Mis-Assembly of Clathrin Lattices on Endosomes Reveals a Regulatory Switch for Coated Pit Formation

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Abstract. The clathrin-coated pit lattice is held onto the plasma membrane by an integral membrane protein that binds the clathrin AP-2 subunit with high affinity. In vitro studies have suggested that this protein controls the assembly of the pit because membrane bound AP-2 is required for lattice assembly. If so, the AP-2 binding site must be a resident protein of the coated pit and recycle with other receptors that enter cells through this pathway. Proper recycling, however, would require the switching off of AP-2 binding to allow the binding site to travel through the endocytic pathway unencumbered. Evidence for this hypothesis has been revealed by the cationic amphiphilic class of drugs (CAD), which have previously been found to inhibit receptor recycling. Incubation of human fibroblasts in the presence of these drugs caused clathrin lattices to assemble on endosomal membranes and at the same time prevented coated pit assembly at the cell surface. These effects suggest that CADs reverse an on/off switch that controls AP-2 binding to membranes. We conclude that cells have a mechanism for switching on and off AP-2 binding during the endocytic cycle.

RECEPTOR-MEDIATED–endocytosis is a constitutive endocytic process that uses clathrin-coated pits as the vehicle for internalization. The membrane coat is a polygonal lattice composed of clathrin triskelion and AP-2 subunits (21). AP-2 holds the clathrin lattice on the membrane by forming a linkage between each triskelion and a high affinity binding site integral to the plasma membrane (24, 30). AP-2 may also modulate receptor clustering within the assembled pit (29). Receptors that are capable of clustering contain a characteristic β-turn motif in the cytoplasmic tail (4, 11).

The steady state number of coated pits on the surface of each cell type is usually constant (2). The fusion of secretory vesicles during exocytosis, however, can stimulate the formation of additional coated pits that retrieve excess membrane. For example, the fertilization of a sea urchin egg causes cortical granules to fuse with the plasma membrane, and this is immediately followed by a massive recruitment of clathrin-coated pits to the membrane (12). Likewise, nerve terminal-derived–coated vesicles contain almost exclusively membrane that was inserted during synaptic vesicle exocytosis (25). These observations raise questions about how the assembly of the pit is regulated and whether or not these regulatory mechanisms operate during each endocytic cycle.

Receptor-mediated–endocytosis can proceed for many hours after cells are incubated in the presence of protein synthesis inhibitors (2) because the component parts of each pit recycle. There are three populations of vesicles that receptors may encounter as they recycle (13, 28): early endosomes, late endosomes, and recycling vesicles. Clathrin and AP-2, by contrast, appear to dissociate from the membrane shortly after the formation of endosomes and remain soluble in the cytoplasm until used to assemble another coated pit (15, 31).

The high affinity AP-2 binding site responsible for holding the clathrin lattice on the membrane appears to be an integral membrane protein(s). Most likely it is internalized and recycled during endocytosis along with membrane proteins like the LDL receptor that reside in coated pits (8). This would require that AP-2 binding activity be turned off after internalization to prevent subunit binding and subsequent assembly of coated pits on endosomal membranes. So far, the only marker for the AP-2 binding site is the presence of the clathrin lattice.

Reagents that inhibit receptor recycling have been useful for identifying different stages in endocytosis (8). The most thoroughly studied group of inhibitors are those that elevate the pH of acidic compartments. They interfere with receptor recycling by inhibiting the dissociation of ligand from receptor, which leads to trapping of the receptor in endosomes (5). A less well understood group of inhibitors are the cationic amphiphilic drugs (9, 19). This class of drugs prevents recycling of receptors for transferrin (19), epidermal growth factor, and α_2-macroglobulin (9). The mechanism of inhibition is not known but probably does not involve the disruption of pH gradients in endosomes (9, 19).

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We now report that cationic amphiphilic drugs cause coated pits to disappear from the cell surface and reappear on endosomal membranes. The assembly of endosomal pits is inhibited at 18°C while at the same time disassembly of plasma membrane pits still takes place. These results suggest that the membrane protein(s) that binds AP-2 with high affinity is switched on and off during each endocytic cycle.

Materials and Methods

Materials

The following reagents were obtained from Sigma Chem. Co. (St. Louis, MO): bovine albumin fraction V (BSA, A-3294), bovine albumin fatty acid free (A-6003), Hepes (H-3375), chlorpromazine (C-8138) sphingosine (S-6879), imipramine (I-7374), calmidazolium (C-3930), chloroquine (C-6628), PMA (P-8139), W-7 (A-3281), and staurosporine (S-4400). The following reagents were from Calbiochem (La Jolla, CA): calphostin C (208725), H-7 (371955), K252a (420300), monensin (475896), and trifluoperazine (642150). The following reagents were purchased from Fluka Chemical Corp. (Ronkonkoma, NY): trinitrophenol (80450) and magnesium acetate (63047). Dextran sulfate was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). Bicinchoninic acid was from Pierce (Rockford, IL). 1,4-diazabicyclo(2.2.2)-octane was obtained from Aldrich (Milwaukee, WI). Glutaraldehyde (16320) and osmium tetroxide (19176) were purchased from Electron Microscopy Sciences (Fort Washington, PA). Goat anti-mouse IgG conjugated to FITC (62-6511) and goat anti-rabbit IgG conjugated to FITC (62-6111) were obtained from Zymed Laboratories, Inc. (South San Francisco, CA). Goat anti-rabbit IgG conjugated to rhodamine was from Fisher Scientific (Pittsburgh, PA). Goat anti-mouse IgG conjugated to 10-nm gold was obtained from Energy Beam Sciences (Agawam, MA). Mouse monoclonal antibodies that recognize the 100-kD α subunit of AP-2 (AP.6) and the clathrin heavy chain (X-22) were kindly provided by Dr. Francis Brodsky. Monoclonal IgG 2001 was used as a control for all monoclonal antibodies (6). A rabbit polyclonal anti-LDL receptor IgG was prepared as previously described (1). Human LDL was prepared and radiolabeled with ^{125}I as described (14).

Buffers and Media

Medium A was Eagle's essential minimum medium without bicarbonate supplemented with 20 mM Hepes (pH 7.4) and 2 mg/ml BSA. Buffer B was 150 mM NaCl, 50 mM Tris (pH 7.4) and 2 mg/ml BSA. Buffer C was buffer B without BSA. Buffer D was 50 mM NaCl, 10 mM Hepes (pH 7.4) and 4 mg/ml dextran sulfate. Buffer E was 100 mM sodium phosphate (pH 7.4), 150 mM NaCl. Buffer F was 68.2 mM KCl, 4.1 mM Mg acetate and 36.4 mM Hepes (pH 7.2). Buffer G was 100 mM sodium phosphate (pH 7.4).

