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Inhibition of Endocytosis From Coated Pits by Acidification of the Cytosol

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Binding and endocytosis of the ligands transferrin, epidermal growth factor (EGF), and ricin were measured in a number of different cell lines after treatment of cells with compounds that react with SH-groups and under conditions where the cytosolic pH was lowered. N-ethylmalemide and diamide irreversibly inhibited endocytosis of all ligands tested, whereas low pH in the cytosol strongly inhibited endocytosis of transferrin and EGF. Data obtained by electron microscopy indicated that the formation of coated vesicles from coated pits is inhibited in acidified cells. Entry of ricin was much less affected, and ricin endocytosed under these conditions was able to intoxicate the cells. At low pH in the cytosol there was a calcium-dependent increase in the number of transferrin receptors at the cell surface. The increase was even larger in the presence of the calcium ionophore A23187, whereas it was completely blocked by the calmodulin antagonists trifluoperazine and W7. The results show that endocytosis from coated pits can be inhibited in a reversible way by acidification of the cytosol and they suggest that a second pathway of endocytosis exists, possibly involving formation of vesicles from uncoated areas of the membrane.

Key words: endocytosis, coated pits, cytosol, transferrin, epidermal growth factor

During recent years there has been an ongoing discussion as to whether endocytosis occurs exclusively by the coated pit/coated vesicle pathway or whether surface-bound ligands and fluid phase markers can enter cells by formation of vesicles from uncoated areas of the membrane. Results obtained by several investigators suggest that endocytosis of surface-bound ligands occurs not only from coated areas, but also from noncoated areas of the membrane. Thus, it was recently shown by Moya et al [1] that endocytosis of the toxic lectin ricin continued when the coated pits had been removed by potassium depletion of the cells. Also, infection of cells with human rhinovirus type 2, which requires passage through a low pH compartment for entry into the cytosol, occurs normally in potassium-depleted cells [2], suggesting

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that transfer of this virus to a low pH compartment occurs even in the absence of coated pits. Furthermore, Morris et al [3,4] have found that pseudomonas toxin and diphtheria toxin are internalized even when endocytosis of the toxins from coated pits is inhibited by addition of amines, and it has been suggested that smooth pits are involved in the uptake of cholera toxin and tetanus toxin [5].

Removal of coated pits by potassium depletion of the cytosol has a number of other effects on the cells, like inhibition of chloride transport, removal of the membrane potential, and cell shrinkage [6]. Alternative methods to inhibit endocytosis are therefore required. In the present work we have studied the effect of a number of treatments on the rate of endocytosis. For this purpose we have measured the endocytic uptake of transferrin and EGF, which by a number of groups have been shown to be endocytosed from coated pits [7–10], as well as the uptake of the lectin ricin, which binds to terminal galactose on both glycolipids and glycoproteins, and which is therefore likely to be endocytosed by all different mechanisms operating (for review, see [11]).

MATERIALS AND METHODS

Materials

[^{14}C]Dimethyloxazolidine 2,4-dione (DMO) was obtained from New England Nuclear (Boston, MA). [^{3}H]Leucine, [^{14}C]sucrose, [^{3}H]H_2O, and Na ^{125}I were from the Radiochemical Centre (Amersham, UK). Transferrin, pronase, epidermal growth factor, 2-(N-morpholino) ethane sulfonic acid (MES), Hepes, Tris, trifluoperazine, nigericin, and valinomycin were obtained from Sigma Chemical Co. (St. Louis, MO). W7 and W5 were purchased from Seikagaku Kogyo Co., LTD (Tokyo, Japan). Amiloride was a gift from Merck, Sharp & Dohme (Drammen, Norway). Transferrin was saturated with iron as described [12], and ^{125}I-labeled ligands were prepared by the iodogen method [13].

Conjugates of ricin and colloidal gold (particle size 5 nm) were prepared by the method of Slot and Geuze [14] and the amount of protein necessary to stabilize the colloidal gold solution was determined by the method of Horisberger and Rosset [15].

