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Visualization of acidic organelles in intact cells by electron microscopy

[3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine/dinitrophenol congener/immunoperoxidase staining/receptor-mediated endocytosis/monensin]

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ABSTRACT **We report the synthesis of a probe that permits the visualization by electron microscopy of acidic organelles in intact cells. This probe, 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine (DAMP), is a basic congener of dinitrophenol that readily diffuses into intact cells. Its primary and tertiary amino groups (apparent pKa, 10.6) allow it to be concentrated in acidic organelles and to be retained there after fixation with aldehydes. The dinitroarene moiety of DAMP can then be localized with mouse monoclonal antibodies directed against dinitrophenol. The antibodies, in turn, can be visualized by light or electron microscopy by reaction with rabbit anti-mouse antibodies coupled to rhodamine or horseradish peroxidase, respectively. We have used these methods to show that DAMP concentrates in a variety of membrane-bound structures in cultured fibroblasts, including classic multivesicular bodies (resembling lysosomes), intermediate-sized vesicles with multiple shapes (resembling endosomes), and an abundant population of very small spherical vesicles. A small fraction of coated vesicles is labeled with DAMP. Labeling with DAMP does not occur when the pH gradient of fibroblasts is disrupted by the ionophore monensin or the weak base chloroquine. DAMP should be a useful probe for exploring the assembly, distribution, and function of acidic organelles by electron microscopy.**

The concept that cellular organelles can maintain a pH lower than that of the surrounding cytoplasm was established by the pioneering studies of lysosomes conducted by deDuve and his colleagues (1). These workers showed that weak bases, such as chloroquine, which become positively charged at acidic pH, are concentrated in lysosomes. Concentration occurs because these chemicals diffuse freely through the hydrophobic membrane into lysosomes in their unprotonated state at neutral pH, but they become positively charged at acidic pH within the lysosomes and, hence, leave these structures only slowly. Through the use of fluorescent weak bases, such as acridine orange, Allison and Young (2) were able to visualize acidic compartments in intact cells by light microscopy. Through the use of fluoresceinated dextran derivatives whose fluorescence spectrum is altered as a function of pH, Okhuma and Poole (3) estimated that the pH of lysosomes is in the range of 4.7 to 4.8 in living cells.

Recent work in several laboratories has demonstrated that lysosomes are not the only organelles with an acidic pH. Tycko and Maxfield (4) used fluorescein coupled to α-2 macroglobulin to demonstrate that molecules that enter cells by receptor-mediated endocytosis are delivered to acidic endosomes before they reach lysosomes. Marsh *et al.* (5) showed that the acidic pH of endosomes is critical to the process by which lipid-enveloped viruses enter the cytoplasm after their cellular uptake by receptor-mediated endocytosis. Stone *et al.* (6) and Forgac *et al.* (7) demonstrated that clathrin-coated vesicles have proton pumps that can mediate their acidification *in vitro*. Glickman *et al.* (8) have made similar findings with regard to the presence of proton pumps in isolated Golgi vesicles.

The above experiments raise the possibility that a variety of organelles may maintain an acidic pH in intact cells. The ultrastructural identification of these acidic compartments has not been possible heretofore, because no method has been available to tag these compartments for visualization by electron microscopy. Here we report the development of such a method through the use of a newly synthesized basic congener of dinitrophenol (DNP). This compound concentrates in acidic compartments of cultured cells, where it can be visualized in the electron microscope after fixation and reaction with appropriately tagged anti-DNP antibodies. We have used this compound to demonstrate directly that cultured fibroblasts do indeed contain a large variety of acidic intracellular organelles in addition to lysosomes.

MATERIALS AND METHODS

Low-density lipoprotein (LDL) was radiolabeled with ^{125}I as described (9). Ferritin-conjugated LDL (LDL–ferritin) was prepared as described (10). A mouse hybridoma clone producing monoclonal antibody directed against DNP conjugated to ovalbumin (clone HDP1) (11) was kindly provided by J. B. Fleischman (Washington University School of Medicine, St. Louis, MO). The hybridoma cells were grown in the peritoneal cavity of mice and an IgG fraction of the anti-DNP antibody was prepared by passage of the ascites fluid over protein A–Sepharose (12). Affinity-purified, tetramethylrhodamine isothiocyanate-labeled anti-mouse IgG was obtained from Zymed [San Francisco, CA (Cat. no. 61-6515)]. Saponin, ovalbumin, and 3,3′-diaminobenzidine tetrahydrochloride were obtained from Sigma. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was obtained from Cappel [Westchester, PA (Cat. no. 3211-0081)].