Cell Culture

Cultured fibroblasts were derived from a skin biopsy obtained from a normal human subject. Cells were grown in monolayer and set up for experiments according to a standard format (14). On day 0, 7×10^4 cells were seeded into each Petri dish (100 × 15 mm) containing 10 ml DME supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (vol/vol) FCS. Fresh medium of the same composition was added on day 3. On day 5 of cell growth, each monolayer received 8 ml DME supplemented with penicillin, streptomycin, 5 μg/ml selenium, 5 μg/ml insulin, 5 μg/ml transferrin (ITS Premix), and 10% (vol/vol) human lipoprotein-deficient serum. Cells were used for all binding experiments on day 7 of cell growth.

Surface Binding of ^{125}I-LDL

LDL receptors were measured using a standard ^{125}I-LDL binding assay performed in a 4°C cold room (14). Cells were incubated in the presence and absence of 50 μM chlorpromazine in medium A at 37°C for the indicated times and placed on ice for 30 min. Each monolayer was quickly rinsed twice with 2 ml ice cold medium A. Each dish then received 2 ml ice cold medium A containing 10 μg/ml ^{125}I-LDL in the presence or absence of 500 μg/ml unlabeled LDL. After incubating at 4°C for 2 h, the medium was removed and each dish was rinsed three times with 3 ml ice cold buffer B. Each monolayer was then incubated twice for 10 min with

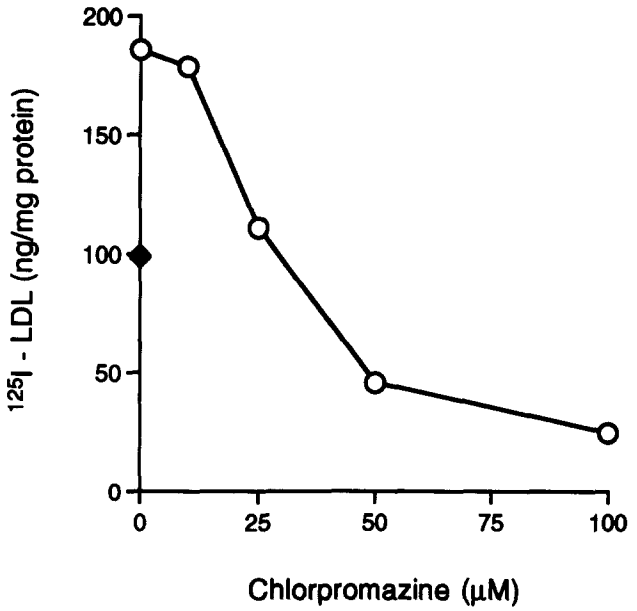


Figure 1. Chlorpromazine induces the disappearance of LDL receptors from the cell surface. Normal human fibroblasts were incubated in the presence of the indicated concentration of chlorpromazine (○) in medium A for 30 min at 37°C. At the end of the incubations, cells were assayed for ^{125}I-LDL binding as described in the methods. A separate set of cells (◆, on the ordinate) was incubated in the presence of 25 μM monensin for 30 min at 37°C before carrying out the ^{125}I-LDL binding assay. All measurements are the average of duplicate values. Non-specific binding was <8%.

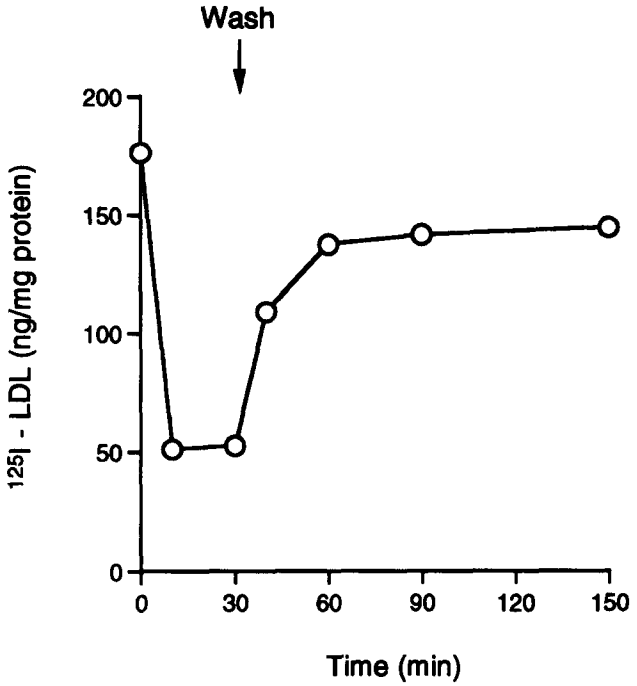


Figure 2. Chlorpromazine reversibly traps LDL receptors inside of the cell. Normal human fibroblasts were incubated in the presence of 50 μM chlorpromazine in medium A for the indicated time. After 30 min of incubation the cells were washed and further incubated in medium A in the absence of the drug for various times. At the end of each time period, the cells were assayed for ^{125}I-LDL binding as described. All measurements are the average of duplicate values. Non-specific binding was <5%.