Cells

Hep-2 cells (human laryngeal carcinoma cells with HeLa markers) were obtained from Dr. P. Boquet (Institute Pasteur, Paris, France). A431 cells were obtained from the American Tissue Type Collection (Rockville, MD). HeLA S3, Vero, and MCF-7 cells are strains that have been growing in this laboratory for years. All cell lines used were maintained as monolayer cultures in minimal essential medium complemented with penicillin, streptomycin, and 10% (v/v) fetal calf serum and in an atmosphere containing 5% CO_2. The day before use the cells were seeded out into 24-well disposable trays, Petri dishes, or into T-25 flasks as indicated in legends to figures.

Measurement of Receptor-mediated Endocytosis of ^{125}I-Transferrin, ^{125}I-EGF, and ^{125}I-Ricin

The amount of surface-bound and internalized transferrin was measured as described by Ciechanover et al [16]. Briefly, cells were incubated with ^{125}I-transferrin at 37°C for the indicated periods of time, washed three times with ice-cold PBS, and

then treated for 1 hr at 0°C with 0.3 ml serum-free medium containing 0.3% (w/v) pronase. Then the cells and the medium were transferred to Eppendorf tubes and centrifuged for 2 min, and the radioactivity in the pellet and in the supernatant was measured.

Endocytosis of ^{125}I-EGF was measured as described by Haigler et al [17]. For this purpose, cells incubated with ^{125}I-EGF were washed five times with ice-cold PBS, and then incubated for 6 min in a solution containing 0.5 M NaCl, 0.2 M acetic acid, pH 2.5, to release surface-bound EGF. The cells were subsequently washed once in the same buffer, then dissolved, and finally the cell-associated radioactivity was measured.

Endocytosis of ^{125}I-labeled ricin was measured as the amount of toxin that could not be removed from the cells with lactose, as previously described [18].

Measurement of pH in the Cytosol

The cytosolic pH was determined from the distribution of the weak acid [^{14}C]DMO as described by Deutsch et al [19]. Cells were incubated with [^{14}C]DMO (5 μCi/ml) for 5 min at 37°C, then washed five times with ice-cold PBS, and the cell-associated radioactivity was measured. The volume of extracellular liquid associated with the cells after washing five times was measured after incubation of the cells with [^{14}C]sucrose (5 μCi/ml). The extracellular space corresponded to 7% of the cell volume. The total volume of the cells in a well was calculated from the amount of [^{3}H]H_2O associated with cells after incubation with 1 μCi/ml [^{3}H]H_2O for 18 hr.

Electron Microscopical Studies

Cells growing in monolayers in T25 flasks were washed with Hepes buffer and incubated with a ricin-gold conjugate (Ri-Au) in Hepes medium for 30 min at 37°C, as previously described [20,21]. Following incubation, the cultures were washed and fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 60 min at room temperature. The fixed cells were scraped off the flasks and centrifuged in buffer for 25 min at 1,600g. Pellets were postfixed with 2% OsO_4 in cacodylate buffer, pH 7.2, for 1 hr at 4°C and treated with 1% uranyl acetate in distilled water for 1 hr at 20°C. Cell pellets were thereafter embedded in Epon, cut at approximately 50 nm, and examined in a JEOL 100 CX electron microscope.

The frequency of coated pits was determined by counting any coated pit located at the plasma membrane and less than three profile diameters away from the plasma membrane.

RESULTS AND DISCUSSION

It is well established that transferrin binds to surface receptors that cluster in coated pits. Ricin, on the other hand, binds diffusely to the cell surface. In fact, when Vero cells are incubated with a conjugate of ricin-Au, the conjugate can be seen both in coated pits and in uncoated invaginations of the membrane. This is expected because of the wide variety of molecules that carry terminal galactose and which can therefore serve as binding sites for ricin (Fig. 1).

It has been reported earlier that compounds that modify SH-groups have an inhibitory effect on endocytosis in macrophages [22]. We therefore studied the effect of such compounds on the endocytosis of transferrin and ricin in Vero cells. The data

