Filipin-sensitive Caveolae-mediated Transport in Endothelium: Reduced Transcytosis, Scavenger Endocytosis, and Capillary Permeability of Select Macromolecules

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Abstract. Caveolae or noncoated plasmalemmal vesicles found in a variety of cells have been implicated in a number of important cellular functions including endocytosis, transcytosis, and potocytosis. Their function in transport across endothelium has been especially controversial, at least in part because there has not been any way to selectively inhibit this putative pathway. We now show that the ability of sterol binding agents such as filipin to disassemble endothelial noncoated but not coated plasmalemmal vesicles selectively inhibits caveolae-mediated intracellular and transcellular transport of select macromolecules in endothelium. Filipin significantly reduces the transcellular transport of insulin and albumin across cultured endothelial cell monolayers. Rat lung microvascular permeability to albumin in situ is significantly decreased after filipin perfusion. Conversely, paracellular transport of the small solute inulin is not inhibited in vitro or in situ. In addition, we show that caveolae mediate the scavenger endocytosis of conformationally modified albumins for delivery to endosomes and lysosomes for degradation. This intracellular transport is inhibited by filipin both in vitro and in situ. Other sterol binding agents including nystatin and digitonin also inhibit this degradative process. Conversely, the endocytosis and degradation of activated α_2-macroglobulin, a known ligand of the clathrin-dependent pathway, is not affected. Interestingly, filipin appears to inhibit insulin uptake by endothelium for transcytosis, a caveolae-mediated process, but not endocytosis for degradation, apparently mediated by the clathrin-coated pathway. Such selective inhibition of caveolae not only provides critical evidence for the role of caveolae in the intracellular and transcellular transport of select macromolecules in endothelium but also may be useful for distinguishing transport mediated by coated versus noncoated vesicles.

CLATHRIN-coated vesicles are the best characterized of the vesicular carriers and with the clustering of receptors at the cell surface provide a specific delivery system for a multitude of ligands from the plasmalemma to endosomes for cellular processing (Goldstein et al., 1985). For many years, the smaller noncoated plasmalemmal vesicles (also known as caveolae) received a second billing to the larger and more structurally impressive coated variety. Although the existence of caveolae has been known for 40 years (Yamada, 1955; Palade, G. E. 1953. *J. Appl. Physics.* 24:1424; and Palade, G. E., *Anat. Rec.* 1958. 130:467), defining their precise function(s) has been somewhat difficult. For decades, noncoated vesicles were thought at best to function only in fluid-phase endocytosis and because of this conception, they were frequently called pinocytic or "drinking" vesicles (for review see Silverstein et al., 1977). One of the interesting functions originally ascribed to these structures upon their discovery in endothelium was a potential role in the transport of "quanta" of molecular cargo from the blood across the endothelium to the tissue interstitium; again, fluid phase uptake was envisioned (Palade and Bruns, 1968). In the interim, more studies have focused on the discovered role of clathrin-coated vesicles in receptor-mediated endocytosis.

Recent studies have brought renewed interest in caveolae because they may be responsible for various important cellular processes ranging from signal transduction to a variety of receptor-mediated transport processes including transcytosis, endocytosis and potocytosis. Distinct endocytosis independent of the clathrin-coated pathway has been described in a variety of cell types for various probes including fluid-phase markers such as sucrose, ferritin, and horseradish peroxidase (Oliver, 1982; Van Deurs and Nicausen, 1982) and membrane-bound probes such as cationized ferritin, ricin (Van Deurs et al., 1990), Con A (Hansen et al., 1991), toxins

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(Montesano et al., 1982; Trans et al., 1987), low-density lipoprotein (LDL)[1] (Mommaas-Kienhuis et al., 1985), viruses (Kartenbeck et al., 1989), and specific antibodies recognizing β-adrenergic receptors (Raposo et al., 1989), glycosylphosphatidylinositol-anchored cell membrane proteins (Keller et al., 1992; Bamezai et al., 1992), and human lymphocyte antigens class I antigens (Huet et al., 1980). It is not at all clear that one type of noncoated vesicle is involved in the endocytosis of all of these molecules.

Caveolae located abundantly on the surface of certain continuous vascular endothelium may be involved not only in fluid-phase and receptor-mediated endocytosis but also transcytosis (for review see Schnitzer, 1993). Binding sites and in some cases specific proteins have been identified on the endothelial cell surface that appear to be responsible for receptor-mediated transcytosis of a variety of blood-borne ligands including insulin (King and Johnson, 1985) and the carrier proteins, transferrin (Wagner et al., 1983; Jeffries et al., 1984) and albumin (Schnitzer and Oh, 1994). Native and modified albumins bind the cell surface (Schnitzer et al., 1988; Schnitzer and Bravo, 1993) via distinct receptor proteins (Schnitzer et al., 1992; Schnitzer and Oh, 1994). Modified albumins bind gp30 and gp18 and are internalized for degradation (Schnitzer and Bravo, 1993). In contrast, native albumin, acting as a carrier for fatty acids and other small ligands (Galis et al., 1988), binds albondin for caveolae-mediated delivery across the cell to the underlying tissue cells (Milici et al., 1987; Schnitzer and Oh, 1994). Anderson et al. (1992) propose that caveolae also participate in a different form of transport called potocytosis wherein small molecules bind to receptors which localize within caveolae to create a specialized environment with a high ligand concentration that facilitates its transmembrane transport directly into the cytoplasm by distinct protein channels.

Caveolae are found most abundantly in certain endothelia of the continuous type but are also found to varying degrees in many, if not all, cell types including fibroblasts, adipocytes, and muscle cells. Cholesterol is a very important component of caveolae that appears to be required to maintain the structural integrity of this vesicular complex. At equilibrium, the plasmalemma contains about 90% of the total cholesterol found in many cells. Caveolae disappear in cells that are depleted of cholesterol and exposure of cells to sterol-binding agents such as filipin preferentially removes cholesterol from the plasmalemma which causes disassembly of caveolae and unclustering of receptors found in caveolae (Rothberg et al., 1990 and 1992). Most endothelia of the continuous type contain a very abundant population of caveolae that have been implicated in the endocytosis or transcytosis of select macromolecules (for review see Schnitzer, 1993). Although many recent studies provide support for a role of caveolae in transport, this function, especially for endothelium, has remained controversial, at least in part because there has not been any way to selectively inhibit this pathway. Therefore, based on this information, we decided to examine the effects of exposing endothelium to filipin on the cell surface density of caveolae and on their putative function in endocytosis and transcytosis both in situ and in vitro.

1. *Abbreviations used in this paper*: BAEC, bovine aortic endothelial cells; BLMVEC, bovine lungs microvascular cells; LDL, low-density lipoprotein; PS, permeability-surface area; RFC, rat epididymal fat pads.

