low pH in the cytosol appears to prevent coated pits from pinching off and forming coated vesicles.

When the cytosol was acidified we found a higher amount of transferrin bound at the cell surface than in control cells. This increase is probably not due to inhibition of endocytosis, since increased binding was already observed at cytosolic pH values that were higher than those required to block endocytosis. Also, at 37°C the total amount of transferrin associated with cells having low cytosolic pH was higher than in control cells incubated with transferrin at 37°C. Recently, redistribution of internal transferrin receptors to the cell surface has been shown to occur upon addition to cells of serum, insulin, insulin-like growth factors, EGF, and platelet-derived growth factor (10, 11, 58). Similarly to the results described in the present paper, Ca2+ in the medium was required for the serum-induced redistribution. It has been suggested that calmodulin or another Ca2+-dependent regulatory protein is involved in an exocytic reaction. Shetler et al. (48) found that trifluoperazine inhibits insulin-induced exocytosis of glucose transporters. Furthermore, both trifluoperazine and W7 inhibit secretion in Paramecium (17). Recently, Adelsberg and Al-Awqati (1) showed that internal low pH induces exocytosis of proton pumps in the turtle bladder epithelium by a Ca2+-dependent mechanism, and it is possible that the increased number of transferrin receptors at the cell surface is due to a related process. Our finding that trifluoperazine and W7 prevent the acidification-induced increase in transferrin binding capacity is in agreement with this suggestion.

In conclusion, we have shown that acidification of the cytosol selectively inhibits the internalization of certain molecules by inhibiting pinching off of coated pits, whereas the uptake of other molecules was only slightly reduced. Our data are compatible with the notion that two endocytic pathways may operate in parallel, and that only one of them, the coated pit pathway, is inhibited at low intracellular pH.

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