Synthesis of 3-(2,4-Dinitroanilino)-3′-Amino-*N*-Methyldipropylamine (DAMP). A solution containing 353 mg of 3,3′-diamino-*N*-methyldipropylamine (Aldrich) and 200 mg of bromodinitrobenzene in 4 ml of acetonitrile was stirred at room temperature in the dark for 3 days. The solvent was evaporated, and the residue was purified by flash chromatography on silica gel (230–400 mesh) by elution with 10% isopropylamine/methyl alcohol ($R_f \approx 0.45$). The product (105 mg) was obtained as a yellow oil: H-NMR ($CDCl_3$) δ (90 MHz), 1.60–2.12 (m, 4H), 2.30 (s, 3H), 2.56 (t, 6H, $J \approx 7$ Hz), 2.80–3.16 (m, 2H), 3.48 (apparent q, 2H, $J \approx 7$ Hz), 6.94 (d, 1H, $J \approx 9$ HZ), 8.20 (dd, 1H, $J \approx 2.9$ Hz), 9.02 (d, 1H, $J \approx 2$ Hz).

Radiolabeled DAMP was prepared by adding 1 mCi of 1-

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Abbreviations: DAMP, 3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine; DNP, dinitrophenol; HRP, horseradish peroxidase; LDL, low density lipoprotein.

fluoro-2,4-dinitro[3,5-^{3}H]benzene (16.6 Ci/mmol; 1 Ci = 37 GBq; Amersham) in 0.3 ml of acetonitrile to a solution of 5 mg of 3,3'-diamino-*N*-methyldipropylamine in 1 ml of acetonitrile. After 3 days at room temperature, 6 mg of unlabeled DAMP was added, and the mixture was purified as described above to give [3,5-^{3}H]DAMP at a specific radioactivity of 18,800 dpm/nmol.

Cell Culture. Cultured fibroblasts, derived from a skin biopsy obtained from a normal subject, were grown in monolayer and set up for experiments in 60-mm dishes according to a standard format (9). All experiments were carried out on day 7 of cell growth in 2 ml of medium (either Dulbecco's modified Eagle medium or Ham's F-12 medium) containing 5% (vol/vol) lipoprotein-deficient serum (9) and the indicated addition.

Indirect Immunofluorescence. Fibroblast monolayers grown on glass coverslips were subjected to the protocol indicated in the figure legends and then fixed for indirect immunofluorescence. To localize DAMP, cells were fixed for 15 min at room temperature in 3% (wt/vol) paraformaldehyde in buffer A (10 mM sodium phosphate/150 mM sodium chloride/2 mM magnesium chloride, pH 7.4), after which they were washed once with 2 ml of 50 mM NH_4Cl and twice with buffer A. Each monolayer was permeabilized with 2 ml 0.1% (vol/vol) Triton X-100 in buffer A for 5 min at −10°C. Each coverslip was placed in a Petri dish (cell side up), covered with 60 μl of monoclonal mouse anti-DNP IgG (50 μg/ml), and incubated for 60 min at 37°C. After 4 washes with buffer A (15 min each), the cells were incubated with 60 μl of tetramethylrhodamine isothiocyanate-conjugated rabbit anti-mouse IgG (40 μg/ml) for 60 min at 37°C. The coverslips were washed as described above, mounted on glass slides, and viewed under a fluorescence microscope (13).

Immunoelectron Microscopy. Fibroblasts were incubated with DAMP as indicated in the figure legends and fixed in 2% paraformaldehyde in buffer B (10 mM sodium periodate/0.75 M lysine/37.5 mM sodium phosphate, pH 6.2). The cells were processed for indirect immunoperoxidase staining of intracellular sites by the method of Louvard *et al.* (14) using 50 μg of monoclonal mouse anti-DNP IgG per ml and 0.5 mg of HRP-conjugated goat anti-mouse IgG per ml. To localize HRP, cells were incubated at room temperature for 10 min with 0.2% (wt/vol) diaminobenzidine and 0.01% (vol/vol) H_2O_2. The cells were fixed in 2% (wt/vol) osmium tetroxide and 1% (wt/vol) potassium ferrocyanide in 0.1 M sodium cacodylate (pH 7.3), dehydrated, released from the dish in propylene oxide, pelleted, and embedded in araldite (15). Sections were cut on a Sorvall MT-2B microtome and viewed without staining in a JEOL 100CX electron microscope.