Materials and Methods

Materials

Reagents and other supplies were obtained from the following sources: FCS, and PBS from GIBCO-BR (Grand Island, NY); gelatin from Difco Laboratories (Detroit, MI); crystallized BSA and [^{14}C]inulin from ICN Biochemicals (Cleveland, OH); filipin, nystatin, digitonin, FITC conjugated BSA, bovine insulin, and ovalbumin from Sigma Chemical (St. Louis, MO); DME from Irvine Scientific (Irvine, CA); Iodogen (1,4,5,6-tetrachloro-3a,6a-diphenylglycouril), Triton X-100, BCA protein assay from Pierce Chemical Co. (Rockford, IL); Na^{125}I from Amersham Corp. (Arlington Heights, IL); α_2-macroglobulin from Boehringer-Mannheim Biochemicals (Indianapolis, IN); ^{14}C-labeled BSA from Du Pont/New England Nuclear (Boston, MA); Kodachrome, Kodacolor, and Tri-X Pan films from Eastman Kodak (Rochester, NY) and all tissue culture plasticware from Costar Corp. (Cambridge, MA) or Corning (Wilmington, DE).

Cell Culture

Microvascular cells, derived from rat epididymal fat pads (RFC) and bovine lungs microvascular (BLMVEC), along with bovine aortic endothelial cells (BAEC) were obtained, grown in culture, and tested periodically for endothelial markers as in our past work (Schnitzer, 1992; Schnitzer and Oh, 1994; Schnitzer et al., 1994).

Albumin Probes

As described and characterized previously (Schnitzer et al., 1992), BSA was modified in the following ways: (*a*) conjugation to 5–10-nm colloidal gold particles (A-Au); and (*b*) maleic anhydride treated (Mal-BSA). These probes along with native, unmodified BSA were radioiodinated using Iodogen as in our previous work (Schnitzer and Pinney, 1992).

Surface Binding Assay

Binding assays were performed as described previously (Schnitzer et al., 1988*a*; Schnitzer and Pinney, 1992). Briefly, confluent cell monolayers grown on 6-well trays were washed extensively, incubated for 10 min with filipin in DME or DME alone, and then incubated for 20 min at 4°C with ^{125}I-labeled BSA, ^{125}I-labeled A-Au or ^{125}I-labeled Mal-BSA in DME. After washing (3 × 1 min) with ice-cold DME, the cells were lysed using 5% Triton X-100 and 1% SDS, scraped from the wells, and then pipetted into vials for counting of radioactivity using a Beckman Gamma 5500B. Duplicate wells were run for each concentration used. Specific binding was quantified and final calculations were performed as in Schnitzer and Pinney (1992).

Cell-associated Binding, Uptake, and Degradation Assay at 37°C

As per our past protocol (Schnitzer and Bravo, 1993), confluent BLMVEC, RFC, and BAEC monolayers were washed extensively before incubation at 37°C for various times with ^{125}I-ligand. For insulin degradation studies, the BLMVEC were kept in DME without fetal calf serum at 37°C for 1 h before starting the assay. At specified times, all of the media from each well was removed and saved. The cells were immediately washed with ice-cold DME, lysed, and counted for radioactivity as described above. The media saved from each well was subjected to 10% TCA precipitation to determine the extent of ligand degradation. As shown in Schnitzer and Bravo (1993), the TCA-soluble counts were degradation products from cellular processing of the radioactive ligand whereas the TCA insoluble counts (pellet) represented the undegraded ligand remaining in the medium.

Fluorescence Microscopy

Washed BLMVEC grown on gelatin-coated glass coverslips were incubated at 37°C for 10 min with DME plus 1 mg/ml BSA as the control or DME plus BSA supplemented with the potential inhibitor (1–5 μg/ml of filipin or 1 mg/ml Mal-BSA). Then, this media was removed and replaced with

FITC-conjugated A-Au (OD at 515 nm = 0.2) in DME plus BSA. After 30 min the cells were washed (3× ice cold DME for 1 min) and fixed in 4% paraformaldehyde in PBS for 1 h. The coverslips with the cells were mounted on a glass slide and then were examined with a Zeiss Axiophot fluorescence microscope set for the appropriate excitation/emission viewing and photography (Kodachrome, Kodacolor, and/or Tri-X Pan films).

Electron Microscopy

After incubation with A-Au for 5, 10, 30 min at 37°C, confluent BLMVEC and BAEC monolayers were washed, fixed, and processed on the 35-mm plastic dishes for standard transmission electron microscopy as in our previous work (Schnitzer et al., 1988*a*). Other BLMVEC monolayers were treated with filipin at 5 μg/ml in DME for 0 to 60 min before processing for electron microscopy. The cell surface density of caveolae for untreated and treated BLMVEC was assessed quantitatively as in Schnitzer et al. (1994*a*). Some BLMVEC (control and filipin treated) were incubated with A-Au before processing for electron microscopy.

In Vitro Transport across Endothelial Cell Monolayers

As in our previous work (Schnitzer and Oh, 1994), BLMVEC seeded onto Transwell filters were used to measure transport across confluent cell monolayers. ^{125}I-labeled insulin, ^{125}I-labeled BSA and [^{14}C]inulin were used as probes to assess transport across BLMVEC monolayers either treated or untreated with filipin in DME for 10 min at 37°C.

Transport and Permeability Measurements in the Rat Lung In Situ

The capillary permeability of BSA in the rat lung was assessed as in our past work (Schnitzer and Oh, 1994). The tissue uptake of ^{125}I-labeled A-Au, ^{125}I-labeled BSA, ^{14}C-labeled BSA, and [^{14}C]inulin was measured similarly by perfusion through the pulmonary artery except that a 3-min perfusion of radiolabeled tracer was used instead of a 2-min perfusion. Briefly, for all tracers, the lung vasculature was flushed for 3 min with oxygenated Ringer's solution containing 30 μM of nitroprusside, for 90 s with Ringer's solution with or without filipin, for 3 min with radioactive tracer in Ringer's, and then for 3 min with Ringer's. Rat lung tissue samples were weighed and measured for radioactivity so that transport and capillary permeabilities could be calculated and compared with control lungs not treated with filipin.

Results

Examination of Modified Albumin Endocytosis in Cultured Endothelial Cells

Recently, we provided evidence that gp30 and gp18 mediate the avid binding, internalization, and degradation of modified albumins (Schnitzer et al., 1992; Schnitzer and Bravo, 1993); however, the intracellular mechanism and pathway for the processing of these ligands remains unclear. Here, we first examined the kinetics of the binding, endocytosis and degradation of modified albumins by cultured monolayers of BLMVEC and BAEC. The BLMVEC are advantageous because relative to other endothelial cells, they have a more abundant population of caveolae (Schnitzer et al., 1994) and interact more avidly with modified albumins. We then focused on A-Au as a typical modified albumin probe to assess its subcellular distribution and movement between specific intracellular compartments.