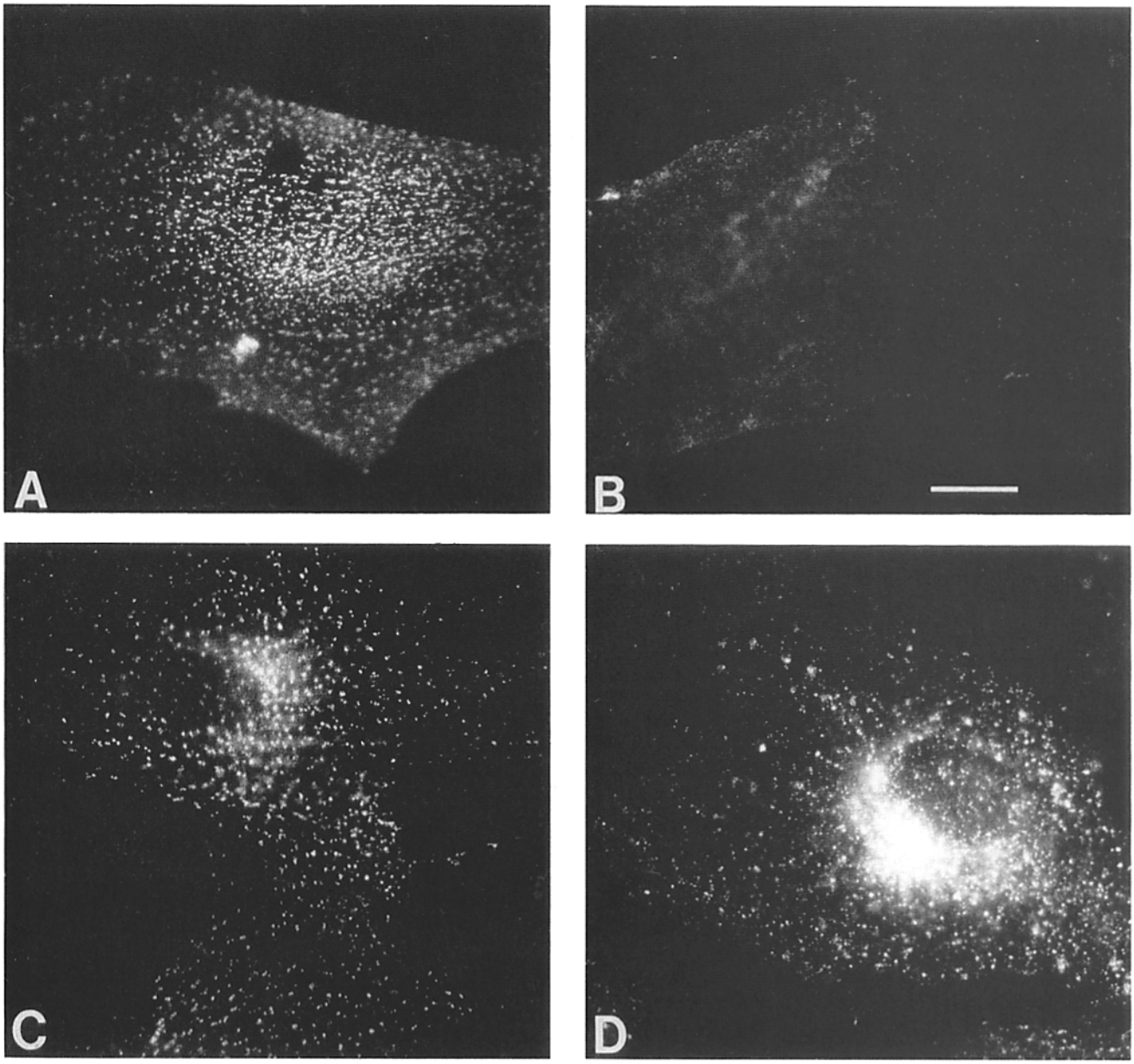


Figure 3. Effect of chlorpromazine on the distribution of LDL receptors in cultured fibroblasts. Normal human fibroblasts were incubated in the absence (*A* and *C*) and presence (*B* and *D*) of 100 μM chlorpromazine for 30 min at 37°C. The cells were then either incubated in the presence of anti-LDL receptor IgG at 4°C before fixation (*A* and *B*) or fixed, permeabilized and incubated with the anti-LDL receptor IgG. Both sets of cells were processed to localize the bound IgG as described. Bar, 15 μm.

3 ml ice cold buffer B, followed by one 10 min wash with 3 ml ice cold buffer C. After the extensive washing procedure, each dish received 2 ml of ice cold buffer D. The dishes were incubated at 4°C on a rotary shaker (40 rotations per min) for 60 min. The buffer was removed and a 1-ml aliquot was used to determine the amount of radioactivity released. The cells on the dish were then solubilized by incubating in 1 ml 0.1 N NaOH at room temperature for 30 min. An aliquot (50 μl) was used for protein determination.

Indirect Immunofluorescence Microscopy

Indirect immunofluorescence was carried out by standard methods (1) using AP.6 and X-22 IgGs at a concentration of 5 μg/ml and purified rabbit anti-LDL receptor IgG at 10 μg/ml. All antibodies were diluted in buffer E containing 1% BSA and spun at 12,000 rpm for 10 min before use. For double staining, cells were incubated with both antibodies simultaneously. After each antibody incubation, cells were washed four times in buffer E containing 0.1% BSA for 15 min. Each coverslip was rinsed with distilled water and mounted on a glass slide with a drop of 2.5% 1,4-diazabicyclo-(2.2.2)octane. Cells were observed and photographed using a Zeiss photomicroscope III with appropriate filter packages.

Immunogold Labeling

Cells were incubated in the presence or absence of chlorpromazine in medium A for the indicated time and then chilled to 4°C. Cells were briefly washed twice with 3 ml ice cold buffer F followed by 5 min in 3 ml of ice cold buffer F containing 20 μg/ml digitonin. Cells were quickly rinsed twice with ice cold buffer F and fixed for 30 min at 4°C in buffer G containing 3% paraformaldehyde, 3 mM KCl, 3 mM $MgCl_2$, and 3 mM trinitrophenol. After a 10-min incubation at room temperature in buffer G containing 100 mM NH_4Cl, cells were incubated sequentially at room temperature with 1% BSA in buffer G for 60 min, 10 μg/ml of either AP.6 or 2001 IgGs in buffer G for 3 h and a 1:50 dilution in buffer G of goat anti-mouse