Other Assays. Protein content of cells and lipoproteins was measured by the method of Lowry *et al.* (16). Binding, internalization, and proteolytic degradation of ^{125}I-labeled LDL by fibroblast monolayers were measured by described methods (9).

RESULTS

Fig. 1 shows the structure of DAMP. This compound has a dinitroarene group that reacts with monoclonal antibodies to dinitrophenol. It also has a primary and tertiary amino group (apparent pKa, 10.6) that become protonated and positively charged at acidic pH. The primary amino group also allows the DAMP molecule to become covalently linked to proteins in the presence of aldehyde fixatives such a formaldehyde, which allows it to be retained in acidic organelles after fixation.

Based on previous experience with weak bases (1, 3), we postulated that DAMP would become concentrated in cellular compartments that have an acidic pH. To test this hypothesis, we incubated human fibroblasts with 30 μM DAMP for 30 min at 37°C. The cells were fixed with formaldehyde and the DAMP was visualized by fluorescence microscopy by reacting cells with a mouse monoclonal anti-DNP antibody followed by a rhodamine-coupled rabbit anti-mouse antibody. The DAMP was present in large perinuclear vacuoles, consistent with the known distribution of lysosomes, as well as in smaller punctate dots scattered through the cytoplasm (Fig. 2*A*). The carboxylic ionophore monensin is known to dissipate proton gradients by exchang-

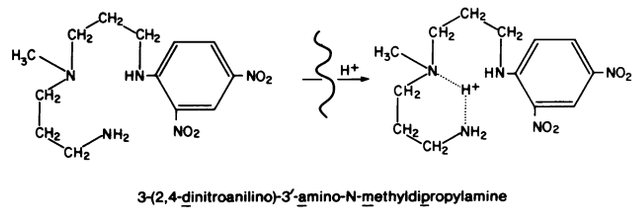

FIG. 1. Structure of DAMP in its unprotonated (*Left*) and protonated (*Right*) forms.

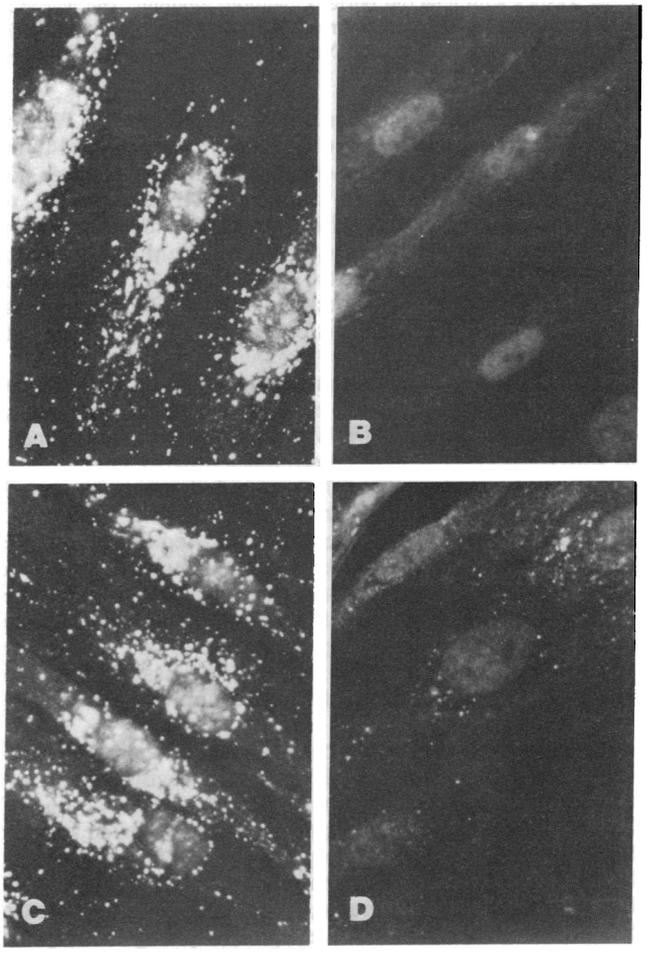

FIG. 2. Visualization by indirect immunofluorescence of the intracellular distribution of DAMP in fibroblasts treated with chloroquine or monensin. (*A* and *B*) Monolayers were incubated with 30 μM DAMP for 30 min at 37°C, after which the cells were washed with medium containing 10% lipoprotein-deficient serum and then treated for 5 min at 37°C in the absence (*A*) or presence (*B*) of 25 μM monensin. (*C* and *D*) Monolayers were treated for 30 min at 37°C in the absence (*C*) or presence (*D*) of 300 μM chloroquine, after which the cells received 30 μM DAMP for 30 min at 37°C. The cells in *A–D* were fixed, washed, and processed for indirect immunofluoresence localization of DAMP. (×400.)