Analysis of Surface Binding, Internalization, and Degradation of Modified Albumins. Modified albumin binding to the surface of BLMVEC and BAEC was assessed at 4°C using confluent monolayers that were incubated with ^{125}I-labeled A-Au or ^{125}I-labeled Mal-BSA. Cellular processing of these ligands was also assessed at 37°C to evaluate uptake and degradation (see Materials and Methods). Because the observed surface binding, internalization, and degradation profiles were very similar to our past findings using the RFC cell monolayers (Schnitzer and Bravo, 1993), we report here only the significant differences which were: (*a*) a slightly slower equilibration time for BLMVEC and BAEC surface binding of ^{125}I-labeled A-Au and ^{125}I-labeled Mal-BSA which required 30–40 min in comparison to the 20–30 min necessary for the RFC monolayers (data not shown); and (*b*) a larger capacity (approximately fivefold) of high affinity binding sites for the BLMVEC with a greater affinity (~5–10-fold) than the RFC cells. Fig. 1 shows the Scatchard analysis of the high affinity binding of Mal-BSA. The apparent equilibrium binding constant (K_d) for the higher affinity binding was 5.7 nM (0.38 μg/ml) with a maximum number of binding sites (B_{max}) of 63 ng/10^6 cells (570,000 binding sites/cell or 65 μg/m^2). Scatchard analysis of ^{125}I-labeled A-Au binding also revealed higher affinity binding with a K_d of 15 nM (1.0 μg/ml), about fourfold more than that for the RFC cells. We also examined BAEC monolayers and found, relative to the BLMVEC, similar high affinity binding with about a twofold decrease in B_{max}. In addition to possible variations among species and in morphological differences between the cells including cell surface densities of caveolae (Schnitzer et al., 1994*a*), our use of bovine albumins in all of the binding experiments may provide at least a partial explanation for the greater affinities and number of binding sites of the bovine cells in comparison with the rat cells.

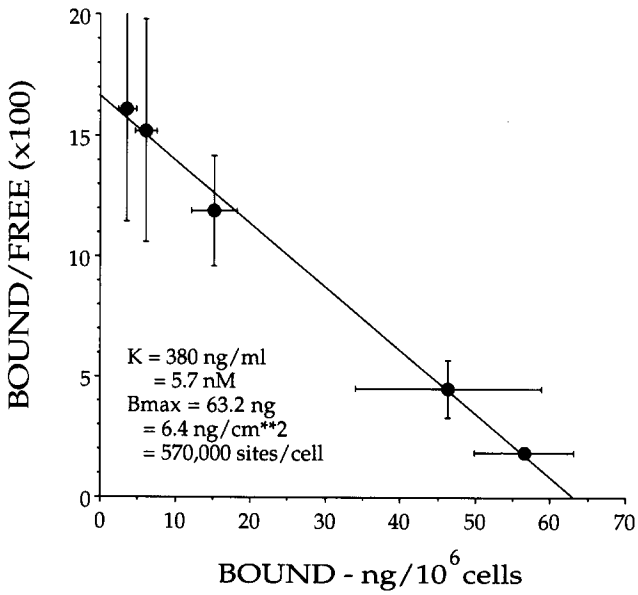

Figure 1. High affinity binding of modified albumins to BLMVEC. Confluent BLMVEC monolayers were assayed for surface binding of Mal-BSA (see Materials and Methods). The data was analyzed using a Scatchard plot. The ratio of bound to free Mal-BSA is presented on the ordinate axis while the abscissa presents the total specific bound Mal-BSA. The linear regression equation for the high affinity binding after subtraction of the moderate affinity binding component is $Y = 16.64 - 0.263 \times$ ($R^2 = 0.995$). Each point represents the mean of multiple observations ($6 \geqslant N \geqslant 2$) with standard deviations given as error bars for both axes.

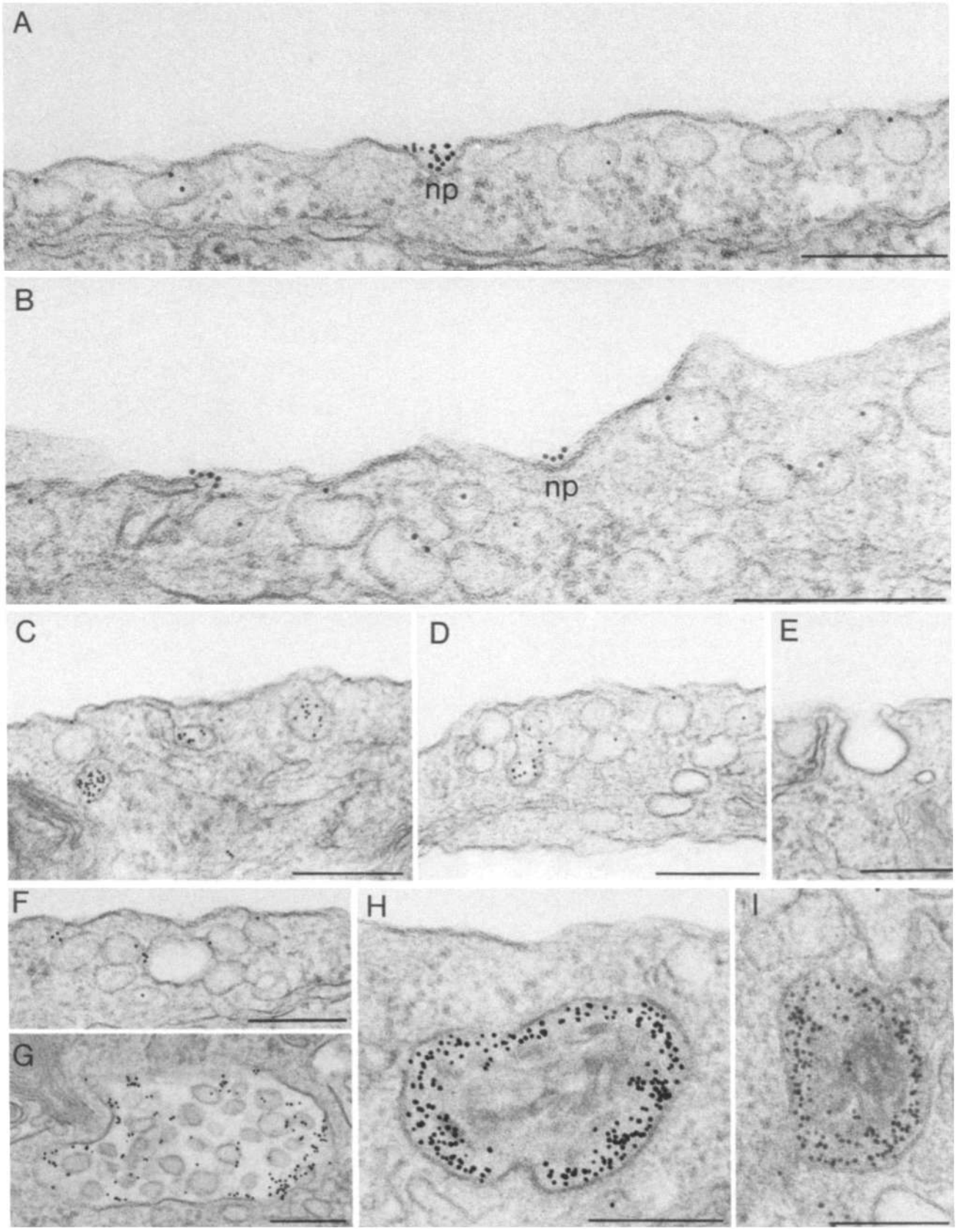

Figure 2. Electron microscopy of cellular processing of A–Au. BLMVEC monolayers were washed and incubated at 37°C with A–Au for 5, 10, or 30 min in the presence of 1 mg/ml of BSA in DME. After washing, the monolayers were processed and sectioned for electron microscopy. (*A*, *B*, and *F*) 5 min A–Au incubation. A–Au detected within noncoated pits (*np*), apparently in the initial stages of forming caveolae and at the introit of formed noncoated plasmalemma vesicles (*pv*), possibly restricted by vesicle diaphragms. The forming plas-