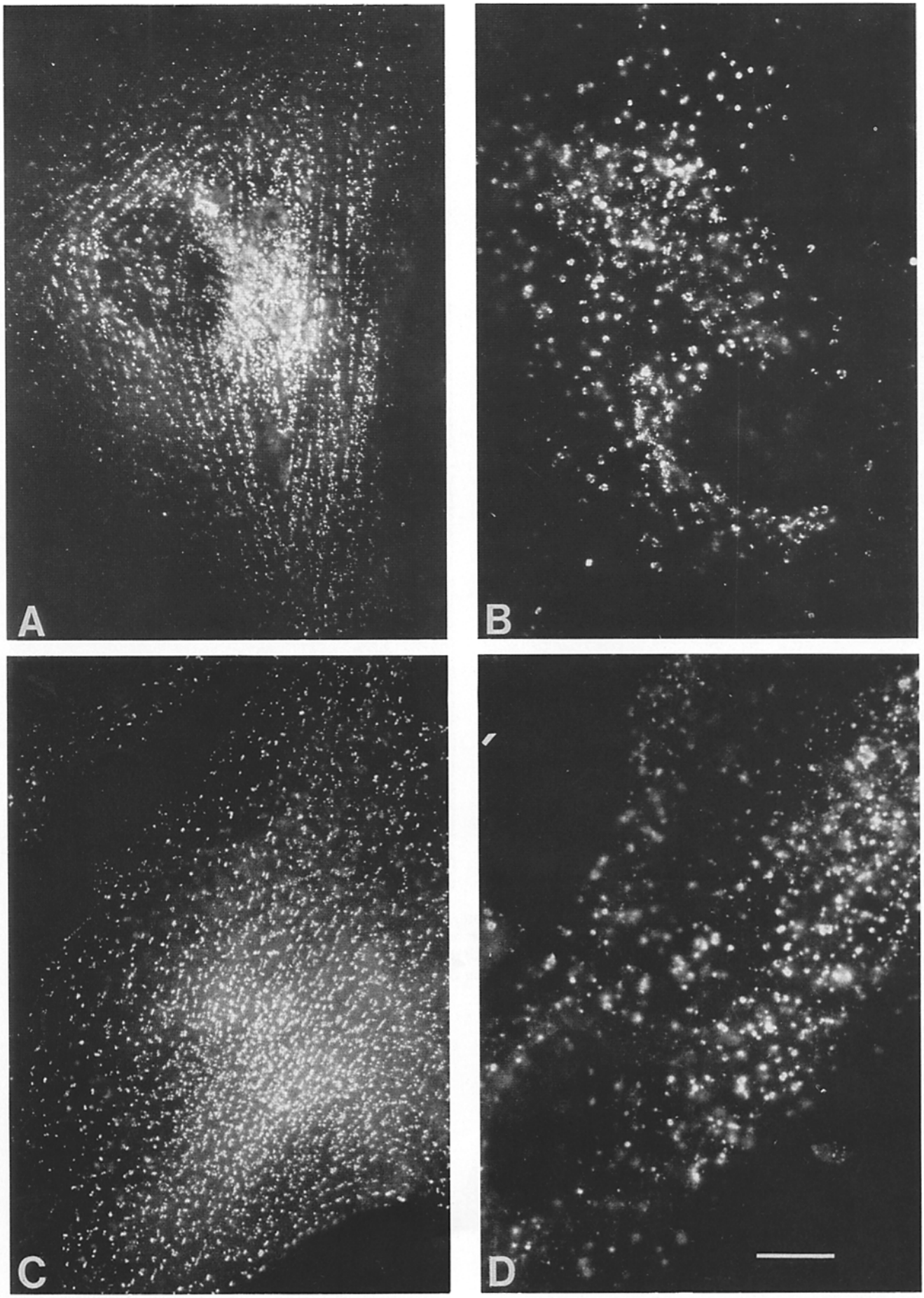
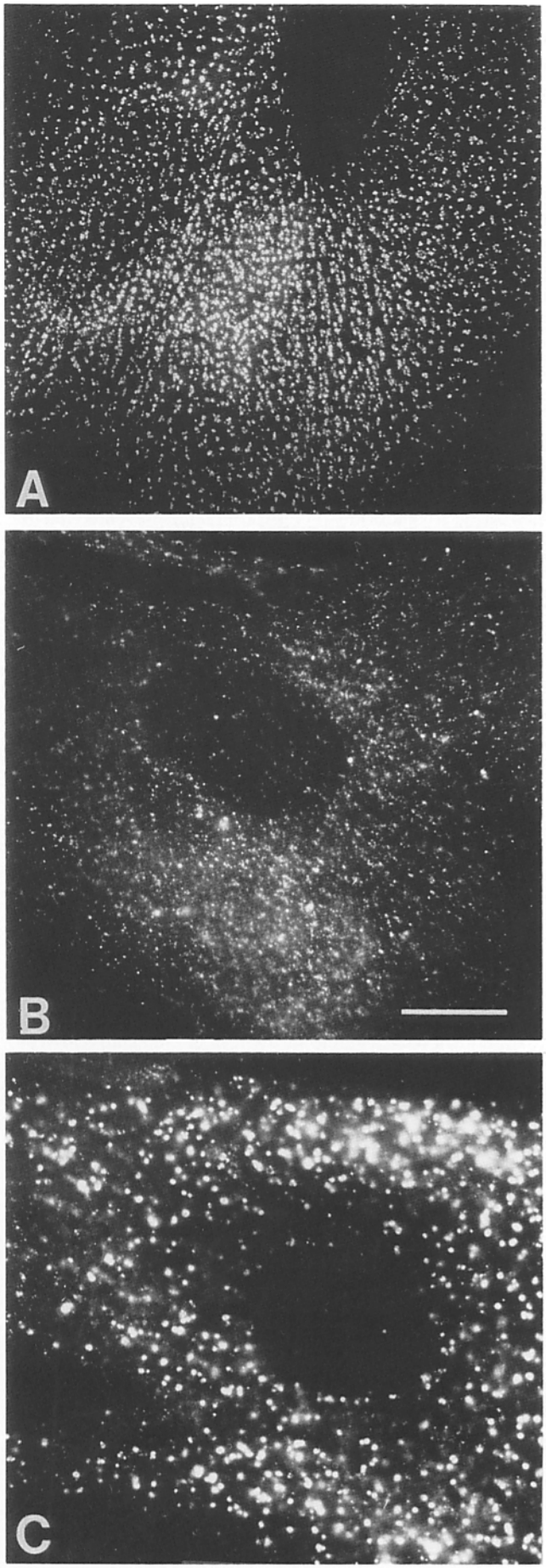


Figure 4. Chlorpromazine causes the relocation of both clathrin (*A* and *B*) and AP-2 (*C* and *D*) from coated pits to vesicles in cultured fibroblasts. Normal human fibroblasts were incubated in the presence of 100 μM chlorpromazine for 30 min at 37°C. At the end of the incubation, cells were fixed, permeabilized, and processed to localize either clathrin (*A* and *B*) or AP-2 (*C* and *D*) by indirect immunofluorescence. Bar, 15 μm.



IgG conjugated to 10 nm gold for 2 h. After each antibody incubation, cells were washed for 15 min at room temperature with three changes of buffer G containing 0.1% BSA. Cells were then fixed with 2.5% glutaraldehyde in buffer G at 4°C for 60 min and washed three times with buffer G. The fixed cells were postfixed at 4°C for 60 min with 1% osmium tetroxide in buffer G containing 1.5% potassium ferrocyanide and washed five times with buffer G. Samples were embedded in Eponate and thin sectioned by standard methods. All pictures were taken with a JEOL 100 CX electron microscope.

Other Methods

Protein determinations were made using the Micro Bicinchoninic Acid assay (35) using BSA as a standard.

Results

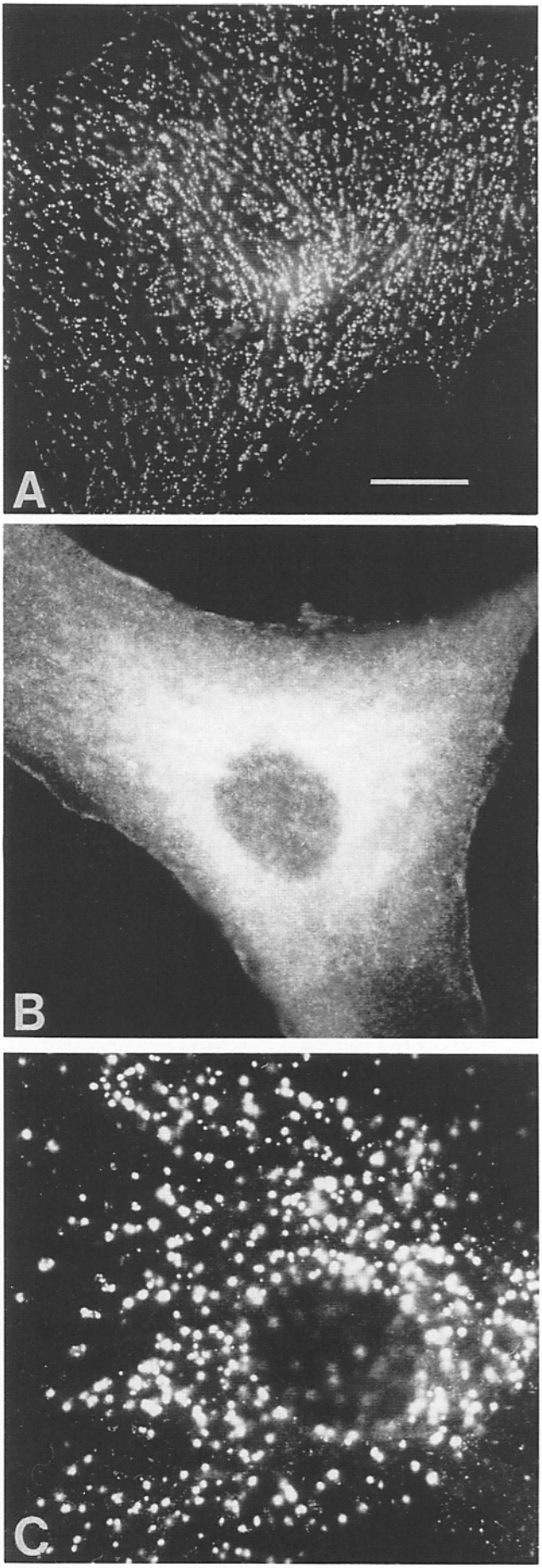
Chlorpromazine Inhibits LDL Receptor Recycling

