Effects of Cytoplasmic Acidification on Clathrin Lattice Morphology

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Abstract. Reducing the internal pH of cultured cells by several different protocols that block endocytosis is found to alter the structure of clathrin lattices on the inside of the plasma membrane. Lattices curve inward until they become almost spherical yet remain stubbornly attached to the membrane. Also, the lattices bloom empty "microcages" of clathrin around their edges. Correspondingly, broken-open cells bathed in acidified media demonstrate similar changes in clathrin lattices. Acidification accentuates the normal tendency of lattices to round up in vitro and also stimulates them to nucleate microcage formation from pure solutions of clathrin. On the other hand, several conditions that also inhibit endocytosis have been found to create, instead of unusually curved clathrin lattices with ex-

traneous microcages, a preponderance of unusually flat lattices. These treatments include pH-"clamping" cells at neutrality with nigericin, swelling cells with hypotonic media, and sticking cells to the surface of a culture dish with soluble polylysine. Again, the unusually flat lattices in such cells display a tendency to round up and to nucleate clathrin microcage formation during subsequent in vitro acidification. This indicates that regardless of the initial curvature of clathrin lattices, they all display an ability to grow and increase their curvature in vitro, and this is enhanced by lowering ambient pH. Possibly, clathrin lattice growth and curvature in vivo may also be stimulated by a local drop in pH around clusters of membrane receptors.

SEVERAL different methods have recently been developed to block receptor-mediated endocytosis. These include potassium depletion (30, 36, 37, 38, 41, 46, 61), hypertonicity (14, 21, 22), and cytoplasmic acidification (15, 28, 60). The first two treatments produce profound changes in the distribution of cytoplasmic clathrin, the protein that normally produces geodesic "cages" around portions of the plasma membrane that undergo endocytosis (4, 19, 52, 53, 68). Clathrin-coated pits in the plasma membrane largely disappear, according to electron microscopic observations (21, 22, 38), while diffuse clathrin immunofluorescence increases throughout the cytoplasm (22, 36). This diffuse fluorescence turns out to result from precipitation of cytoplasmic clathrin into unusually small "microcages" (21, 22), similar to the polymers that form during dialysis of pure clathrin into acidic media (32, 64, 69, 73). Hence, potassium depletion and hypertonicity appear to induce clathrin to come out of solution in a form that can no longer participate in the production of normal coated pits.

Previous electron microscopy of acidified cells has not detected such structural changes (15, 60): coated pits remain visible and microcages have not been recognized. It has therefore been suggested that acidification somehow "paralyzes" coated pits so that they can no longer pinch off from the plasma membrane to form coated vesicles (60). In this study we apply the freeze-dry replica technique (24) to the analysis of acidified cells. This provides further evidence for a "paralysis" of coated pits in that lattices with increased curvature but persistent attachment with the plasma membrane

do indeed accumulate during acidification. In addition, however, acidification is also found to cause the same precipitation of clathrin microcages seen after hypertonicity and potassium depletion, indicating that all these treatments have certain common features.

In the course of this study it has also become clear that it is possible to manipulate the degree of curvature of clathrin lattices. Thus, flat lattices will round up "spontaneously" in vitro after cells are broken open, a process accelerated by acidic buffers and thus analogous to the increased curvature of lattices seen after in vivo acidification. Conversely, pH "clamping" the cytoplasm at neutrality leads to the accumulation of unusually flat clathrin lattices, a process that is promptly reversed upon subsequent acidification both in vivo and in vitro. These observations indicate that clathrin lattice behavior on the plasma membrane largely reflects the known properties of clathrin in solution: namely, that lowering ambient pH promotes both clathrin assembly and curvature. In addition, they indicate that assembly and curvature can be separable phenomena and can be induced to occur sequentially, substantiating the idea that molecular rearrangements can occur within the assembled lattice to bring about curvature (20, 31).

Materials and Methods

Cell Culture

3 × 3-mm pieces of No. 1 glass coverslip precleaned in hot chromic acid, washed extensively with water, and sterilized with ethanol before use. The cells were maintained in MEM supplemented with 10% FCS, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. After 1 d of culture at 37°C, at a time when the cells had spread and reached subconfluence on the small glass squares, they were removed from the CO_2 incubator and washed four times at 37°C in a standard avian Ringer's solution before further experimental manipulation.

Methods of Cell Acidification

Four different methods were used to acidify cells. Details of each method and documentation of the degree of acidification produced (as determined by carboxyfluorescein UV spectrofluorimetry) will be presented in a subsequent report. (a) "Ammonia prepulse" developed by Boron and colleagues (8, 9, 58): Cells are exposed to 25 mM NH_4Cl in normal Ringer for 15 min at 37°C, which loads them with ammonium ions and free ammonia; they are then transferred to sodium-free (and low Cl) medium composed of 135 mM N-methylglutamine neutralized to pH 7.2 with powdered Hepes (free acid form) containing 2 mM KCl, 5 mM $MgCl_2$, 3 mM EDTA, 0.5 mM KH_2PO_4, and 10 mM glucose. The absence of NH_4Cl from this medium promotes outward diffusion of accumulated ammonia from the cells, but since NH_4^+ is in rapid equilibrium with NH_3 and NH_3 is much more membrane permeable, most ammonia exits in the latter form. This leaves behind protons and rapidly acidifies the cells. Thereafter, acidification is maintained as long as the Na/H exchanger or "antiporter" is blocked by elimination of external Na. (b) Nigericin "pH clamp" developed by Thomas (66, 67): cells are washed in a medium where K^+ replaces all Na^+; they are then exposed to nigericin ($10^{-5}M$). This ionophore supports electroneutral K^+/H^+ exchange (54) so that when the K^+ concentration is made equal across the plasma membrane, H^+ concentration soon equalizes as well. Intracellular pH thus becomes "clamped" at the value imposed by the external buffer. The specific composition of the isotonic K^+ medium is also designed to prevent Donnan swelling via chloride imbalance in that it contains 50 mM K^+ gluconate, 50 mM KCl, 30 mM MES brought to pH 6.3 with KOH, 5 mM $MgCl_2$, 3 mM EGTA, 0.5 mM KH_2PO_4, and 10 mM glucose. (c) Direct acidification via application of weak acids as developed by Rogers et al. (56, 57): Cells are exposed to 10 mM acetic acid in normal Ringer's solution. The undissociated form of this acid promptly penetrates the plasma membrane and dissociates in the cytosol, thereby lowering cytoplasm pH. To provide a high concentration of the penetrable form, the pH of the Ringer's is allowed to drop to 6.0 when acetate is added. (d) Simple acidification of the medium: As known for some time (7, 15, 35), intracellular pH in most cells follows extracellular pH to some extent, usually lagging by 0.5–1.0 pH unit. Thus, severe acidification of normal Ringer's by substituting its usual Hepes buffer with MES buffer at pH 5.5 creates significant cellular acidification.

In experiments not detailed below, we have found that all four treatments have comparable effects on endocytosis, verging on total inhibition of bulk HRP uptake at an internal pH 6.3 or less, and all induce the changes in clathrin lattices described below. Grossly, cells treated by the various protocols can be distinguished because treatment a (NH_4Cl prepulse) also causes severe vacuolization of the endosomal/lysosomal system (the others do not), while treatment b (nigericin clamp) is alone in causing a dramatic nucleolar condensation.