Table I. A–Au Interaction with the Endothelial Cell Surface and Its Invaginated Coated and Noncoated Vesicles

	Number examined	Number with A–Au	Percent with A–Au	A–Au/vesicles	A–Au/μm*	Enriched‡
Caveolae	342	133	39	3.8	9.8	9.0
Coated vesicles	22	1	4.5	.09	.39	.36

BLMVEC were incubated with A–Au for 5–10 min and processed for electron microscopy. Random pictures of each sample were taken in 20 different cells. We examined 110 μm of luminal-equivalent cell surface membrane (non-plastic side) and counted invaginated vesicles as in Schnitzer et al. (1994).
* Average number of A–Au detected per total membrane length for total vesicles examined.
‡ Relative to examination of plasmalemma proper (no invaginations) which revealed 1.1 A–Au/μm.

Electron Microscopy. The cellular processing of A–Au at 37°C was examined by standard electron microscopy. Fig. 2 shows typical results demonstrating the endocytosis of A–Au by noncoated plasmalemma vesicles. After just 5-min incubation of A–Au with the cells at 37°C, most of the A–Au is located at the cell surface with small levels of accumulation within apparent endosomes inside the cell (range of 1–5 A–Au per occupied endosome; most endosomes were unoccupied). Noncoated vesicles with A–Au can be found on the cell surface in various stages of invagination from noncoated pits to fully formed, flask-shaped plasmalemma vesicles. The A–Au is found: (*a*) as single particles on the outer surface of the diaphragms of clearly formed caveolae (one to two gold particles per diaphragm); (*b*) as clusters of three to seven particles not usually attached to each other as aggregates but attached individually to the cell surface, all within small "dimples" in the membrane that could be noncoated pits forming noncoated plasmalemma vesicles; (*c*) within a subset of fully formed noncoated plasmalemma vesicles which are either attached directly to the cell surface or adjacent to the plasmalemma within 1,000Å; and (*d*) as random solitary particles on the plasmalemma proper. Larger numbers of A–Au were found in noncoated pits than in fully formed plasmalemma vesicles. The large size of the A–Au and the small radius of the introit to the vesicles appears to prevent easy access to fully formed, flask-shaped vesicles, especially those with diaphragms. A similar profile is observed when A–Au is interacted with the cells for 30 min at 4°C including: (*a*) solitary gold particles on the cell surface and the outside of vesicle diaphragms; and (*b*) very few formed caveolae containing more than one gold particle (data not shown). With longer incubations of 10 min at 37°C, more caveolae with many more gold particles (≥5) are found, suggesting that, if this accumulation and clustering of A–Au within vesicles is a dynamic process, then vesicles may have formed from the noncoated pits in this time frame. After a 10-min incubation, multivesicular bodies and endosomes contain not only A–Au more frequently but also much more A–Au than detected after just a 5-min incubation with the cells. At 30-min incubation, there is ample A–Au visible within small to large endosomes, multivesicular bodies and lysosomes. Quantitative analysis of the A–Au subcellular distribution indicates that after short exposure times, A–Au is associated primarily with the cell surface, especially caveolae, and with longer incubations is progressively delivered to endosomes, multivesicular bodies and lysosomes (see Fig. 7).

At all time points, coated pits and vesicles contain A–Au infrequently and when labeled, only one to two particles were found. Fig. 2 *E* shows a typical coated vesicle without A–Au. Table I shows our quantitative analysis of the interaction of A–Au with the cell surface and its invaginated coated and noncoated vesicles. In examining each distinct vesicle population as a whole, we find significant enrichment of nearly 10-fold for A–Au localization to caveolae relative to the plasmalemma proper whereas almost a threefold decrement in relative binding to coated vesicles is noted. Direct comparison of vesicular types shows about 25-fold more A–Au binding to caveolae than to coated vesicles. Because a significant portion of the caveolae do not appear to interact with A–Au, there may be differences in caveolae so that subpopulations of caveolae may exist. If so, then the surface binding density for that subpopulation reactive with A–Au is actually greater than that for the general population. With a density of about 25 A–Au/μm, the enrichment found in these select caveolae relative to the plasmalemma proper would increase from 10- to nearly 25-fold. Regardless of mathematical manipulation, it is clear that A–Au preferentially interact with caveolae.

It is curious to note that we found less A–Au at the cell surface after a 30-min incubation than a 10-min incubation even though these studies were not "warm-up" protocols but constant 37°C incubations. This observation suggests the possibility that the A–Au receptors and/or the vesicular carriers may be diminished at the cell surface after ligand internalization. A–Au binding could initiate a strong vectorial transport of itself into the cell with slow recycling of the vesicles and/or A–Au receptors back to the cell surface. Unfortunately, we cannot definitively address this issue until monospecific antibodies are produced to the surface receptors

malemma vesicles have several A–Au particles, many of which are bound next to the cell membrane. A–Au in pv not directly attached to the cell membrane is noted. Generally, pv contain one to two A–Au particles. Possible initial stages of pv fusions with forming early spherical endosomes (see *F*). The pv appear to be docking with larger vesicle, possible early endosome. (*C* and *D*) 10 min A–Au incubation. More extensive accumulation of A–Au in formed plasmalemma vesicles at cell surface (See *C*) and apparently within cytoplasm (see *D*) with many A–Au/vesicle. (*E*) 10 min A–Au incubation. Represents typical coated vesicle without A–Au. (*G*) 10 min A–Au incubation. Multivesicular body (*mvb*) studded with A–Au. Note many A–Au associated with internal vesicles. This micrograph represents most A–Au seen in mvb at this time point. (*H* and *I*) 30 min A–Au incubation. Very extensive accumulation of A–Au within multivesicular bodies (*H*) and lysosomes (*I*). Bars, 0.2 μm.

along with markers specific for the vesicles. We did, however, notice in the 30-min incubations that the plasmalemma directly adjacent to small and large endosomes containing ample A–Au was in almost all cases devoid of caveolae. This was not true for the cell membrane "above" empty endosomes found in cells exposed for 5 or 10 min to the A–Au and many endosomes found in cells not incubated with A–Au. Our observations suggest that: (*a*) caveolae containing A–Au can detach from the cell surface and deliver their contents to endosomes; (*b*) A–Au binding may initiate vesicle formation and rapid detachment of vesicles from the cell surface for vectorial delivery to endosomes; and (*c*) these vesicles with their A–Au receptors may not be recycled quickly from the endosomes back to the cell surface.