LDL receptors become trapped in endosomes when cells are incubated in the presence of monensin (5). To see if cationic amphiphilic drugs also affect LDL receptor recycling, we incubated cells at 37°C for 30 min in the presence of different concentrations of chlorpromazine, and then assayed for ^{125}I-LDL surface binding at 4°C (Fig. 1, ○). We observed a progressive decline in the number of receptors on the cell surface as the drug concentration was increased. Only ~13% of the receptors remained after incubation in 100 μM chlorpromazine. By contrast, 25 μM monensin reduced surface receptors by <50% during the same incubation period (Fig. 1, ◆ on the ordinate).

Almost immediately after we exposed the cells to 50 μM chlorpromazine, the receptors began to disappear from the surface (Fig. 2, ○). Maximum loss occurred after 30 min of incubation. Receptors rapidly reappeared on the cell surface when we removed the drug (wash, Fig. 2). Eighty-three percent of the original number of receptors were on the cell surface after a 30-min incubation at 37°C in the absence of the drug.

We used indirect immunofluorescence to determine the fate of receptors during drug treatment (Fig. 3). Cells were incubated in the presence (Fig. 3, *B* and *D*) or absence (Fig. 3, *A* and *C*) of the drug at 37°C for various times and assayed for the presence of either surface (Fig. 3, *A* and *B*) or internal (Fig. 3, *C* and *D*) LDL receptors by indirect immunofluorescence. Initially most of the receptors were on the cell surface, arranged in a typical punctate pattern (Fig. 3 *A*). Thirty minutes of incubation in the presence of chlorpromazine caused receptors to leave the cell surface (compare Fig. 3 *A* with 3 *B*) and become located in small vesicles in the cytoplasm (compare Fig. 3 *C* with Fig. 3 *D*). We also noted that the amount of anti-receptor IgG binding to the Golgi area of the cell tended to increase (Fig. 3 *D*). After we removed the drug, most of the cells regained a normal surface staining pattern (data not shown).

Figure 5. Time course for the chlorpromazine-induced redistribution of AP-2 in fibroblasts. Normal human fibroblasts were incubated for 0 min (*A*), 10 min (*B*), or 30 min (*C*) in the presence of 100 μM chlorpromazine at 37°C. At the end of each incubation, the cells were fixed, permeabilized, and processed for indirect immunofluorescence localization of AP-2. Bar, 15 μm.



Chlorpromazine Causes Coated Pits to Assemble on Endosomes

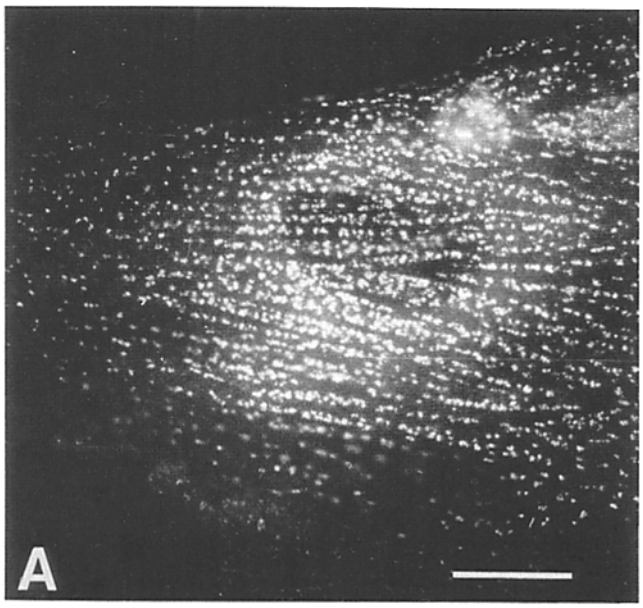
Monensin inhibits receptor recycling by trapping receptors in endosomes but it does not affect the distribution of clathrin-coated pits (5). This was not the case for chlorpromazine. When we treated cells with this drug for 30 min at 37°C, fixed, and then stained with anti-clathrin IgG (Fig. 4), the clathrin was no longer present on the cell surface in coated pits (compare Fig. 4 *A* with *B*). Instead, staining was associated with numerous vesicles deep within the cytoplasm of the cell. Often the staining was organized into quadrants at the periphery of each vesicle, suggesting that clathrin was only partially coating the membrane. The exact same staining pattern was seen when we replaced the anti-clathrin IgG with an IgG specific for AP-2 (compare Fig. 4 *C* with 4 *D*).

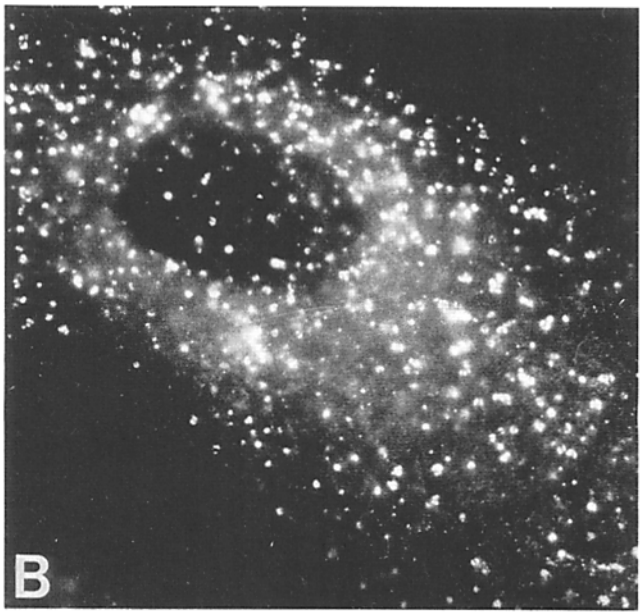
Immunofluorescence shows that anti-AP-2 IgG stains almost exclusively coated pits in human fibroblasts (Fig. 4 *C*), which indicates that this subunit is not normally present on early endosomes and must dissociate soon after coated vesicles form. To determine if chlorpromazine prevented the uncoating of the clathrin coat after vesicle formation, we used immunofluorescence to follow the distribution of AP-2 in cells that were incubated in the presence of chlorpromazine for various times at 37°C (Fig. 5). The earliest change observed (5–10 min of incubation) was the disappearance of AP-2 from the cell surface (compare Fig. 5 *A* with 5 *B*) and a concomitant increase in diffuse as well as particulate fluorescence in the cytoplasm of the cell. This was followed by the appearance of distinct vesicle staining after 15–30 min of incubation (Fig. 5 *C*).