Preparing Carbon–Platinum Replicas of the Inner Cell Surface

At the end of the treatments outlined above, cells on the small coverslips were exposed for 5 s to 0.3 mg/ml poly-L-lysine (35–50 kD; Sigma Chemical Co., St. Louis, MO) dissolved in the appropriate experimental solution. Cells were then washed for 10 s in hypotonic medium, prepared by mixing one part of the experimental solution with two parts of distilled water. Cells were then transferred to buffer A (70 mM KCl, 30 mM Hepes, pH 7.2, 5 mM $MgCl_2$, 3 mM EGTA) and immediately broken open by placing an ultrasonic microprobe (Kontes Co., Vineland, NJ) 4 mm away from the surface of the coverslip and delivering 15% of maximum power for 1 s. Sonicated cells were immediately transferred to 2% glutaraldehyde in buffer A for 30 min at room temperature. After fixation, the coverslips were washed exhaustively with distilled water. Thereafter, each coverslip was placed on a slab of aldehyde-fixed rabbit lung and attached to the plunger of a "Cryopress" freezing machine (Med-Vac, Inc., St. Louis, MO). Excess water was blotted from the coverslip and the sample was immediately frozen by abrupt contact with a copper block cooled with liquid helium, as previ-

ously described (20, 26). The frozen coverslip was then transferred to a freeze-etch machine (model No. 301 or 400; Balzers S. p. A., Milan) and freeze-dried at −80°C for 15 min. A replica of its exposed surface was prepared by rotary evaporating 2–3 nm of platinum at an angle of 24° above the horizontal, followed by 10 nm of carbon at a 75° angle. The replica was then separated from the coverslip by immersion in full strength hydrofluoric acid, washed twice in distilled water, and cleaned by flotation on household bleach (5% sodium hypochlorite) for 5–10 min. Finally, it was washed several times in distilled water and picked up on a 400-mesh Formvar-coated grid for electron microscopy.

Fluorescence Microscopy

To localize clathrin distribution by indirect immunofluorescence, human fibroblast monolayers grown on 22-mm square glass coverslips were subjected to the experimental treatments described above and then fixed with 2% (wt/vol) paraformaldehyde in buffer B (100 mM NaCl, 20 mM Hepes buffer, pH 7.2, with 2 mM $CaCl_2$). The fixative was then quenched by washing the cells briefly in 50 mM NH_4Cl and 50 mM lysine in buffer B. Cells were then washed twice with buffer B alone and permeabilized with 0.1% (wt/vol) NP-40 in buffer B for 5 min at 25°C. Each coverslip was then covered with 100 μl of mouse anti-clathrin IgG (50 μg/ml of monoclonal anti–heavy chain antibody "×22", kindly provided by Dr. Francis Brodsky; cf. reference 10)] and incubated for 60 min at 25°C. After four washes (15 min each) with buffer B, the cells were incubated with 100 μl of goat anti–mouse IgG conjugated to fluorescein isothiocyanate (50 μg/ml; No. F-3008; Sigma Chemical Co.) for 60 min at 37°C. The coverslips were finally washed and mounted on glass slides in 0.1 M N-propyl-gallate (Sigma Chemical Co.) in 90% glycerol/10% buffer B. Finally, they were viewed with a Leitz epifluorescence photomicroscope.

Internalization of Horseradish Peroxidase

Horseradish peroxidase (HRP)[1] uptake was analyzed as previously described (25). Cultured chick or human fibroblasts grown on 22-mm square coverslips were subjected to the above treatments, then incubated for 30 min at 37°C with 5 mg/ml HRP (type VI; Sigma Chemical Co.) while still in the experimental solution, and finally fixed with 2% formaldehyde and 0.25% glutaraldehyde in buffer B for 1 h. The cells were then processed for visualization of internalized peroxidase with 0.05% diaminobenzidine and 0.01% H_2O_2 in buffer B as previously described (25).

Preparation of Pure Clathrin for In Vitro Assembly Experiments

Coated vesicles were prepared from calf brains by sucrose density centrifugation, and clathrin was stripped from them with 0.5 M Tris, pH 7, as described (32, 34). Purification was achieved by ammonium sulfate precipitation and chromatography on Superose 6B also as described previously (76). Molecular intactness was assessed with SDS-PAGE (32) and deep-etch electron microscopy of clathrin adsorbed to mica (23). Protein concentration was determined by absorbance at 280 nm.

Electron Microscopy

All electron micrographs were prepared using a JEOL 200CX electron microscope operating at 100 KV. Stereo viewing and digitization and quantification of the micrographs was carried out as previously described (17, 21, 22, 27, 34).

Results

Spontaneous Increase in Clathrin Lattice Curvature In Vitro

Because clathrin lattices are found with many different degrees of curvature when normal cells are examined in the electron microscope (20), their individual sequence of assembly and curvature must be relatively unsynchronized.

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1. *Abbreviation used in this paper:* HRP, horseradish peroxidase.

Any changes that occur in experimentally altered cells thus need to be assessed relative to this intrinsic variability. Here, we first determined that the various lattice configurations seen in whole cells fixed in glutaraldehyde (20, 27) were also encountered when cells were broken open by sonication and then fixed (Fig. 1). When, however, fixation in glutaraldehyde was delayed for several minutes after breaking cells open, significant changes were seen (Fig. 2). As detailed in Table I, clathrin lattices on broken membrane fragments became progressively more curved with time.

This spontaneous increase in lattice curvature required no obvious energy source, occurring even when Mg-ATP was removed with apyrase, with hexokinase and glucose, or with EDTA. The only conditions that inhibited it were low temperature (10°C slowed it and 4°C stopped it) and ionic media that approached lattice-depolymerizing conditions (i.e., buffers <20 mM ionic strength or > pH 8; cf. references 32, 45, 73). Fortunately, however, lattice curvature increased slowly enough that it was not a problem when cells were manipulated in vivo and fixed immediately after sonication. It only became a problem when sonicated cells were manipulated for some time in vitro; any lattice changes observed under these conditions were superimposed on an inevitable increase in lattice curvature.

Effects of Acidification on In Vitro Lattice Curvature

Complementary to the inhibitory effects of in vitro alkalinization mentioned above, in vitro acidification of the media around exposed clathrin lattices caused them to curve much more intensely and much more rapidly than usual (Fig. 3 and Table I). The apparent threshold for this phenomenon was between pH 6.3 and 6.5 (not shown). A further effect of in

vitro acidification was to cause some lattices to break up into sharply curved sublattices (Fig. 4). This bizarre result occurred particularly frequently in regions of cells that happened to be adherent to the underlying coverslip, due to brief pretreatment with polylysine before sonication. It was not seen in unattached regions of the same cells nor in the absence of in vitro acidification. Thus it appeared to reflect a competition between an abnormal resistance to membrane curvature (due to the polylysine) and an abnormal tendency for lattice curvature (due to the low pH of the medium). As in the spontaneous curvature described above, low pH-induced curvature also was not dependent on ATP availability and could only be inhibited by cooling to <10°C.

In Vivo Acidification of Living Cells

Acidifying the cytoplasm of whole cells by any of the several protocols outlined in Materials and Methods led to an abrupt arrest of HRP uptake in chick and human fibroblasts (Fig. 5), but as noted by other investigators (15, 60), did not involve the loss of clathrin lattices. Two lines of evidence confirmed this: first, immunofluorescent localization of clathrin in acidified cells displayed normal numbers of bright surface spots (Fig. 6); second, replicas of acidified cells displayed normal numbers of clathrin lattices (Table I). Thus, coated pits in acidified cells do indeed appear to become "paralyzed". A similar interpretation has been given to the persistence of coated pits in A431 cells during mitosis (55), at which time endocytosis is also inhibited (48, 59, 72).