Sterol Binding Agents Reversibly Inhibit Caveolae-mediated Endocytosis and Degradation

Filipin is a macrolide pentene polyene antibiotic, a class of drugs that binds sterols such as cholesterol (Bolard, 1986) and can disrupt caveolae in fibroblasts and smooth muscle cells (Severs and Simons, 1986; Rothberg et al., 1990; Davis and Shivers, 1992). It is now clear that endothelial cells can endocytose and degrade modified albumins apparently via specific interaction with gp30 and gp18 (Schnitzer and Bravo, 1993) and that this scavenger endocytosis is mediated by caveolae (see above). BLMVEC have an abundant population of caveolae (Schnitzer et al., 1994) of which a subpopulation avidly endocytose modified albumins for degradation and apparently others transport native albumin transcellularly (Schnitzer and Oh, 1994). Therefore, we decided to investigate the possible effects of filipin on caveolae and their processing of modified albumins.

Filipin Reduces BLMVEC Surface Density of Caveolae. Confluent BLMVEC were incubated with filipin and examined by electron microscopy to assess caveolae density at the cell surface. Fig. 3 shows that filipin treatment of BLMVEC reduces the cell surface density of caveolae in a time dependent manner. Both 30- and 60-min incubations decrease the number of noncoated plasmalemma vesicles per length of cell membrane by about 90%. Even exposures as short as 5 min result in about a 50% reduction. The caveolae density at the zero time point agrees well with our previous results (Schnitzer et al., 1994).

Inhibition of A–Au Uptake and Degradation but Not Surface Binding. To assess biochemically filipin's effects on caveolae-mediated processing, BLMVEC treated or untreated with filipin were incubated with ^{125}I-labeled A–Au. Fig. 4 shows a dose response curve illustrating that filipin greatly inhibits both the internalization and degradation of ^{125}I-labeled A–Au by BLMVEC in a concentration dependent manner. The A–Au associated with the cells after a 30-min incubation at 37°C was maximally decreased by 70%. Degradation of A–Au was decreased by a maximum of more than 80%. A concentration as low as 0.5 μg/ml decreased degradation by greater than 70% and was nearly as effective as 5 μg/ml. The effective inhibition of caveolae-mediated processing at these concentrations is quite consistent with our morphological observations that caveolae begin to disassemble in just minutes at 5 μg/ml of filipin (see above).

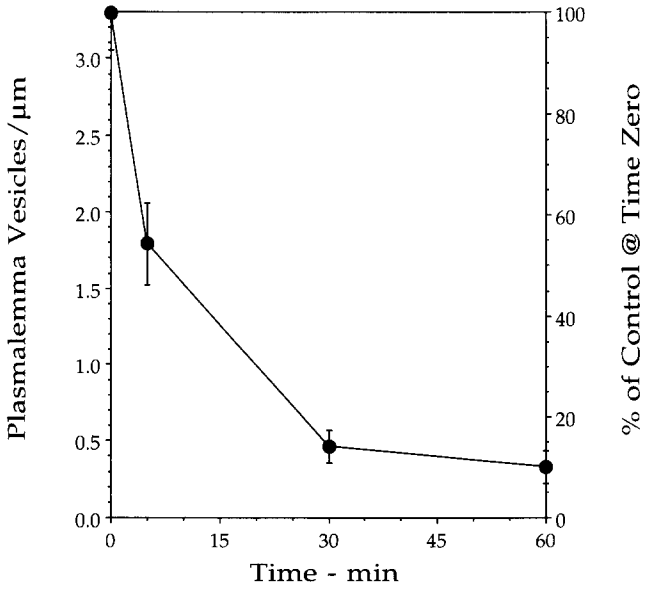

Figure 3. Filipin reduces the cell surface density of noncoated plasmalemma vesicles.BLMVEC monolayers were treated with 5 μg/ml of filipin in DME for the indicated times, processed, and examined by electron microscopy for quantitation of cell surface density of caveolae as described previously (Schnitzer et al., 1994). The results are given as a mean value with a standard error bar for both the density of caveolae (number per μm (left Y-axis)) and the percentage of the zero time point (right Y-axis).

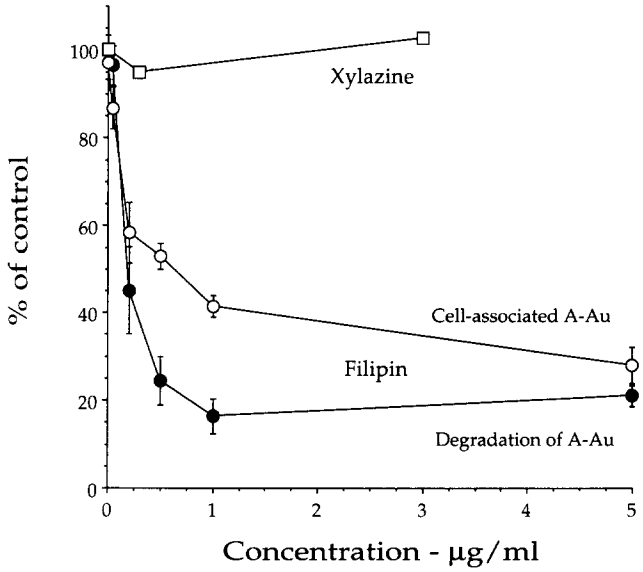

Figure 4. Dose response curve for filipin inhibition of endocytosis and degradation of A–Au. Confluent RFC, BAEC, and BLMVEC monolayers in six-well trays were washed, preincubated for 10 min at 37°C in DME containing the indicated concentration of filipin or xylazine as a control, and then incubated fo 30 min with ^{235}I-labeled A–Au. After washing, the cells and media were processed as usual to determine the cell-associated and -degraded A–Au (see Materials and Methods). The results were normalized to the control without filipin. They were quite similar for each endothelial cell type and therefore, were combined. Each point represents the mean of multiple observations ($4 \leq N \leq 11$) with SD given as error bars ($N = 2$ for each xylazine point).

Because filipin's inhibitory effect potentially could result from its direct interactions with the receptor and/or ligand so as to prevent cell surface binding, we tested the effects of filipin on A–Au interaction with the BLMVEC surface. These experiments were performed by preincubating the cells with filipin for 10 min at 4°C or at 37°C before performing the usual binding assay at 4°C. We found no evidence for interference with the binding. In fact, we observed a 25–50% increase in detected binding with filipin treatment which is consistent with the possible unmasking of receptors normally found inside fully formed noncoated plasmalemma vesicles. As discussed earlier in our electron microscopy studies, direct access of A–Au to fully formed plasmalemma vesicles may be limited so that filipin-induced flattening or "devagination" of the vesicles to the cell surface may cause more direct exposure of the A–Au binding sites. Furthermore, consistent with these results, we found that filipin does not affect direct A–Au binding of gp30 and gp18 electrotransferred onto nitrocellulose from gels after SDS-PAGE of cell lysates (data not shown).