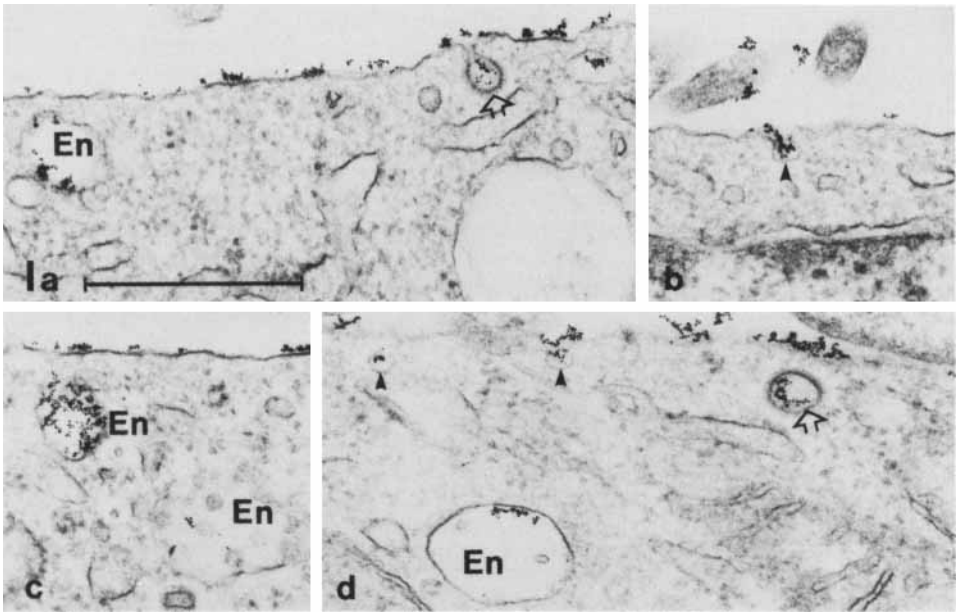

Fig. 1. Surface binding and endocytosis of Ri-Au in Vero cells. Incubation with Ri-Au for 30 min at 37°C. Ri-AU is seen scattered on the cell surface, in coated pits (open arrows) and smooth pits (arrowheads) as well as in endosomes (En). **a–d** ×70,000.

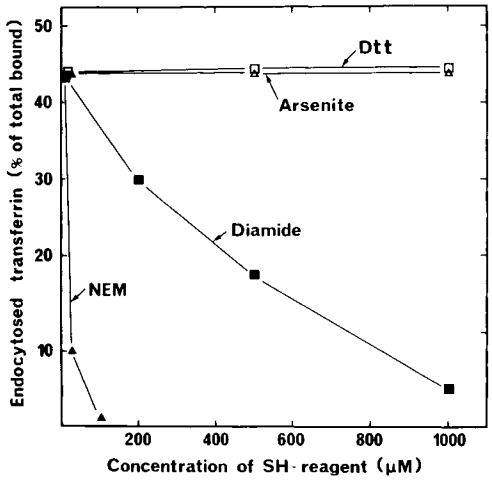

Fig. 2. Ability of SH-reagents to inhibit endocytosis of transferrin. Vero cells growing in 24-well disposable trays were incubated for 10 min at 37°C in Hepes medium (pH 7.2) with and without N-ethylmalemide (NEM), arsenite, diamide, and dithiothreitol (Dtt). ^{125}I-transferrin (200 ng/ml, 38,600 cpm/ng) was then added and, after a 10-min incubation, surface bound and endocytosed transferrin was measured as described in Materials and Methods.

in Figure 2 show that N-ethylmalemide and diamide in submillimolar concentrations strongly inhibited the endocytosis of transferrin. There was no effect of arsenite at similar concentrations, but at a higher concentration (5 mM), arsenite also reduced endocytosis (not demonstrated). On the other hand, dithiotreithol did not have any effect. Also the endocytic uptake of ricin was strongly reduced by N-ethylmalemide and diamide (data not shown) and sulfhydryl reagents are therefore not suitable to study differences in the uptake mechanisms of the two ligands.

We then tested whether or not acidification of the cytosol has different effects on the endocytosis of different ligands. For this purpose, Vero cells, Hep2 cells, and A431 cells were acidified by prepulsing with NH_4Cl. During incubation of the cells with NH_4Cl, presumably both NH_3 and NH_4^+ enter the cytosol from the surrounding medium, but the cells under this incubation are able to regulate the cytosolic pH by using the Na^+/H^+ exchanger. Upon subsequent transfer to medium without NH_4Cl, NH_4^+ in the cytosol will dissociate to NH_3 and H^+, and the membrane permeant NH_3 will then diffuse out of the cells and leave the protons behind [21]. To prevent normalization of the internal pH by the Na^+/H^+ exchanger, the experiment was either carried out in Na^+-free buffer or in the presence of amiloride. As shown in Figure 3, endocytosis of transferrin and EGF was strongly reduced under these conditions.