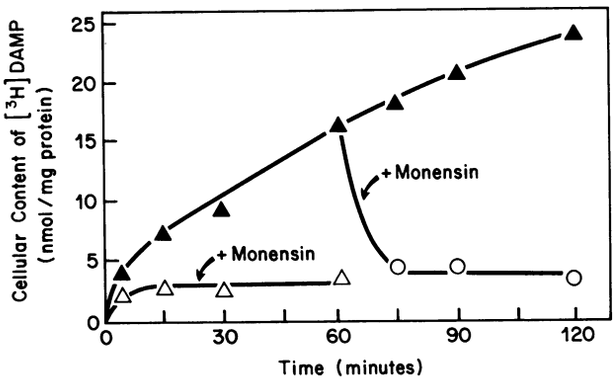

FIG. 3. Effects of monensin on the accumulation of [^{3}H]DAMP in fibroblasts. All monolayers were incubated at 37°C with 25 mM Hepes (pH 7.4) and 30 μM [^{3}H]DAMP (18,800 dpm/nmol). One set of monolayers received no monensin (▲). A second set of monolayers received 50 μM monensin at either zero time (△) or at 60 min (○). At the indicated time, each monolayer was washed 3 times (3 ml per wash) with ice-cold buffer C (0.15 M NaCl/2 mg of bovine serum albumin per ml/50 mM Tris chloride, pH 7.4), followed by two additional washes with ice-cold buffer C without albumin. The washed cells were dissolved in 1 ml of Triton X-100, and an aliquot (0.5 ml) was placed in 10 ml of Aquasol (New England Nuclear) for scintillation counting. Each value is the average of duplicate incubations.

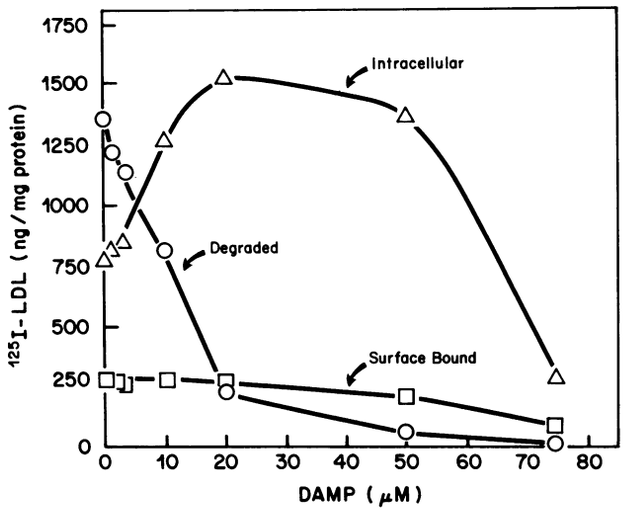

FIG. 4. Effect of various concentrations of DAMP on the surface binding, internalization, and degradation of ^{125}I-labeled LDL in fibroblasts. Duplicate monolayers of fibroblasts received the indicated concentration of DAMP and 10 μg of protein per ml of ^{125}I-labeled LDL (197 cpm per ng of protein) in the absence or presence of 400 μg of protein per ml of unlabeled LDL. After incubation at 37°C for 2 hr, the specific amount of surface-bound ^{125}I-labeled LDL (□), intracellular ^{125}I-labeled LDL (△), and degraded ^{125}I-labeled LDL (○) were determined by subtracting the amount of ^{125}I-labeled LDL in the presence of unlabeled LDL from that in the absence of unlabeled LDL.

ing protons for potassium ions across membranes (17). When the cells were allowed to take up DAMP, washed, and then exposed to monensin for a 5-min period, the DAMP was no longer visualized within the cell (Fig. 2*B*). Chloroquine, a weak base, increases the pH of intracellular compartments (1, 2). When cells were incubated with 300 μM chloroquine, uptake of DAMP was prevented (Fig. 2 *C* and *D*). The results of Fig. 2 suggest that the accumulation of DAMP, and its retention in cell organelles, is dependent on a pH gradient across the membrane of these organelles.