To determine the possible structural basis of such a "paralysis" of coated pits, freeze-etch replicas of acidified cells such as Fig. 7 were examined. These displayed an unusually great abundance of nearly spherical clathrin lattices, similar

Table I. Experimental Alteration of the Curvature of Clathrin Lattices beneath Coated Pits In Vivo and In Vitro

Treatment	Total No. of lattices evaluated*	Relative curvature of clathrin lattices[‡]			
		Flat	Gently curved	Hemispherical	Almost completely spherical
Whole cells, fixed	47	0.10	0.40	0.20	0.30
Fixed immediately after breakage	118	0.15	0.35	0.25	0.25
Sat 5' at pH 7.2 after breakage	87	0.10	0.05	0.45	0.35
Sat 5' at pH 6.2 after breakage	68	0.05	0.10	0.00	0.80
pH 6 clamped with nigericin	81	0.15	0.15	0.10	0.50
"Rebound" acidity after NH₄ prepulse	73	0.10	0.15	0.20	0.60
pH 7 clamped with nigericin	91	0.55	0.15	0.10	0.20
Control for pH 7 clamp (neutral KCl medium)	49	0.25	0.30	0.15	0.25
Prolonged polylysine exposure	41	0.80	0.05	0.00	0.15

* Representing all of the lattices that were present on a total of 50 μm^2 of membrane surface area, i.e., on 20 electron micrographs taken at 50,000×, of fields chosen only for their technical quality.
‡ These values represent fractions of the total number of lattices evaluated, rounded off to the nearest 0.05, so that the total is not necessarily 1.00. To assess curvature, stereo pairs were analyzed to obtain unambiguous visual cues of depth. For the nonstereo images presented in this paper, degree of curvature is indicated by the relative whiteness of the image: the more domed the lattice, the more platinum is deposited on it and, in reverse contrast, the whiter it appears (cf. Figs. 1 and 2, showing the range between flat and sharply curved lattices).
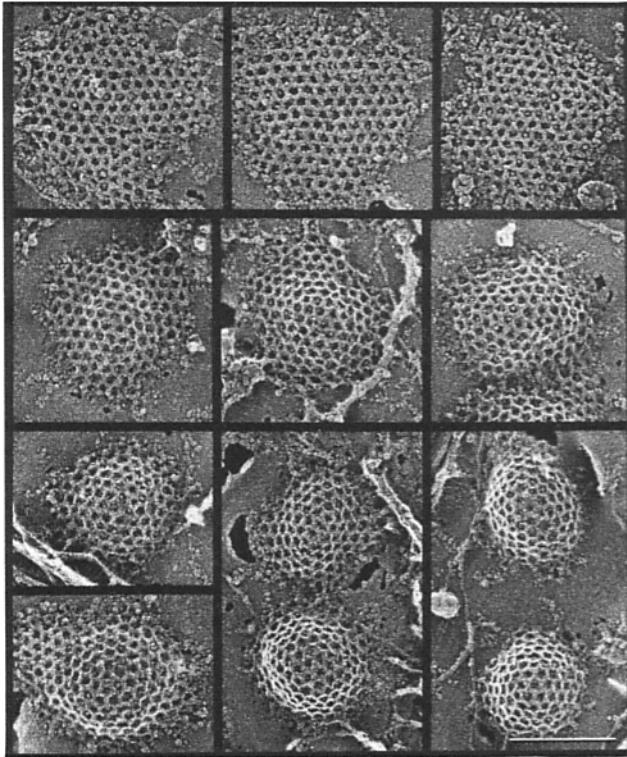
Figure 1. Gallery of coated pit profiles on the inner surface of normal chicken fibroblasts growing in culture, illustrating the range of lattice curvature seen normally. Bar, 0.2 μm.
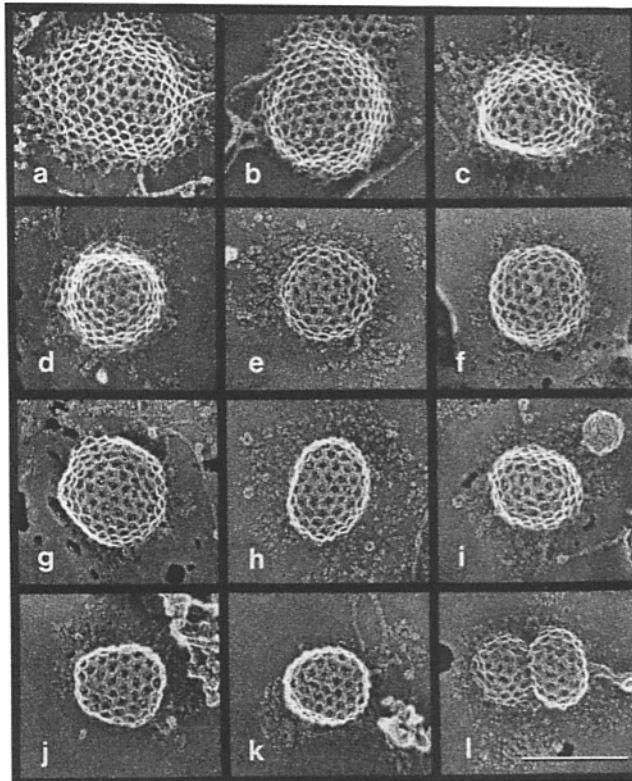

Figure 2. Gallery of the more rounded types of clathrin coated pits seen in normal chick fibroblasts (*a–d*), compared with the unusually rounded appearance of coated pits on membrane fragments derived from cells that were broken open and allowed to remain in pH 7 buffer for 10 min at 25°C before fixation and freeze drying (*e–l*). Bar, 0.2 μm.
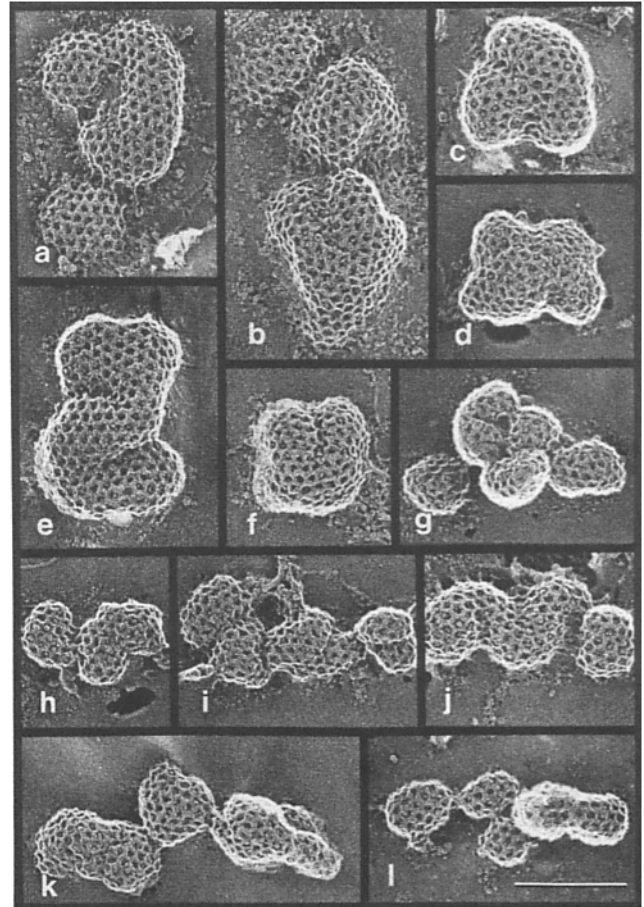
Figure 3. Gallery of relatively large clathrin lattices allowed to round up in vitro before fixation and freezing. The initially large size of these particular lattices caused them to form multiply curved domains. *a–f* were from membrane fragments exposed to neutral media, while *g–l* were from membrane fragments exposed to pH 6.2 media. This clearly exacerbated their tendency to curve and to parcel up into multiply curved domains. Bar, 0.2 μm.
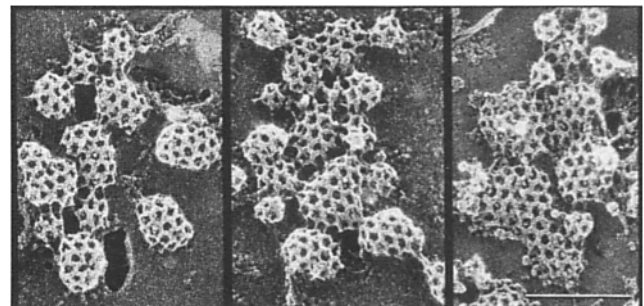