The extent of acidification obtained was calculated from the distribution of the weak radioactive acid [^{14}C]DMO. We found that preincubation of Vero cells with 10 mM NH_4Cl and subsequent removal of the amine reduced the pH from 6.9 to 6.5, whereas preincubation with 25 mM NH_4Cl reduced the internal pH to approximately 6.0. The data therefore indicate that to reduce the endocytosis significantly, the internal pH must be below pH 6.5. It should be noted that the concentration of NH_4Cl required to give a certain acidification varied to some extent from one day to another.

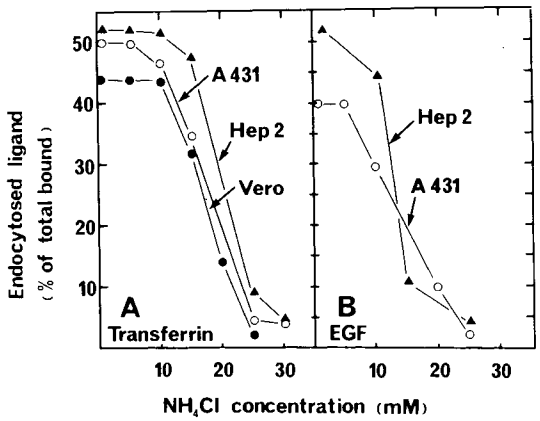

Fig. 3. Effect of acidification of the cytosol by NH_4Cl prepulsing on endocytic uptake of transferrin and EGF. The indicated cell lines growing in 24-well disposable trays **(A)** or in Petri dishes (diameter 35 mm) **(B)** were incubated for 30 min at 37°C in Hepes medium (pH 7.0) with and without the indicated concentrations of NH_4Cl. The medium was then removed, and 0.2 ml **(A)** or 0.5 ml **(B)** 0.14 M KCl containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride, and 20 mM Hepes (pH 7.0) was added. After an additional 5-min incubation, ^{125}I-labeled transferrin (200 ng/ml, 38,600 cpm/ng) **(A)** and EGF (7 ng/ml, 15,000 cpm/ng) **(B)** were added, and endocytosed transferrin and EGF were measured after 10 min or 15 min, respectively.

Electron microscopical studies showed that there was a similar number of coated pits in cells with acidified cytosol and in control cells (not shown). Furthermore, in experiments with a HRP-labeled second antibody against an antitransferrin receptor antibody, it was demonstrated that the coated pits in acidified cells contained transferrin receptors. The data therefore suggest that the reduced endocytosis is due to inhibition of formation of coated vesicles from coated pits.

When we measured endocytosis of ^{125}I-ricin in acidified cells, we found that it was only reduced to 55–89% of that in control cells. This was confirmed by electron microscopy. Furthermore, ricin endocytosed under conditions where uptake of transferrin and EGF was inhibited intoxicated cells to about the same extent as in control cells. This suggests that the acidification-resistant endocytosis is also important for the intoxication obtained with ricin under normal conditions.

To further investigate whether it is the acidification as such that is responsible for the inhibition of endocytosis from coated pits, we also acidified the cytosol by two other methods. In one case we incubated cells in a medium with pH 5.0 and added low concentrations of acetic acid, which can diffuse through the cell membrane and acidify the interior of the cell. Also in this case the endocytosis of transferrin and EGF was blocked, whereas there was little effect on the entry of ^{125}I-ricin.

The third method applied involves incubation of cells in a buffer containing the ionophores nigericin and valinomycin and isotonic concentrations of KCl. Owing to the ability of nigericin to exchange K^+ for H^+, the pH in the cytosol will become the same as that in the surrounding medium when the concentration of K^+ is the same at the two sides of the membrane. As shown in Figure 4, the endocytosis of transferrin and EGF was also blocked under these conditions, and the results confirm that strong inhibition of endocytosis is only obtained when the pH is below 6.5.