The qualitative conclusions of the immunofluorescence experiments were supported by quantitative measurements of the uptake of [^{3}H]DAMP. When fibroblasts were incubated with [^{3}H]DAMP at 37°C, there was a progressive increase in the cellular content (Fig. 3). Accumulation was prevented by the inclusion of monensin in the medium at zero time. Moreover, when the cells were first allowed to accumulate [^{3}H]DAMP and then incubated with monensin, there was a rapid loss of [^{3}H]DAMP from the cell, even though the [^{3}H]DAMP was maintained in the culture medium. These data indicate that monensin prevents the uptake and retention of DAMP and not its fixation in the tissue, since the radioactivity measurements did not require that the cells be fixed with aldehydes.

Inasmuch as DAMP is a base, its accumulation in lysosomes should increase the pH and block degradation of incoming proteins. Fig. 4 shows that low concentrations of DAMP (20 μM) block the degradation of ^{125}I-labeled LDL in fibroblasts and cause the undegraded lipoprotein to accumulate in the cell. Higher concentrations of DAMP (>50 μM) block ^{125}I-labeled LDL uptake, presumably because DAMP, like other bases, can prevent receptor recycling and trap the LDL receptor within the cell (13). The results of Fig. 4 with DAMP are similar to previous results that we have obtained with chloroquine (13).

To visualize intracellular DAMP by electron microscopy, we incubated fibroblasts with DAMP, fixed the cells with formaldehyde, permeabilized them with saponin, and incubated them with the mouse monoclonal anti-DNP antibody, followed by a HRP-coupled rabbit anti-mouse antibody. The peroxidase was visualized for electron microscopy by incubating the cells with diaminobenzidine and H_2O_2. Many different membrane-bounded compartments concentrated the DAMP (Fig. 5A). Included in this category were typical multivesicular bodies resembling lysosomes (Fig. 5*B*). These multivesicular bodies did not contain DAMP when the cells were incubated in the presence of monensin after exposure to DAMP (Fig. 5*C*). In addition to multivesicular bodies, the DAMP accumulated in intermediate-sized spherical or tubular vesicles resembling endosomes (Fig. 5*D*) and in very small spherical vesicles (Fig. 5*E*). We also noted that an occasional coated vesicle concentrated the DAMP (Fig. 5*F*); however, under the conditions of the current experiments, most of the apparent coated vesicles (Fig. 5*A*, arrows) did not contain peroxidase reaction product.

To determine whether some of the acidic compartments participated in receptor-mediated endocytosis of plasma LDL, we incubated cells with LDL–ferritin at the same time that they were exposed to DAMP. The results showed that the multivesicular bodies that contained DAMP also contained numerous ferritin particles (Fig. 5*G*). These structures did not contain peroxidase reaction product when the cells were incubated with LDL–ferritin in the absence of DAMP (Fig. 5*H*).

DISCUSSION

Experiments in this paper describe a new electron microscopic probe for studying acidic organelles in intact cells. Taking advantage of the previous demonstration that weak bases are concentrated in acidic compartments (1, 3), we synthesized a basic congener of DNP, referred to as DAMP. The virtue of this particular compound is that it can be fixed in the tissue with aldehydes and then visualized with anti-DNP antibodies by both light and electron microscopy.

The accumulation of DAMP in intracellular organelles was dependent on a pH gradient. Monensin, which disrupts pH gradients, and chloroquine, which neutralizes acidic compartments, prevented the uptake of DAMP by fibroblasts. Moreover, monensin caused previously accumulated DAMP to leave cells within minutes. These findings suggest that the DAMP establishes an equilibrium across membranes of cel-