Figure 4. Examples of clathrin lattices that were induced to curve by exposure to pH 6.2 media after cell rupture, but under conditions in which the membrane was first stuck to the underlying coverslip by pretreatment with polylysine. This physically prevented the normal curvature of the lattices and resulted in elaborate partitioning into multiple subdomains, each of which appear in stereo to be roughly hemispherical. Bar, 0.2 μm.
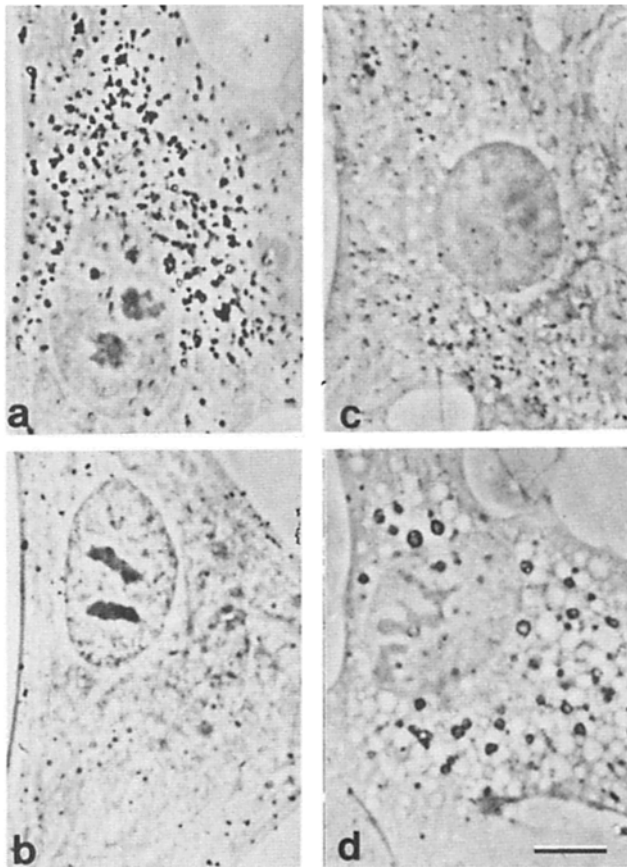
Figure 5. HRP uptake in control and treated chick fibroblasts in culture, as seen by phase-contrast light microscopy. *a* illustrates normal uptake after exposure to 5 mg/ml HRP for 20 min at 37°C. *b* and *c* illustrate severe inhibition of HRP uptake resulting from acidification of the cytoplasm. In *b*, this was accomplished by exposure to 10^{-5} M nigericin in an isotonic K$^+$ medium at pH 6.3, while in *c* it was accomplished by ammonia "prepulse" (see Materials and Methods). In other experiments, these treatments also inhibited receptor-mediated uptake of specific ligands and toxins, as expected (42). Note that acidification with nigericin (*b*) also caused a characteristic condensation of the two nucleoli. *d* illustrates that nigericin applied at neutral pH, e.g., pH clamping, also partially inhibits HRP uptake. In this case, internalized tracer ends up in swollen endosomes. Bar, 10 μm.

Figure 6. Fluorescence microscopy of human fibroblasts reacted with Brodsky's ×22 anti–clathrin antibody (10). *a* and *b* display control cells illustrating the characteristically punctate distribution of clathrin at the cell surface seen normally (5, 6, 16, 18, 39). Presumably, this is clathrin at coated pits. *c* and *d* illustrate clathrin distribution in fibroblasts acidified with nigericin for 30 min. Surface staining appears unaltered at this low magnification; the only difference is inside, where bright rings of fluorescence now appear. These may be organelles that failed to uncoat because low pH inhibited the 70-kD uncoating ATPase (50, 63) or they may be clusters of clathrin microcages, as seen in hypertonically treated cells (21, 22) and in metabolically inhibited cells (44, 49). Bar, 10 μm.

to those seen after in vitro acidification, as well as an unusual prevalence of the form of clathrin lattice illustrated in Fig. 8. This form was superficially reminiscent of the partitioned lattices seen after in vitro acidification (Fig. 4), but differed significantly in that the partitions were generally smaller, always more uniform in diameter (50–60 nm), and almost perfectly spherical. (This made them appear very white in contrast-reversed images because the platinum on their upper and lower surfaces became superimposed during photography.) Such tiny, closed lattices of clathrin have been termed "microcages" in previous reports (21, 27). They clearly display the appropriate polygonal construction for clathrin, with the predominance of pentagons over hexagons expected for unusually small lattices (12, 13, 23, 31, 51, 70, 71). They do not, however, contain any internal membranes: no comparable membrane invaginations have been seen in the envi-
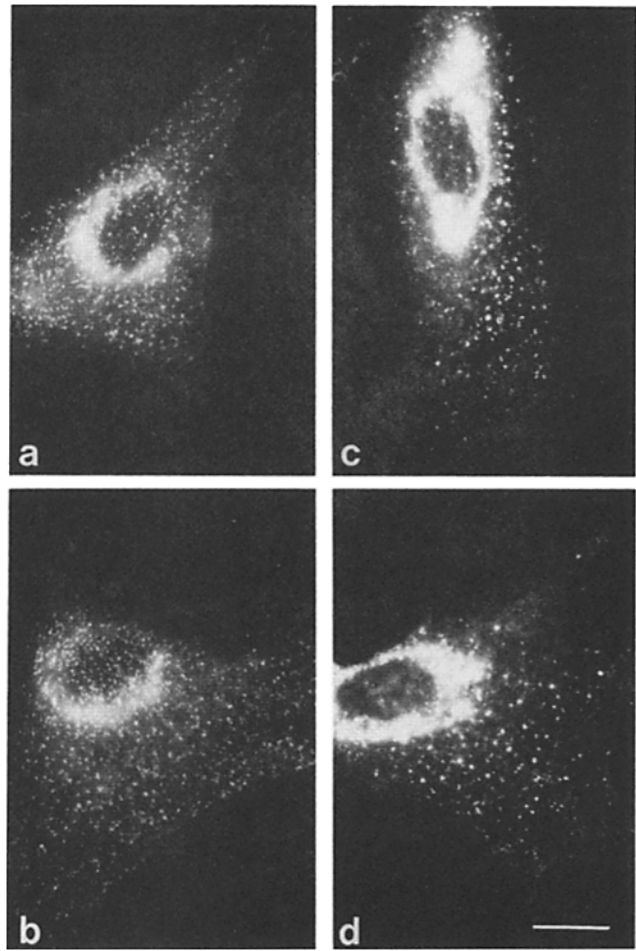
rons of coated pits in previous thin sections of acidified cells (15, 60) nor were they seen when we freeze-fractured through the membranes of coated pits in acidified cells (data not shown). Thus they appear to be abnormally empty polymers, incorrectly attached to the plasma membrane.