These results suggested that chlorpromazine was not preventing the uncoating of coated vesicles. This was confirmed when we incubated cells in the presence of the drug at 18°C for 30 min (Fig. 6) and found that rather than being bound to a membrane, AP-2 was diffusely distributed in the cytoplasm of the cell (Fig. 6, compare *B* with *A*). If we maintained a set of these cells in the drug while shifting the temperature to 37°C, the diffuse AP-2 staining promptly changed to the vesicle staining pattern (Fig. 6, compare *C* with *B*). The same result was obtained with anti-clathrin IgG (data not shown). Therefore, the loss of clathrin/AP-2 from the plasma membrane and appearance on vesicles appear to be separate events.

The effect of chlorpromazine on the distribution of clathrin/AP-2 was reversible (Fig. 7). Cells incubated in the presence of chlorpromazine for 30 min at 37°C displayed AP-2 on vesicles instead of coated pits (Fig. 7, compare *A* with *B*). When we washed away the drug, a normal coated pit staining pattern was observed after just 30 min of incubation (Fig. 7, compare *B* with *C*).

Figure 6. The effects of chlorpromazine on AP-2 distribution at 18°C (*B*) vs 37°C (*C*). Either cells were not treated (*A*) or were incubated in the presence of 100 μM chlorpromazine for 30 min at 18°C (*B* and *C*). One set of these cells was maintained at 18°C (*B*) while the other (*C*) was shifted to 37°C before both were incubated in the presence of chlorpromazine an additional 30 min. The cells were all fixed, permeabilized, and processed for indirect immunofluorescence localization of AP-2. Bar, 15 μm.





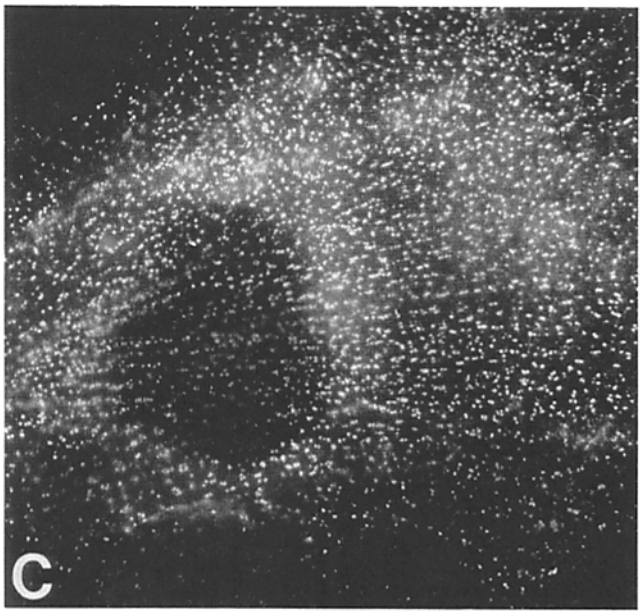


Table I. Effect of Various Drugs on Coated Pit Staining

Drug tested	Amount	Coated pit staining	Vesicle staining
Monensin	25 μM	+	
Sphingosine	20 μM		+
Trifluoperazine	25 μM		+
Imipramine	200 μM		+
Chlorpromazine	50 μM		+
W-7	100 μM		+
PMA	1 μM	+	
Chlorpromazine/PMA	50/1 μM		+
Calphostin C	1 μM	+	
H-7	50 μM	+	
Staurosporine	1 μM	+	
K252a	1 μM	+	
Calmidazolium	2 μM	+	
Chloroquine	200 μM	+	

Normal human fibroblasts were incubated in the presence of the indicated drug for 30 min at 37°C. At the end of the incubation, the cells were processed for immunofluorescence as described. Each sample was scored for either coated pit staining (Fig. 4 *A*) or vesicle staining (Fig. 4 *B*) as shown in Fig. 4.

To better define the mechanism of action of chlorpromazine, we assayed a number of other drugs for their effects on clathrin/AP-2 distribution (Table I). All of the phenothiazines, as well as sphingosine, caused clathrin/AP-2 redistribution. Compounds that elevate the pH of intracellular compartments (e.g., monensin) had no effect. Drugs that tend to inhibit calmodulin-dependent functions (e.g., W-7) caused mis-assembly while those that specifically inhibit protein kinase C (e.g., Calphostin C) did not affect AP-2 distribution. One exception was calmidazolium, which had no effect even though it specifically inhibits calmodulin dependent functions (7, 16).

The vesicle population decorated with clathrin/AP-2 was identified by electron microscopy (Fig. 8). We first incubated fibroblasts in the presence of chlorpromazine for 30 min, and then disrupted the cells and applied anti-AP-2 IgG followed by immunogold. Like normal cells, chlorpromazine treated cells had numerous multivesicular bodies (late endosomes). A portion of the outer membrane of many of these vesicles was decorated with a fuzzy coat that had the characteristic appearance of a clathrin lattice (*arrows*, Fig. 8, *A–C*, *E*). Some vesicles had more than one coated region (*arrow*, Fig. 8 *E*). Usually the coat was much larger than the typical size of a surface coated pit (Fig. 8, *A–C*, *E*). Each coat was also positively stained with anti-AP-2 IgG specific immunogold (*arrows*, Fig. 8, *A*, *C*, and *E*) but not nonimmune IgG (Fig. 8 *B*). We did not see any other membrane compartments that were labeled with either anti-AP-2 IgG gold or fuzzy coats. In addition, fuzzy coats were never seen on endosomal structures of untreated cells (Fig. 8 *D*).

Figure 7. Chlorpromazine induced relocation of AP-2 is reversible. Normal human fibroblasts were either not incubated (*A*) or incubated in the presence of 100 μM chlorpromazine for 30 min at 37°C (*B* and *C*). One set of the chlorpromazine incubated cells (*C*) was further incubated for 30 min at 37°C in the absence of the drug. All cells were fixed and processed for indirect immunofluorescence as described. Bar, 15 μm.

LDL Receptor and AP-2 Are Associated with Different Vesicles

Chlorpromazine could inhibit LDL receptor recycling because the receptor becomes entangled in endosomal coated pits. Therefore, we used indirect immunofluorescence to colocalize receptors and AP-2 at various times after treatment with chlorpromazine (Fig. 9). The two markers were initially together in coated pits at the cell surface (Fig. 9, *A* and *B*). Cells incubated for 10 min in the presence of chlorpromazine had LDL receptors in small vesicles that were devoid of anti-AP-2 IgG staining (Fig. 9, compare *C* with *D*). After 30 min of incubation, prominent endosomal staining of AP-2 was observed, but none of these structures were positive for anti-LDL receptor staining (Fig. 9, compare *E* with *F*). Therefore, AP-2 binding sites and LDL receptors are trapped in different compartments in response to chlorpromazine.