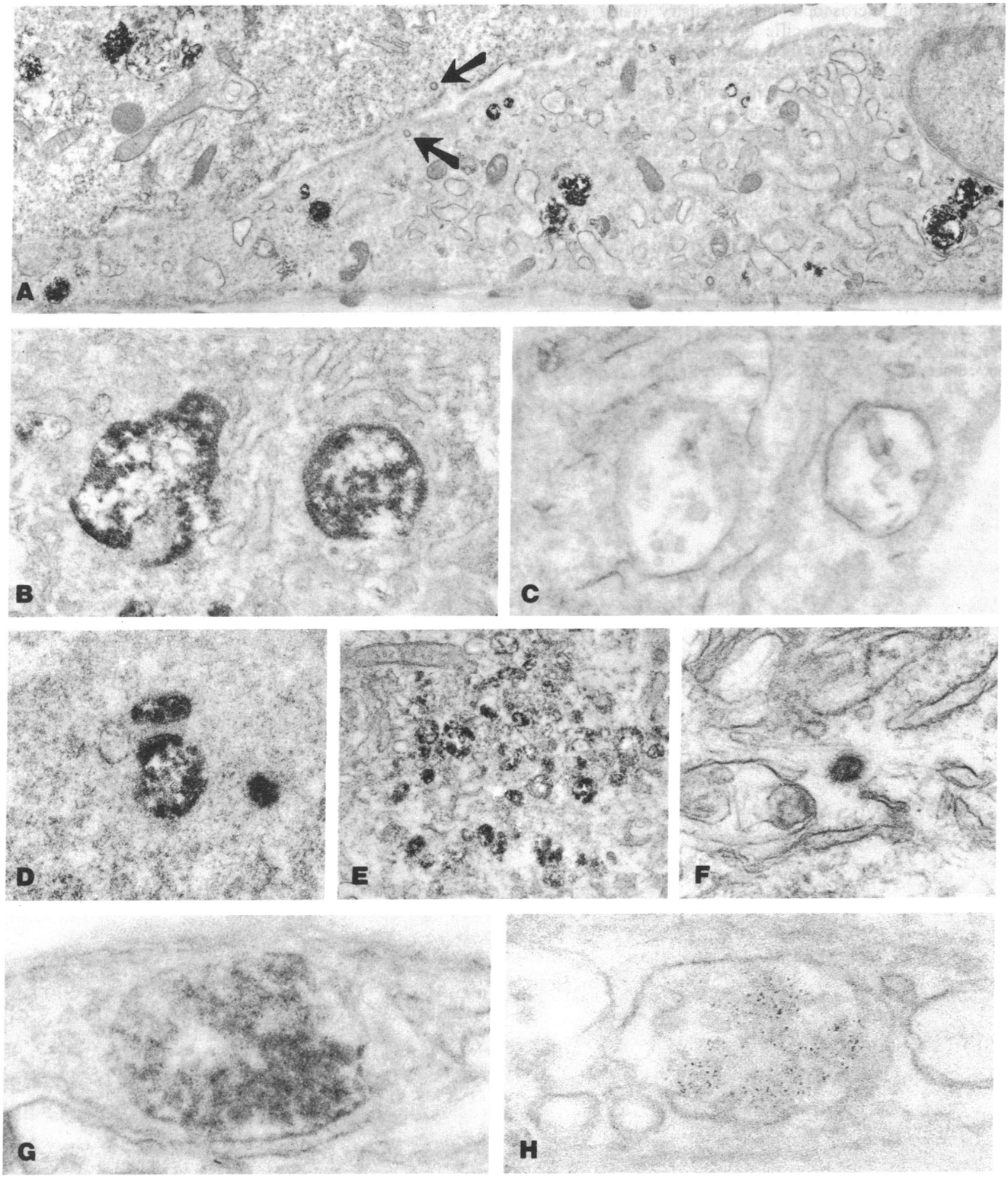

FIG. 5. Electron microscopic localization of DAMP and LDL–ferritin in fibroblasts. (*A–E*) Monolayers of fibroblasts were incubated with 50 μM DAMP for 30 min at 37°C, after which one set of cells (*C*) was washed and incubated for an additional 10 min in the presence of 25 μM monensin. (*F*) Monolayers were incubated with 30 μM DAMP for 15 min at room temperature. (*G* and *H*) Monolayers were incubated with 75 μg of LDL–ferritin per ml in the absence (*H*) or presence (*G*) of 30 μM DAMP for 60 min at 37°C. All cells in *A–H* were fixed, washed, and processed for indirect immunoperoxidase localization of DAMP. (*A*, ×11,075; *B*, ×26,850; *C*, ×42,435; *D*, ×38,415; *E*, ×17,050; *F*, ×60,615; *G*, ×84,280; *H*, ×83,300.)

lular organelles and that its concentration in acidic compartments reflects a steady-state balance between rates of entry and exit. Increasing the pH of the organelle shifts the equilibrium so that the DAMP rapidly leaves the structure.

The stage is now set to determine specifically which organelles within intact cells are acidified. For this purpose, we plan to combine electron microscopic immunocytochemistry (using antibodies against known organelle constituents)

with electron microscopic histochemistry (using enzyme markers) to identify specific organelles that concentrate DAMP. We are presently focusing our attention on the Golgi apparatus to determine whether one can demonstrate *in vivo* which segments of this organelle maintain an acidic pH.

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