Finding that such microcages form during acidification in vivo but not in vitro raised the obvious possibility that they derive from a cytoplasmic pool of clathrin. In vitro lattices would of course not have the opportunity to nucleate polymerization of clathrin in response to a drop in pH even if they had the tendency to do so, simply because the cytoplasmic pool would be totally washed away. The following experiments demonstrate that in vitro lattices in acid do indeed display a clathrin-nucleating propensity.
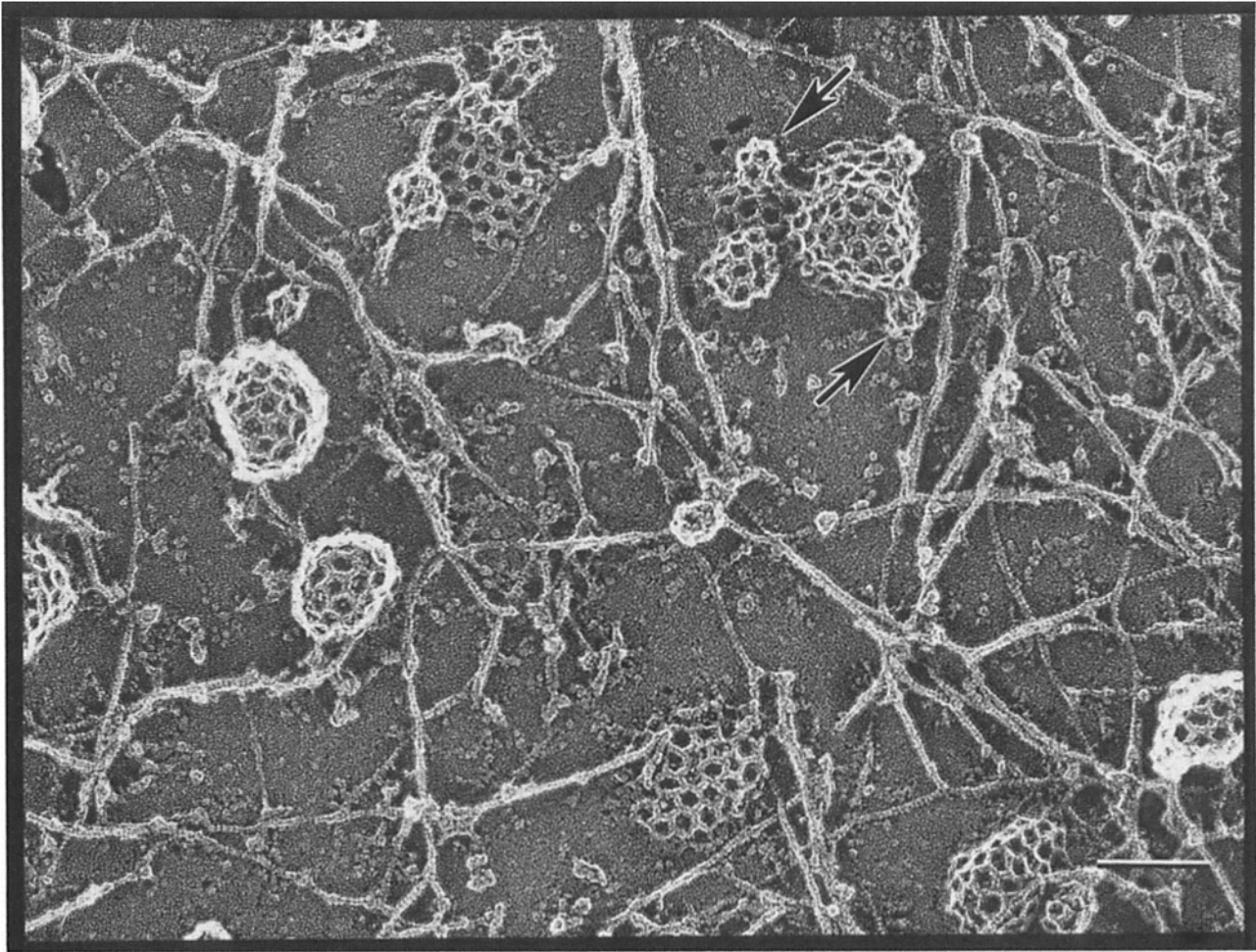
Figure 7. Survey view of a human fibroblast acidified by nigericin treatment at pH 6.3 for 2 min at 37°C. This results in several changes in the clathrin lattices, including excessive rounding and the appearance of adjacent foci of extremely intense curvature (two of these "buds" are indicated at the arrows). Bar, 0.1 μm.

Lattice Edges Act as Clathrin Nucleating Sites

When normal cells were sonicated open and the remaining membrane fragments were bathed in 0.1 mg/ml pure clathrin (initially dissolved in 50 mM Tris, pH 7.5, to keep it soluble), nothing happened to their exposed clathrin lattices, beyond their usual slow rounding up. However, when the medium containing soluble clathrin was subsequently acidified by injecting a 1/10 vol of 1 M MES buffer at pH 6.0 with 20 mM MgCl₂, the exposed lattices acquired microcages that looked exactly like those seen after in vivo acidification. Fig. 9 (*g–l*) illustrates this. After 5 min of acidification at 25°C, numerous small clathrin cages appeared around the edges of all coated pits, which as usual had become curved as a result of the 5 min of in vitro incubation at low pH. These looked identical to the microcages that form around clathrin lattices during in vivo acidification (Fig. 8 and Fig. 9, *a–f*). Hence, under pH conditions in which clathrin is induced to come out of solution in the form of small cages (13, 32, 64, 69, 73, 74), the edges of existing coated pits appear to nucleate this phenomenon, presumably because the edges of normal lattices are incomplete and thus display uncommitted triskelion legs that can induce the polymerization. This in vitro result

substantiates the idea that microcage formation during in vivo acidification involves a similar nucleation phenomenon, but in that case from the normal cytoplasmic pool of clathrin.

The Fate of Acid-induced Microcages

When cytoplasmic pH was held at 6.0–6.3 for prolonged periods (30–60 min), clathrin microcages continued to be found in contact with the persistent "paralyzed" clathrin lattices, as well as elsewhere on the inner surface of the plasma membrane. Subsequent restoration of normal intracellular pH resulted in their prompt dissolution; within 5 min at 37°C they were completely gone. Interestingly, dissolution of microcages also occurred in vitro within 5 min of breaking cells open in buffers of pH 7.2 or higher. Hence, to preserve them once they were formed, it was necessary to fix cells immediately after sonication or else to maintain them in acidic buffer between sonication and fixation.