From these results, it would appear that at 37°C the 70% decrease in cell-associated A–Au after filipin treatment is consistent with a significant prevention of ligand endocytosis but not cell surface binding. We have noted previously that about 25% of A–Au associated with the cells at 37°C is normally cell surface bound and sensitive to Pronase digestion whereas at 4°C greater than 90% is Pronase sensitive (Schnitzer and Bravo, 1993). When we performed similar experiments with filipin-treated cells, we found a significant increase in the cell-associated A–Au that was sensitive to

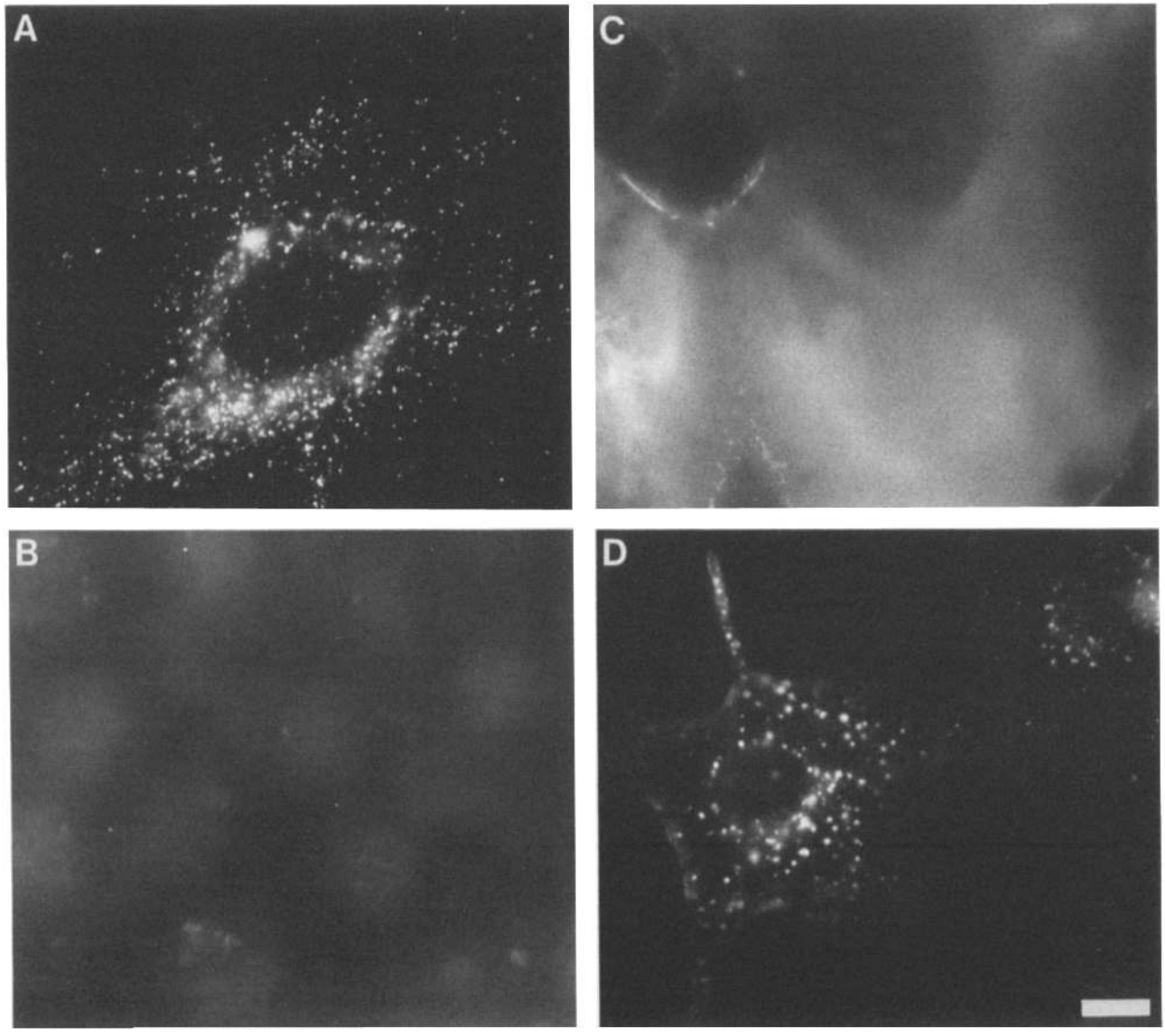

Figure 5. Fluorescence microscopy of processing of A–Au. BLMVEC were incubated at 37°C for 30 min with DME containing FITC–A–Au and 1 mg/ml of either native albumin BSA (*A*) or the modified albumin Mal–BSA (*B*). After washing, the cells were fixed and processed for fluorescence microscopy (see Materials and Methods). In *C*, the cells were pretreated with filipin (5 μg/ml) before the addition of the FITC–A–Au. In *D*, immediately after the filipin treatment, the cells were incubated in DME containing 10% FCS for 30 min to reverse the effects of filipin before examining FITC–A–Au processing. Note that in both *B* and *C*, the exposure time for the film was two to three times that for the control. Bar, 30 μm.

Pronase digestion (87.9 ± 6.4%). As in the studies performed at 4°C, this Pronase sensitivity indicates a lack of cellular uptake to a Pronase-protected compartment consistent with a predominant cell surface distribution.

Fluorescence Microscopy. The effect of filipin on the cellular processing was examined by fluorescence microscopy using A–Au made from FITC conjugated to albumin (FITC–A–Au). Fig. 5 shows that incubation of BLMVEC with FITC–A–Au creates a punctate signal consistent with its accumulation in endosomes or lysosomes as shown earlier by electron microscopy. There are also finer punctate signals, especially visible in the peripheral regions of the cells, which may represent clusters of A–Au within caveolae (also as shown by electron microscopy). As expected from our radiobiochemical assays (Schnitzer and Bravo, 1993), the modified albumin Mal–BSA is an able competitor and prevents binding and internalization of the FITC–A–Au as indicated by the lack of signal on or in the cells (note that all experiments are performed in the presence of 1 mg/ml of BSA which is not inhibitory). With filipin treatment, both large and fine punctate signals disappear and a rather weak diffuse signal is apparent on the cell surface. There is little, if any, evidence for clustering, internalization, and accumulation of the probe which is quite apparent in the control.

Electron Microscopy. We have shown earlier that A–Au interacts preferentially with caveolae, resulting in endocytosis. Here, we use electron microscopy to show directly that filipin inhibits this uptake by caveolae. Fig. 6 shows control and filipin-treated BLMVEC incubated with A–Au. Clearly, the number of caveolae is decreased tremendously and the A–Au associated with the cells is not found, as in the control, within caveolae at the cell surface or even inside the cells but bound only to the outside of the cell at the plasmalemma proper. Fig. 7 shows a graphical summary of our quantitative analysis performed on cells incubated with A–Au for 5 and 30 min. There is a significant difference in the subcellular distribution of A–Au processed by control and filipin-treated cells. After filipin treatment of the cells, A–Au interaction is limited to the cell surface with a very significant decrease in cellular uptake as indicated by a lack of A–Au found in caveola, endosomes and multivesicular bodies. After 5-min incubation of the BLMVEC with A–Au at 37°C, both treated and untreated cells have greater than 80% of the A–Au associated with the cell surface. However, even at this early time point, there is a major difference in the distribution at the cell surface. For control cells, most A–Au is found associated with caveolae attached to the cell membrane or underneath it; however, for the treated cells, A–Au is almost exclusively bound to the plasmalemma proper with only a small percentage of the total A–Au found in the few caveolae that remain after treatment. Even at this early time point, significant reductions with filipin treatment are evident in delivery to endosomes and multivesicular bodies. Longer incubation of the cells with A–Au to 30 min extensively