Earlier studies have indicated that cells have an internal pool of transferrin receptors and that various factors such as serum [24], insulin [25], and SH reagents [22] can induce a redistribution of these receptors resulting in an increased number at the cell surface. Furthermore, such a redistribution has been shown to be dependent on calcium in the medium. We noted that when cells were acidified by NH_4Cl deprivation or by addition of acetic acid more transferrin was bound than to control cells (Fig. 5). Scatchard analysis [26] showed that the increased binding was due to an increased number of receptors on the cell surface and not due to an increased affinity for transferrin. The increase was found to occur at pH values higher than those required to inhibit endocytosis, suggesting that the increase is due to an increased transport of receptors to the cell surface rather than to inhibition of internalization. Adelsberg and Al-Awqati [27] have found that low cytosolic pH can induce exocytosis of H^+ pumps, possibly by a related mechanism.

The data in Figure 5 also show that the increase in transferrin receptors at the cell surface could be abolished by removal of calcium from the medium, and that it was increased when the Ca^{2+} ionophore A23187 was added. It should be noted that addition of A23187 (10 μM) to Vero cells lowered the cytosolic pH value with about 0.07 pH units (not shown). However, this cannot be the reason for the observed effect since the absolute increase in receptor number was larger than that obtained at the highest NH_4Cl concentrations in the absence of the ionophore.

It is well established that the insulin-stimulated exocytosis of glucose transporters can be inhibited by trifluoperazine [28], and it has been suggested that calmodulin or another calcium-binding protein is involved in exocytosis. As shown in Figure 5,

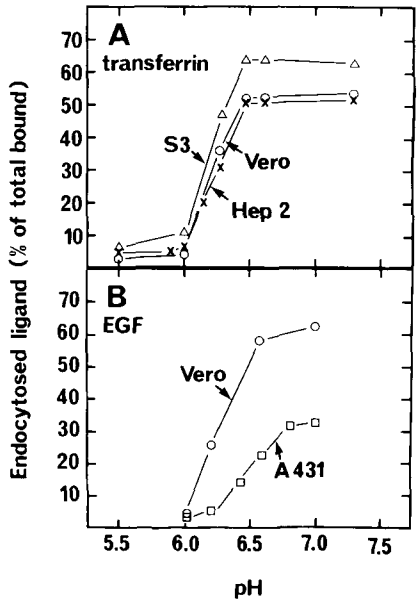

Fig. 4. Effect of pH-equilibration with nigericin and isotonic KCl on endocytic uptake of transferrin and EGF. Cells were growing in 24-well disposable trays to measure endocytic uptake of transferrin **(A)** and in Petri dishes (35 mM) for studies of EGF endocytosis **(B)**. After removal of the growth medium, 0.2 ml **(A)** or 0.5 ml **(B)** of 0.14 M KCl containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 μM nigericin, 10 μM valinomycin, and adjusted to the indicated pH values with Tris, was added. After a 5-min incubation at 37°C, ^{125}I-labeled transferrin (200 ng/ml, 38,600 cpm/ng or ^{125}I-EGF (7 ng/ml, 15,000 cpm/ng) was added, and the amount of endocytosed ligand was measured after 10 min (transferrin) or after 15 min (EGF).

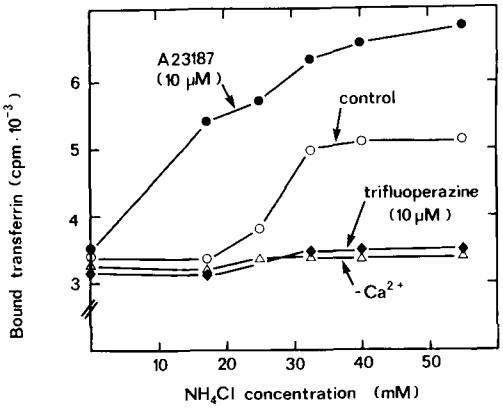

Fig. 5. Effect of calcium deprivation, trifluoperazine, and A23187 on the ability of low internal pH to induce increased binding of transferrin. Vero cells growing in 24-well disposable trays were incubated for 30 min at 37°C in Hepes medium (pH 7.0) with and without the indicated concentrations of NH_4Cl. The cells in some of the wells were incubated with trifluoperazine, while others were incubated in the absence of $CaCl_2$. The medium was then removed and 0.2 ml of buffer (0.14 M KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM amiloride, and 20 mM Hepes) was added. When indicated, $CaCl_2$ was omitted from the buffer. A23187 (10 μM) was added to some of the cells at this point. After an additional 5-min incubation the cells were chilled to 0°C, and ^{125}I-labeled transferrin (200 ng/ml, 38,600 cpm/ng) was added in a $CaCl_2$-containing medium. After a 1-hr incubation at 0°C, the cells were washed three times with cold medium, dissolved in 0.1 M KOH, and the amount of bound ^{125}I-transferrin was measured.

the increase in transferrin binding could also be abolished by trifluoperazine. Furthermore, W7 had a similar inhibitory effect, whereas the inactive analogue W5 had no effect. This supports the view that calmodulin or a similar regulatory protein is involved in the redistribution of transferrin receptors.