Clamping of Cells at Neutral pH

One set of control experiments for the nigericin technique of acidifying cells entailed exposing cells to an isotonic K⁺ medium that did not contain nigericin and was not acidic;
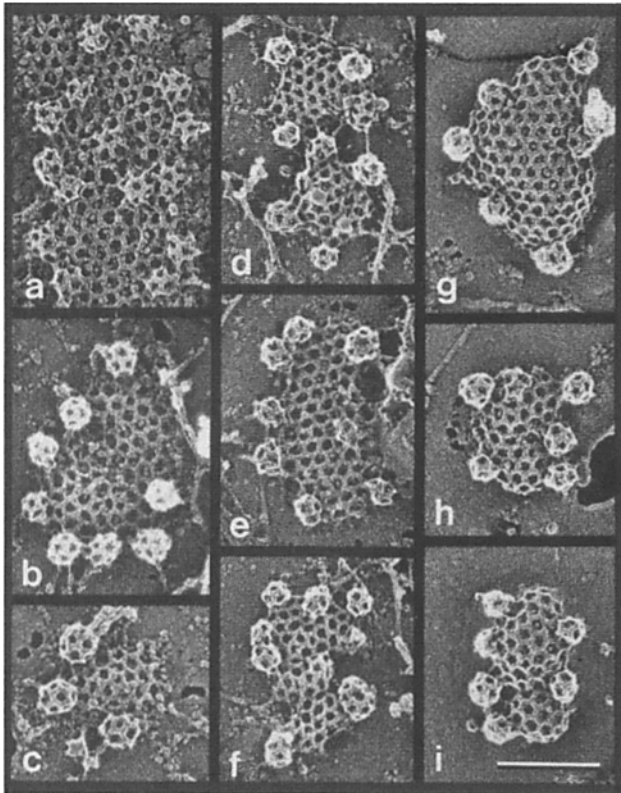
Figure 8. Gallery of selected views of the characteristic microcage nucleation that occurs around clathrin lattices in acidified cells. *a–c* illustrate chick cells acidified by ammonia prepulse, *d–f* illustrate human cells acidified by nigericin at pH 6.3 for 2 min, and *g–i* illustrate chick cells acidified by nigericin treatment at pH 6.3 for 5 min, all at 37°C. Bar, 0.2 µm.
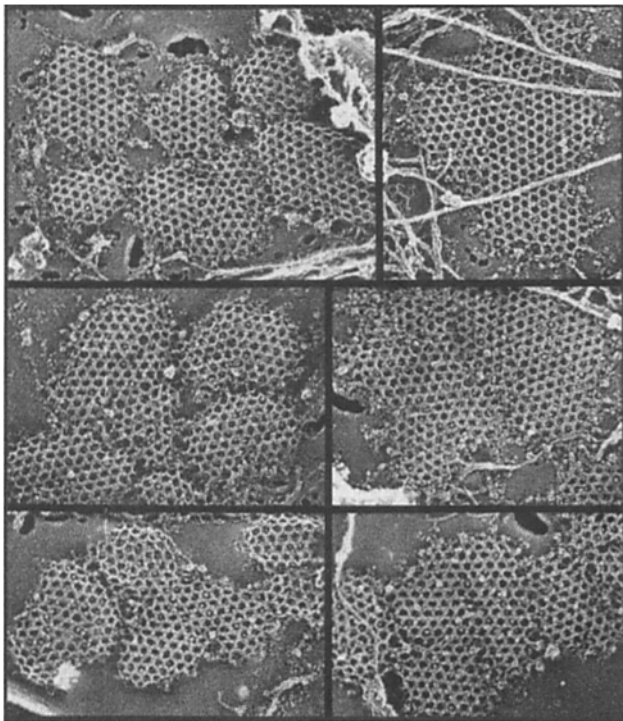

Figure 10. Gallery of typical examples of the appearance of clathrin-coated pits in chick fibroblasts exposed to nigericin in neutral media (pH clamped) for 30 min at 37°C. Lattices under these conditions become unusually flattened and display a tendency to coalescence.
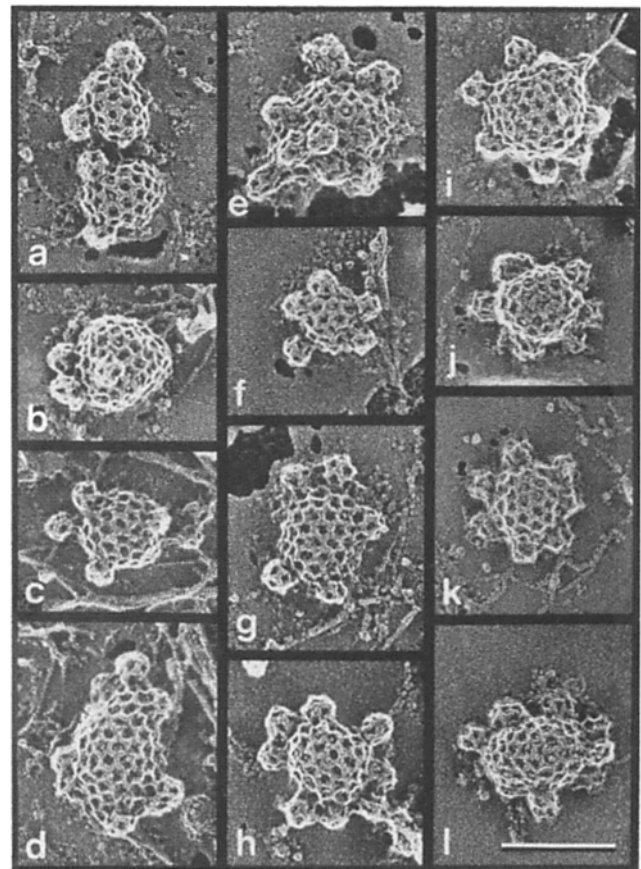

Figure 9. Gallery of selected views of clathrin lattices that display microcage nucleation around their edges from whole cells acidified with nigericin at pH 6.3 (*a–f*), compared to in vitro nucleation of clathrin assembly onto preexisting clathrin lattices (*g–l*). In the latter instances, cells were broken open and exogenous clathrin (0.1 mg/ml) was provided before acidifying their environment. Small clathrin lattices tend to form in suspension as a result of this acidification; however, on the membrane fragments in question, such lattices are found only at the margins of coated pits. Bar, 0.2 µm.

this had no obvious effect on endocytosis or on clathrin organization (not shown). However, when nigericin was added to this neutral K⁺ medium, yet a different change in clathrin was observed: membrane associated lattices became virtually flat (Table I). Often, they also became unusually large and confluent (Fig. 10 and Fig. 11*a*, *b*), approaching the extreme of flatness seen relatively infrequently in normal cells (Table I). Associated with this change, HRP uptake became restricted to a small number of very distended endosomes (Fig. 5 *d*), though was not as inhibited as after acidification (Fig. 5, *b* and *c*).