Discussion

Chlorpromazine causes the loss of coated pits from the surface of the cell and the appearance of clathrin coats composed of the same subunits on endosomal membranes. Chlorpromazine has revealed a previously unrecognized regulatory step in receptor-mediated-endocytosis: the membrane binding of AP-2 and the subsequent formation of clathrin lattices. A likely site of regulation is the binding of AP-2 to its high affinity binding site. These results are in agreement with the recent studies of Seaman et al. (33) who found that AP-2 binding to membranes and coated pit assembly can be modulated in broken cell preparations by either calcium or GTPγS.

AP-2 Receptor

Virshup and Bennett (36) were the first to show that membranes from both coated vesicles and whole brains could bind isolated APs. AP-binding activity was further defined by Mahaffey et al. (24) who discovered a membrane protein(s) within coated pits that binds AP-2 with high affinity. The AP-2 subunit consists of two distinct domains, referred to as the core and the appendage (18, 22), that can be separated after proteolysis. Only the core domain binds to the AP-2 binding site (30). In addition, this is the domain that binds clathrin during lattice assembly (30). Treatment of membranes with proteases removes the binding site (30), which prevents AP-2 binding and lattice assembly.

These data suggest that active AP-2 binding sites are the controlling element for clathrin lattice assembly. These sites appear to be integral membrane proteins and most likely recycle because other plasma membrane proteins that enter cells through coated pits recycle (8). To avoid making coated pits in recycling compartments, the AP-2 binding site must be switched off shortly after coated vesicle formation. It must then be switched back on upon returning to the cell surface. Chlorpromazine appears to be affecting the switching mechanism.

Cells incubated in the presence of chlorpromazine at 18°C lost surface clathrin/AP-2 but after the temperature was shifted to 37°C the subunits appeared on endosomes. Other workers have shown that endosomal fusion is retarded at 18°C while ligand internalization, receptor recycling, and coated vesicle uncoating are unaffected (10, 17). Perhaps the initial loss of clathrin/AP-2 in the above experiment is a consequence of the normal uncoating process while the chlorpromazine inactivated all of the AP-2 binding sites. When the temperature was shifted to 37°C in the presence of the drug, only the endosomal binding sites were activated. This is consistent with in vitro experiments showing that either Ca^{++} or GTPγS can switch lattice assembly from the plasma membrane to endosomes (33).

The LDL receptor and clathrin/AP-2 did not colocalize after cells were incubated in the presence of chlorpromazine. Therefore, the AP-2 binding site and the LDL receptor become trapped in different vesicle populations of the endocytic pathway. This implies that the cytoplasmic tail of receptors like the LDL receptor are not the high affinity AP-2 binding site. On the other hand, transfected cells that over express transferrin receptors appear to have an increased number of clathrin lattices (20, 26), which suggests that receptor tails can influence pit assembly.

We conclude that chlorpromazine activates a population of recycling AP-2 binding sites that normally are inactive. The natural cationic amphiphilic molecule sphingosine also causes anomalous binding of clathrin/AP-2 on endosomes when added exogenously to cells (Table I). Endogenous sphingosine-1-phosphate appears to regulate cell motility, tumor cell invasiveness, and cell proliferation (23, 32). Recent experiments with clathrin deficient *Dictyostelium* have suggested that clathrin-coated pits are important for proper chemotactic motility (27). These observations raise the possibility that endogenous CADs might control the direction of cell movement by regulating coated pit assembly.

Mechanism of Action of CAD

CADs affect the activity of many different enzymes. For example, they inhibit phosphatidic acid phosphohydrolase, CTP:phosphocholine cytidyltransferase, protein kinase C, Ca^{++}/calmodulin-dependent enzymes, Na^{+},K^{+}-ATPase, but activate phospholipase D (23). Protein kinase C (3) and calmodulin (16) have both been found to affect receptor traffic during receptor-mediated-endocytosis.

PMA, an activator of protein kinase C, stimulates the hyperphosphorylation and the internalization of transferrin receptors (for review see 34). Trifluoperazine, a CAD that causes clathrin/AP-2 assembly on endosomes (Table I), has the same effect as PMA (19). In contrast to these results, we found that PMA had no effect on clathrin/AP-2 distribution (Table I). Even pretreatment of cells with PMA before chlorpromazine did not prevent the relocation of clathrin/AP-2 to endosomes. Calphostin C, a potent inhibitor of protein kinase C, also did not affect clathrin distribution (Table I). These results suggest that protein kinase C is not involved in regulating pit assembly.

Inhibitors of calmodulin function also impair transferrin receptor recycling (16). We had less success obtaining direct information on the role of calmodulin in clathrin/AP-2 relocation. Two specific inhibitors of Ca^{++}/calmodulin-dependent enzymes that were tested are W-7 and calmidazolium. The former caused clathrin/AP-2 relocation to endosomes while the latter had no effect (Table I). The recent finding by Seaman et al. (33) that high Ca^{++} can cause the misassembly of coated pits on endosomal membranes in vitro