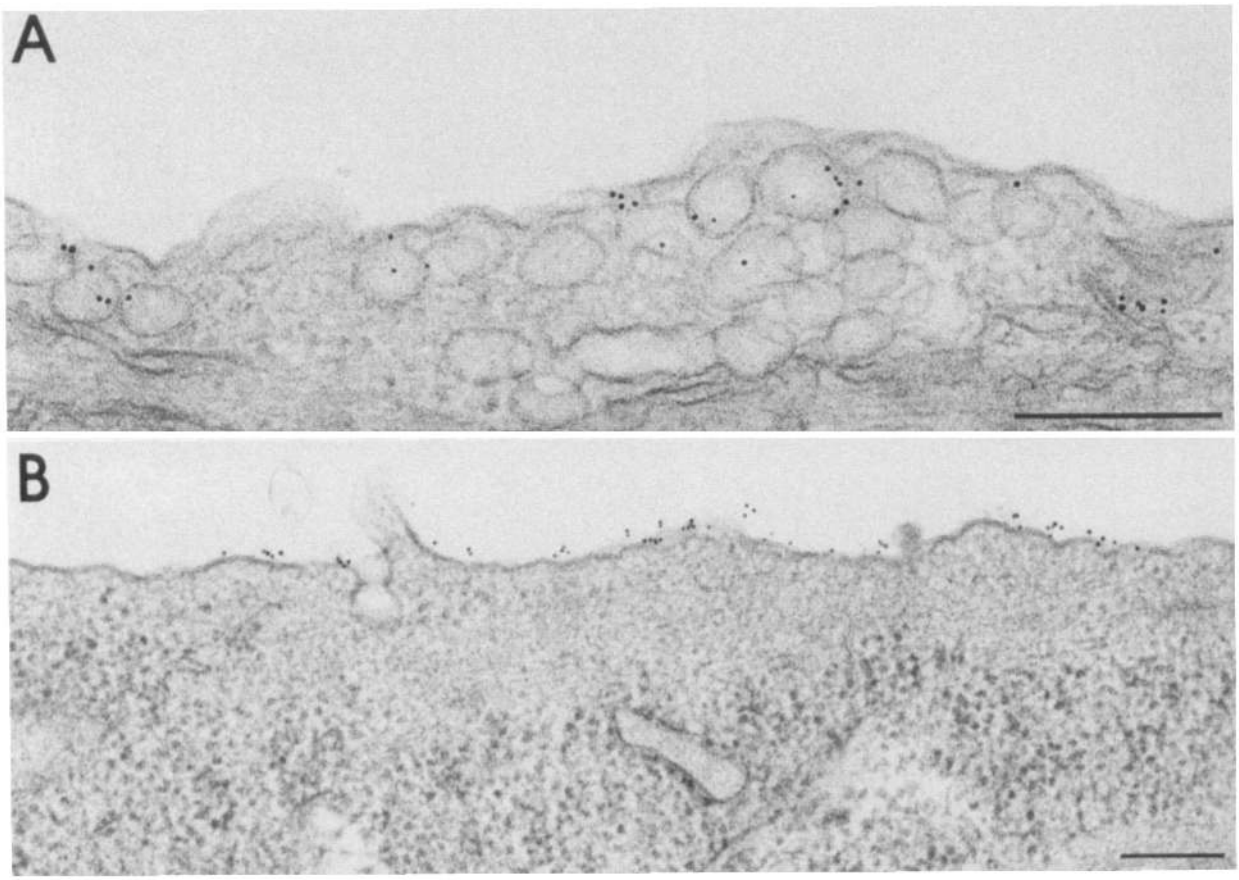

Figure 6. Electron microscopy of A–Au endocytosis by control and filipin-treated BLMVEC. BLMVEC exposed for 20 min to DME alone (*A*) or DME plus 5 μg/ml of filipin (*B*) were incubated with A–Au in DME for 5 min, washed, fixed, and processed for electron microscopy.

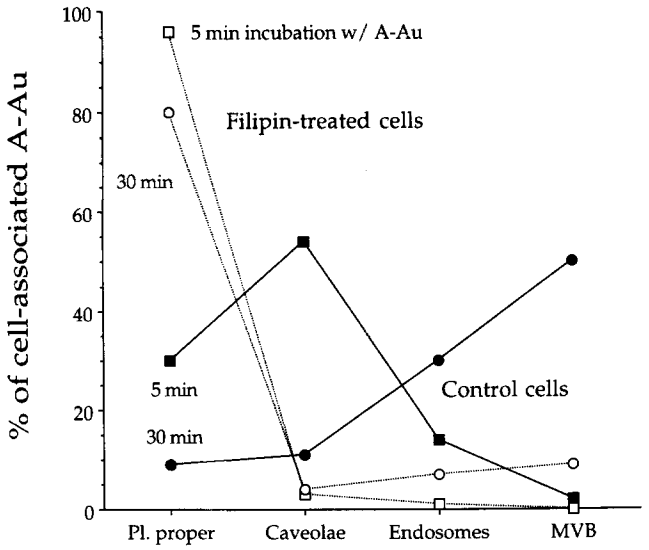

Figure 7. Filipin's effect on A–Au processing by BLMVEC. Before incubation with A–Au for the indicated times, BLMVEC were exposed for 20 min to DME alone (*control*; *solid line* and *symbols*) or DME plus 5 μg/ml of filipin (*dotted line* and *empty symbols*), and then fixed and processed for electron microscopy. Quantitative analysis of the subcellular distribution of A–Au was performed by counting and localizing each A–Au to the plasmalemma proper, caveolae, endosomes or multivesicular bodies/lysosomes (*MVB*). Results for each category are expressed as the mean percentage of the total cell-associated A–Au.