Two experiments demonstrated that the unusually flat lattices in nigericin pH-clamped cells were still capable of curving. First, when such cells were sonicated open and left for several minutes before fixation, their lattices rounded up in the same fashion as untreated cells (not shown). Second, when such cells were challenged by brief (1–2 min) acidification immediately before preparation for electron microscopy, their large flat lattices were replaced by more curved ones that displayed regions of the sort of fragmentation seen during in vitro acidification (Fig. 11, *c–e*) and displayed many examples of the sort of polymerization of microcages
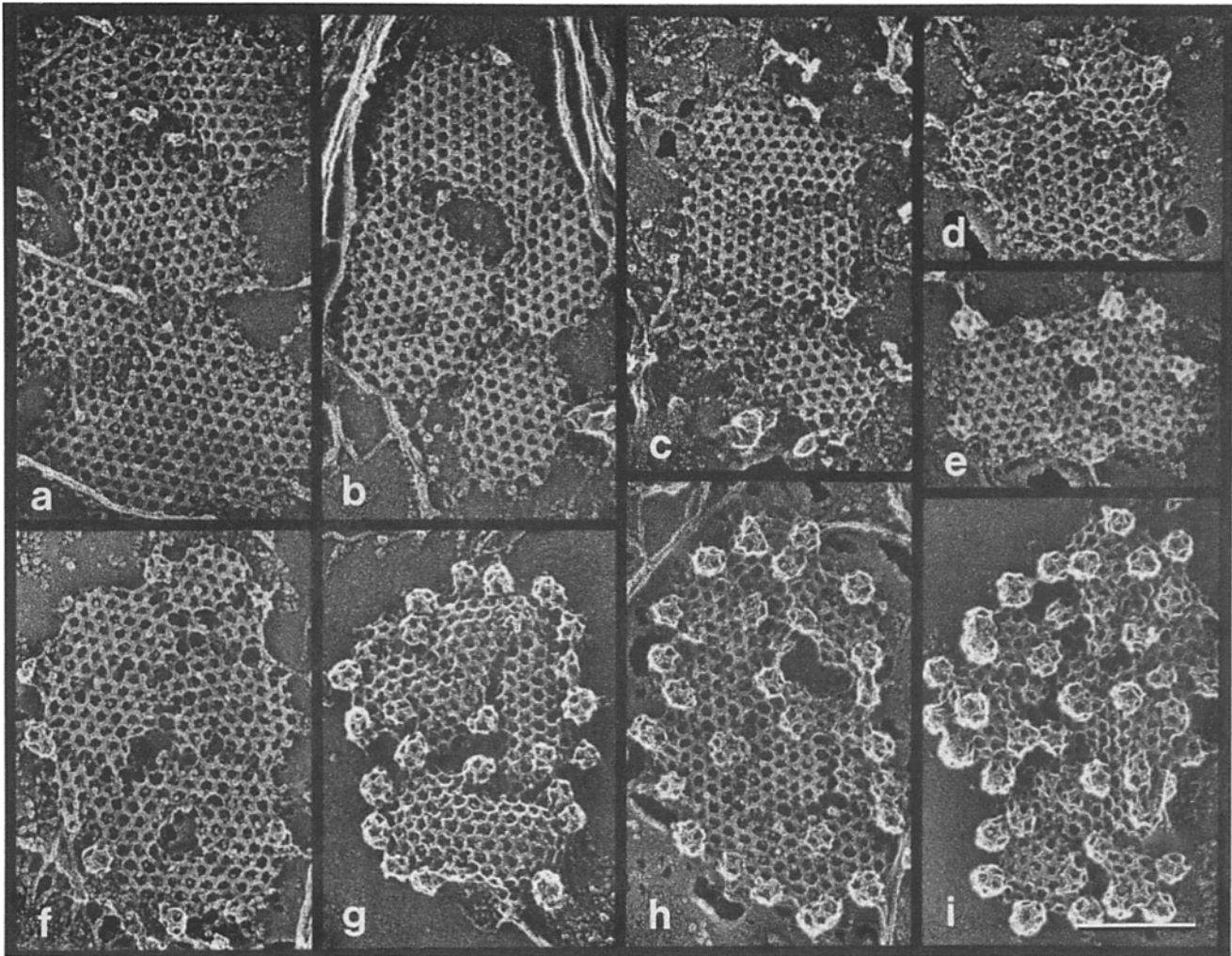
Figure 11. Gallery of typical clathrin lattices seen on the inner surfaces of chick fibroblasts exposed to nigericin at neutral pH without subsequent treatment (*a* and *b*) or followed by brief acidification of the extracellular medium before fixation and freeze drying (*c–i*). In *c–e*, acidification was applied for 1 min. In *f–i*, acidification was applied for 2 1/2 min. The sections are thus arranged to illustrate a progressive increase in the extent of microcage nucleation around the edges of lattices that start out unusually flat (due to the initial pH clamp). This plate thus summarizes the extremes of clathrin lattice configuration that can be generated by manipulation of intracellular pH. Bar, 0.2 μm.

seen during in vitro clathrin decoration (Fig. 11, *f–i*). These results show that the unusually flat lattices that accumulate in pH clamped cells are just as capable of rounding up and nucleating clathrin polymerization as are normal lattices. Thus, it is not clear why clathrin lattices accumulate in flat forms during cytoplasmic clamp at pH 7.

Other Means to Flatten Clathrin Lattices

The above accumulation of flat lattices raised the question of whether clathrin lattices present at the outset of the experiment were flattening out after they were made, or whether the original population of clathrin lattices was being replaced by a new population of lattices that were starting off flatter than usual. Support for the former possibility came from finding two additional methods to produce flat clathrin lattices, both of which were extremely abrupt (occurring in less than the turnover time expected for coated pits). The first entailed exposing living cells to 0.1 mg/ml polylysine for 1 min;

this glued the bottoms of most cells down to the coverslip and, in the process, flattened out most of their coated pits (not shown here, but also documented in reference 45). Preexisting coated pits could also be flattened out promptly by swelling cells with very hypotonic media (1 min in 5 mM phosphate buffer pH 7.0). The latter treatment was sufficiently abrupt to create numerous "faults" in the lattices as they flattened, faults much like the ones found in a cartographer's projection of the earth. Still, even these lattices were able to "heal" their faults and curve up into spheres in vitro; given 5 min between sonication and fixation, the lattices on hypotonically swollen cells became spherical as usual (not shown).

Effects of Gross Cell Damage

Although the in vivo changes described above were in most cases fully reversible, the severity of some of the experimen-

tal conditions occasionally killed a small proportion of the cells in certain experiments. Such cells could be recognized by, among other things, the unique change in clathrin distribution illustrated in Fig. 12. This amounted to a massive accumulation of relatively small and relatively flat lattices. This unusual condition could be readily distinguished from all the other lattice changes described above, dispelling concern that the above changes were also agonal ones. However, what causes this agonal accumulation of lattices remains to be determined.