In the course of these studies, we noticed that low pH in the cytosol had a marked effect on the Golgi complexes that often became very vesiculated, with accumulation of numerous small, apparently coated, vesicular structures, which appeared to be connected to Golgi cisterns (Fig. 6). It is also possible that pinching off of coated vesicles in the Golgi apparatus is inhibited by the low cytosolic pH. However, further experiments are required to determine if this phenomenon is related to the effect on endocytosis.

In conclusion, the results presented here show that low pH in the cytosol inhibits endocytosis of transferrin and EGF in a reversible way, and that at low internal pH there is an increase in the number of transferrin receptors at the cell surface. Furthermore, as measured by studying endocytosis of ^{125}I-ricin, the results suggest that an alternative pathway of endocytosis exists, possibly involving uptake from noncoated areas of cell surface membrane and that the toxin ricin is able to utilize such a pathway on its way to the cytosol.

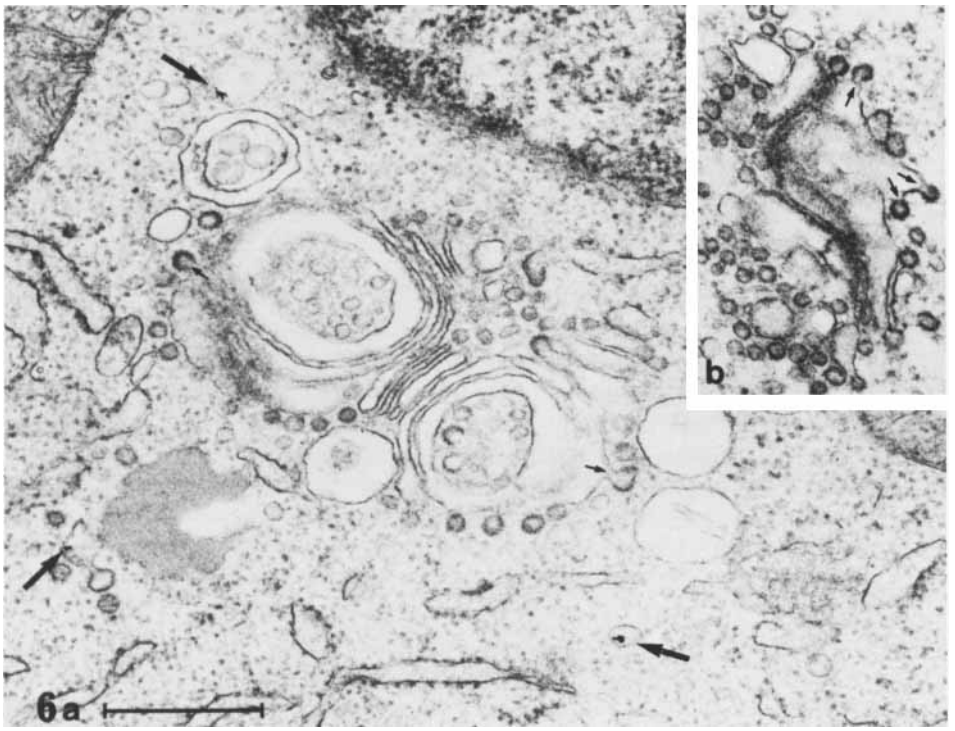

Fig. 6. Typical appearance of Golgi complexes in acidified Vero cells. **(a)** Acidified by incubation with nigericin and valinomycin in isotonic KCl pH 5.9. **(b)** Acidified by prepulsing with 25 mM NH_4Cl. **(a)** is from an experiment with Ri-Au (a few Ri-Au particles are seen in small vesicles; large arrows). **(b)** is from an experiment with Tf-Au. In both **(a)** and **(b)** vesiculated Golgi elements are distinct. Many small vesicles are connected to dilated cisterns (small arrows). a and b ×51,000.

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