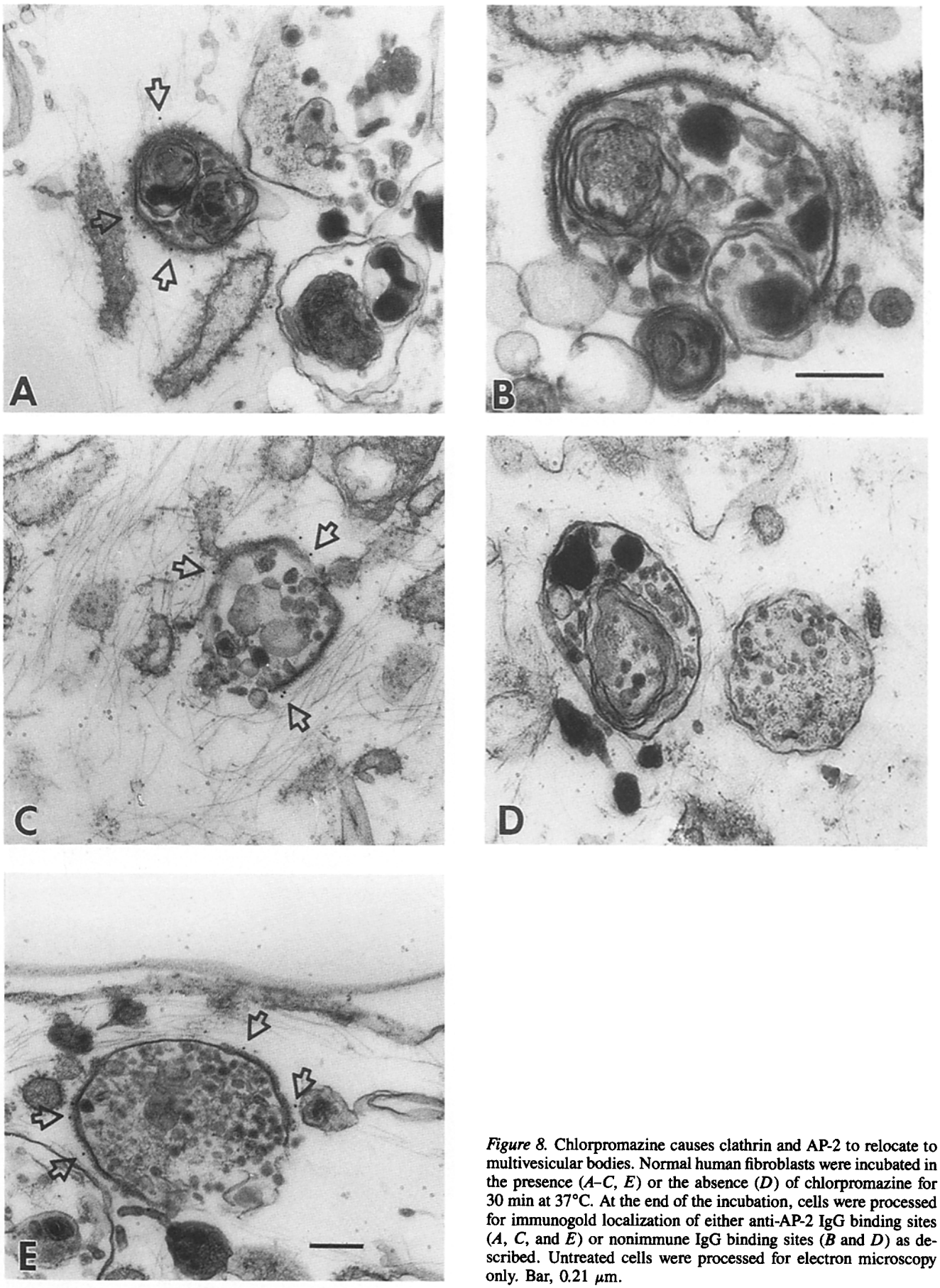
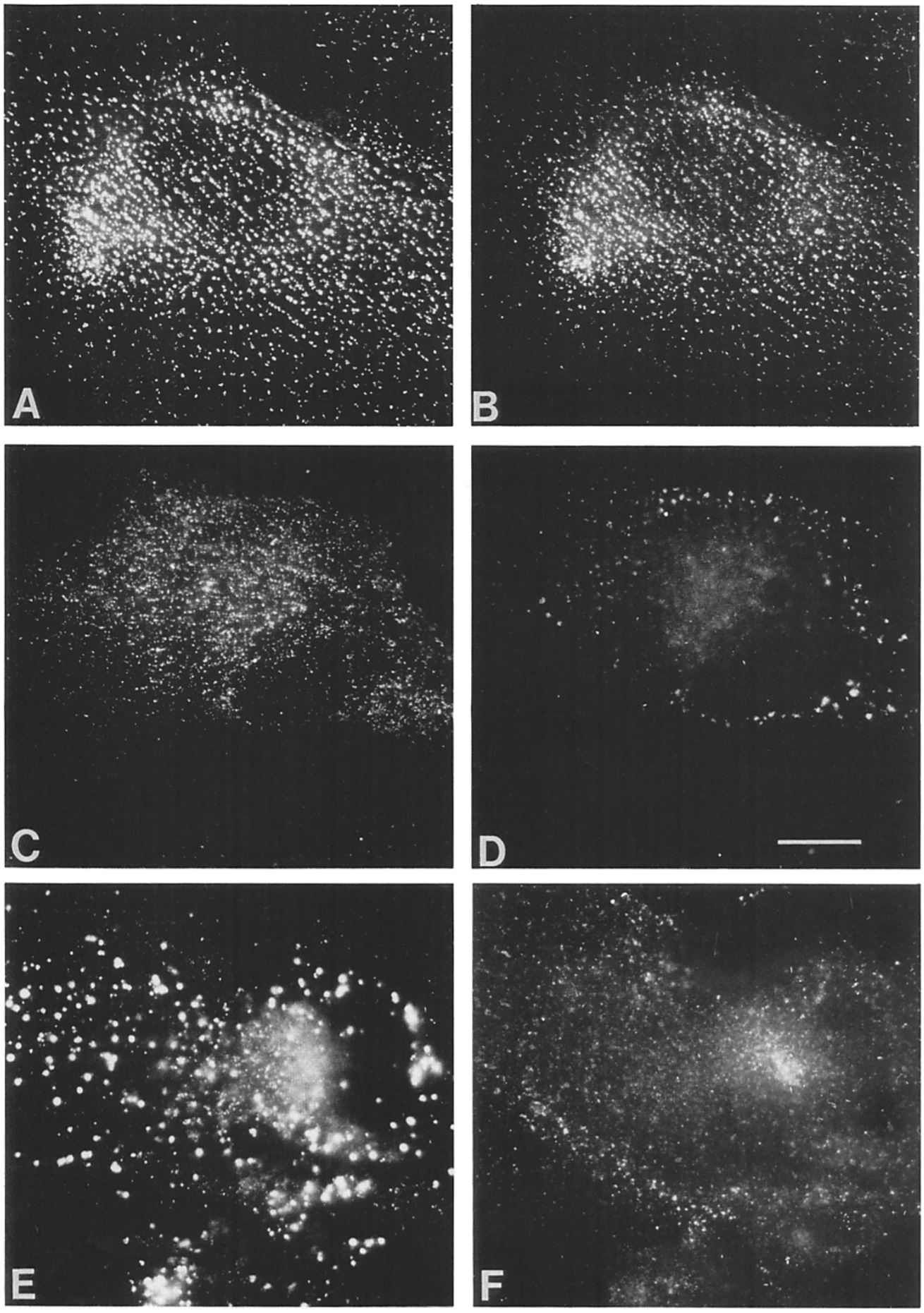


Figure 8. Chlorpromazine causes clathrin and AP-2 to relocate to multivesicular bodies. Normal human fibroblasts were incubated in the presence (*A–C*, *E*) or the absence (*D*) of chlorpromazine for 30 min at 37°C. At the end of the incubation, cells were processed for immunogold localization of either anti-AP-2 IgG binding sites (*A*, *C*, and *E*) or nonimmune IgG binding sites (*B* and *D*) as described. Untreated cells were processed for electron microscopy only. Bar, 0.21 μm.


A
B
C
D
E
F

suggests that a Ca^{++} regulated step might be involved in AP-2 binding.

Control of Endocytosis

Chlorpromazine has revealed a regulatory site for controlling coated pit assembly. The effects of the drug indicate that normally the activation of pit assembly at the cell surface is somehow synchronized with the inactivation of pit assembly on newly formed endosomes. This suggests the existence of a molecular switch, possibly one that is controlled by calcium or GTP (33), that turns on and off AP-2 binding to its binding site. This switch may play an important role in regulating coated pit formation in instances where massive recruitment of membrane to the cell surface occurs during exocytosis.

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Figure 9. Colocalization of AP-2 (*A*, *C*, and *E*) and LDL receptor (*B*, *D*, and *F*) before (*A* and *B*) and after (*C–F*) treatment with chlorpromazine. Normal human fibroblasts were either not incubated (*A* and *B*) or were incubated in the presence of 50 μM chlorpromazine for 10 min (*C* and *D*) or 30 min (*E* and *F*). At the end of the incubations, the cells were fixed and processed for immunofluorescence as described. Bar, 15 μm.