Discussion

The data presented here indicate that acidification has three distinct effects on clathrin lattices. First, it stimulates lattice curvature, an effect that can be seen both in vitro and after cytoplasmic acidification in vivo (Table I). Second, it promotes the polymerization of soluble cytoplasmic clathrin into highly curved microcages, using the edges of preexisting lattices as major nucleating sites (Figs. 8 and 9). Third, it prevents curved lattices from going on to form coated vesicles; e.g., it prevents them from pinching off the plasma membrane and thereby stops endocytosis abruptly and completely (Fig. 5).
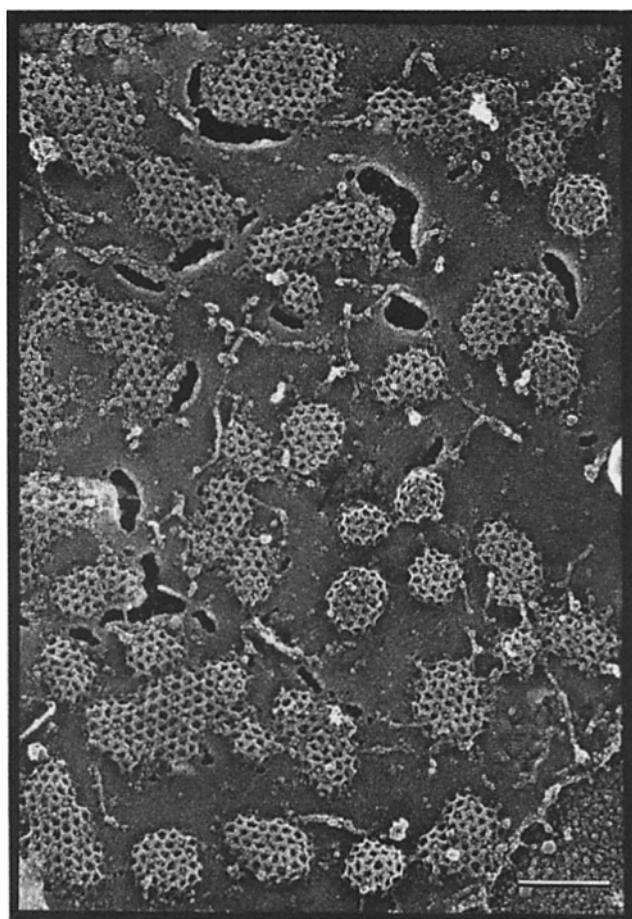

Figure 12. Survey view of a dying fibroblast, illustrating the typical recruitment of flat clathrin lattices and loss of membrane-associated actin that occurs just before cell rupture. This illustrates that the lattice changes presented in the above figures were not agonal changes. Bar, 0.2 μm.

Interpreting these changes is complicated by the fact that electron microscopy does not permit the examination of any particular lattice both before and after treatment, it provides only a series of statistical samples of many lattices at different time points. Thus, the question arises of whether lattices present at the outset of the experiment have themselves changed, or whether they have been replaced by a new population of lattices that are different. Sometimes, the experimental protocol used to manipulate the lattices before electron microscopy will permit a reasonable answer to this question. Thus, for example, when membrane fragments containing clathrin lattices are manipulated in vitro and in simple buffers that do not contain the substrates necessary for new lattice formation, one can be relatively certain that the lattices present at the moment of cell rupture have themselves gone on to change. Moreover, when changes are produced in whole cells on a time scale that is much more rapid than the expected turnover time of clathrin lattices, one can again be relatively certain that preexisting lattices have themselves changed. The latter conclusion is more problematic, however, because the normal rate of turnover of clathrin lattices is not known with any degree of certainty. The usual estimate is 1–5 min (53), but this is based on somewhat indirect evidence (4–6). In this respect, one of the major effects of the treatments applied here is to slow the rate of coated pit turnover or stop it entirely, hence most of the observed changes are likely to have happened to preexisting coated pits. Nevertheless, we cannot rule out the possibility that any changes seen in vivo represent a replacement of the original population of lattices with a new population that is different from the original. Only time-lapse electron microscopy could ever properly distinguish between these two alternatives.

Clathrin Lattice Curvature

In any case, it is clear that after a normal cell is broken open, its exposed clathrin lattices round up in an ATP-independent fashion. This indicates that the capacity to curve is normally built into clathrin lattices, even when they start out relatively flat. One of the effects of acidic pH is thus to enhance this natural tendency to curve. On the other hand, failure of lattices to curve in pH clamped cells suggest an active inhibitory influence. One such influence might be simply an alteration in the surface/volume ratio of the cell; clathrin lattices might be unable to round up until a sufficient amount of membrane was provided by exocytosis (cf. reference 25), and this process might be blocked by pH clamping. Such a constraint would of course be relieved once a cell was broken open and the lattices were left clinging to membrane fragments.

Microcage Formation

It is of interest that three apparently different treatments, hypertonicity, potassium depletion, and acidification, all bring about the formation of clathrin microcages during inhibition of endocytosis (this paper and references 21, 22). This could be because all three treatments, not just the last, create some degree of cytoplasmic acidification (28, 40, 62). It is well documented that in vitro, acidic conditions induce the polymerization of clathrin into small empty cages (32, 64, 69, 73). The abnormal formation of such structures in vivo

could deplete the pool of unpolymerized clathrin that normally participates in the formation of coated pits or participates in the final closure of coated pits into coated vesicles. Alternatively, microcage formation around the edges of coated pits might stearically hinder lattice completion. These are only two of the possible explanations for why coated pits become paralyzed during cytoplasmic acidification. There could also be major effects on ATP availability (11, 65) or direct membrane changes (15, 60). For example, acidification could somehow inhibit membrane fusion at the necks of coated pits after they have rounded up, and thus prevent them from finally pinching off to become coated vesicles.

Physiological Relevance of the pH Effects Seen Here

It is unlikely that cells normally modulate their overall endocytotic rates by titrating the pH of their cytoplasm to the extremes employed in this study. Nevertheless, overall physiological conditions or cell cycle transitions that alter intracellular pH more subtly or transiently might change the equilibrium between polymerized and nonpolymerized clathrin throughout the cell, as well as the overall tendency for clathrin lattices to curve, and thereby alter the ongoing rate of coated vesicle formation. One example of this seems to be during mitosis when endocytotic rates drop substantially (48, 55, 59, 72); it will be interesting to learn what happens to the internal pH of cells during this natural inhibition.

It also becomes of interest to consider whether pH variation might be a control mechanism employed locally by the cell as well. An intriguing possibility is that pH alterations on a local level could modulate individual coated vesicle dynamics. The origins of such pH changes can only be imagined, but they could involve membrane receptors. It is well known that membrane receptors tend to cluster in coated pits (5, 6, 16). Possibly, proton channels are drawn into coated pits along with receptors. Alternatively, the membrane-spanning domains of receptors themselves could form proton channels when brought into close proximity, analogously to the formation of membrane conductances by aggregated F_c receptors (74, 75) or IgE receptors (15a, 30a). Proton fluxes through individual clusters of channels might create an acidification that would be sufficiently local to promote clathrin assembly at the same sites, as well as stimulate lattice curvature, and thereby promote the internalization of the specific membrane domains that bore the clusters.

The Mechanism of Clathrin Lattice Curvature

The results in this study bear on another key question in the cell biology of clathrin. It has been known for some time that cells in culture commonly carry a certain amount of polymerized clathrin in the form of flat lattices (20, 43, 47). One model of coated vesicle formation suggests that flat lattices are the initial phase of clathrin polymerization and that they are induced to curve secondarily, by some other aspect of receptor or membrane activity (20, 31, 38). An alternate model is that curvature is obligately built into the clathrin lattice as it forms, like a virus coat (19, 33, 53). From this latter viewpoint, flat lattices have been interpreted as technical artifacts (53), or frustrated attempts at endocytosis (1–3), or entirely different functional entities such as cell adhesion sites (43, 47). On the contrary, the present work demonstrates that

flat lattices, even the ones that accumulate in pH-clamped cells, can be induced to curve in vitro. This supports the idea that lattice curvature can occur after assembly and that the flat lattices seen in normal cells are not de facto "dead ends", but are more likely to be stages between assembly and curvature. It thus remains a formidable challenge to explain how a lattice of the complexity of clathrin could rearrange its bonds internally, as it would have to do if it were to convert the flat hexagons of a planar array into the puckered pentagons needed for a curved array.

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