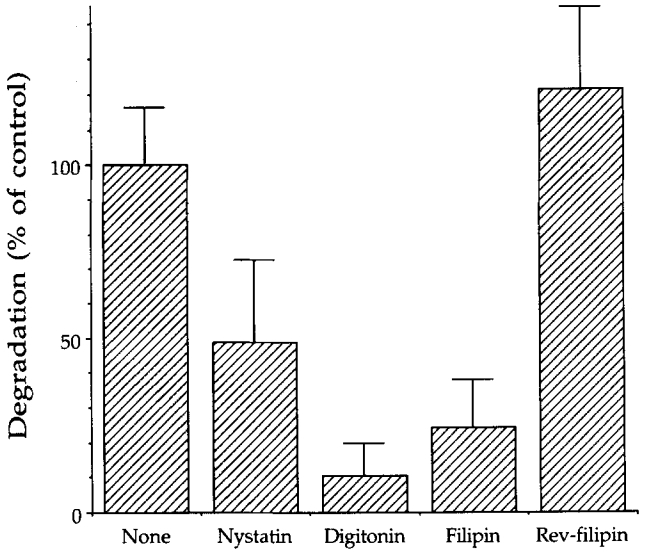

Figure 8. Filipin reversibility and effect of other sterol binding agents on A–Au degradation. Confluent RFC, BAEC, and BLMVEC monolayers in six-well trays were preincubated for 10 min at 37°C in DME alone or containing 5 μg/ml of filipin, nystatin, or digitonin and then processed as in the legend of Fig. 2. Rev-filipin represents the degradation achieved after attempting to reverse the filipin effects using a 30-min incubation of the cells with DME plus 20% FCS as described in the text. The results were normalized to the controls without filipin. Each bar gives the percentage of degradation detected in the control and represents the mean of multiple observations ($2 \leq N \leq 6$) with standard deviations given as error bars.

changes the distribution profile for A–Au associated with the control but not filipin-treated cells. BLMVEC normally accumulate A–Au within endosomes, multivesicular bodies and lysosomes (Fig. 7); however, with treatment, A–Au remains predominantly associated with the cell surface with greatly reduced levels of endocytosis and delivery to intracellular compartments. These results show that filipin-treated cells: (*a*) have fewer caveolae; (*b*) can still bind A–Au at the cell surface but in this case primarily to the plasmalemma proper and not caveolae; and (*c*) have a significant reduction in A–Au internalization. Our results to date indicate that filipin's ability to disassemble caveolae can indeed prevent the endocytosis and degradation of caveolae-specific ligands such as A–Au.

Inhibitory Effect Is Reversible. We examined the reversibility of the effects of filipin treatment on the cells. BLMVEC monolayers treated with filipin for 15 min were washed, incubated for 30 min in standard cell culture media containing 20% FCS and then processed through the usual assay examining A–Au degradation. Fig. 8 shows that this short reversal treatment resulted in restoration of normal, if not slightly elevated, levels of A–Au degradation by the cells. Similar reversibility was also demonstrated by fluorescence microscopy (see Fig. 5 *D*).

Other Cholesterol-binding Agents Inhibit A–Au Degradation. We also tested other sterol/cholesterol binding agents. Like filipin, nystatin is also a polyene antibiotic (Bolard, 1986) whereas digitonin is a cardiac glycoside (Elias et al., 1978). Both agents bind cholesterol and can remove it from cell membranes. Fig. 5 shows that nystatin and digitonin inhibited A–Au degradation by more than 50 and 85%, respectively. Other agents known to interact with lipids in the cell membrane such as xylazine (see Fig. 4) and polymyxin B (data not shown) were ineffective.

Inhibition of α_2-Macroglobulin Endocytosis and Degradation. Activated α_2-macroglobulin is internalized by receptor-mediated endocytosis via clathrin-coated pits/vesicles (Dickson et al., 1981; Trans et al., 1987). Fig. 9 shows that under equivalent conditions, filipin inhibits significantly the endocytosis and degradation of A–Au by about 70% but does not reduce the uptake and degradation of methylamine-activated α_2-macroglobulin. These results suggest that the coated vesicular degradative pathway with its associated coated pits, vesicles, endosomes, and lysosomes is not detrimentally altered by filipin. The compartments of this pathway are functionally intact, unlike the early stages of the noncoated vesicular pathway.

Partial Inhibition of Insulin Endocytosis but Not Degradation. Insulin classically is thought to be internalized by the clathrin pathway but more recent evidence indicates that at least in endothelium it can be endocytosed by both clathrin-coated and noncoated vesicular pathways (Roberts and Sandra, 1992; King and Johnson, 1985). Interestingly, Fig. 9 shows that filipin partially prevents BLMVEC uptake of insulin by about 50%; however, insulin degradation is not diminished. These results are consistent with past observations that coated pits internalize insulin for degradative purposes and that in endothelium, insulin can be internalized not only for degradation but also for other purposes such as transcytosis (King and Johnson, 1985; Rabkin et al., 1993). The

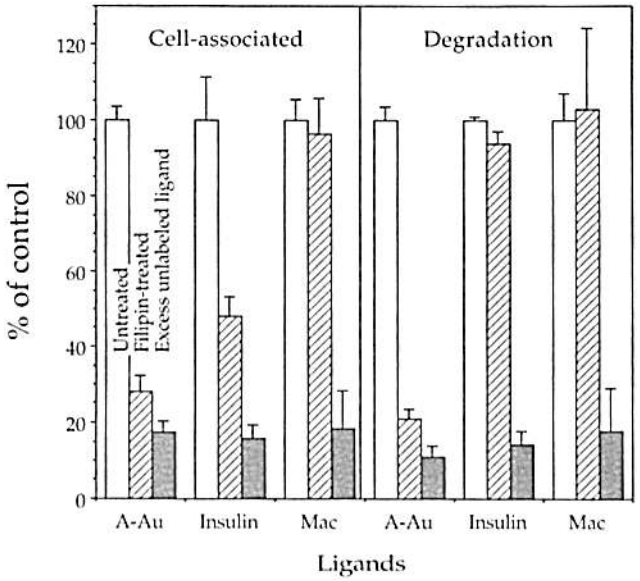

Figure 9. Selectivity in filipin's inhibition of endocytosis and degradation of various macromolecules by BLMVEC monolayers. Confluent BLMVEC monolayers in six-well trays were preincubated for 10 min at 37°C in DME with 1 mg/ml of BSA (DBSA) or DBSA containing either 5 μg/ml of filipin or 1 mg/ml of unlabeled ligand (for ^{125}I-labeled A-Au, Mal-BSA was used). The cells were incubated for 30–60 min at 37°C with ^{125}I-labeled ligands (A-Au, insulin, or methylamine-activated α_2-macroglobulin [Mac]) in DBSA supplemented as in the pre-incubation period, washed, and then processed to assess cell association and degradation (see Materials and Methods). The results were normalized to the controls (DME alone; *untreated*). Each bar gives the percentage of degradation detected in the control and represents the mean of multiple observations ($3 \leq N \leq 6$) with standard deviations given as error bars.

filipin-resistant coated pits/vesicles could be responsible for insulin endocytosis and degradation whereas caveolae, which disassemble after filipin treatment, could internalize insulin not for degradation but ultimately for transcytosis (see below).

Filipin Inhibits Caveolae-mediated Transcytosis

Endothelial cells form an attenuated monolayer lining blood vessels that in many tissues is the critical barrier controlling the transport of molecules from the blood to the interstitium harboring the tissue cells. Receptors located on the endothelial cell surface may play a very important role in the selective delivery of essential nutrients to the underlying tissue cells. Receptors for carrier proteins such as transferrin, albumin, orosomucoid, and ceruloplasmin have been defined kinetically through binding experiments and in some cases specific binding proteins have been identified (for review see Schnitzer, 1993). Other studies have shown that insulin receptors contribute signficantly to the transendothelial transport of insulin by demonstrating that antibodies recognizing insulin receptors on the endothelial cell surface inhibit not only insulin internalization but also its transport across cultured endothelium (King and Johnson, 1985). Since endothelium can have a very abundant population of caveolae, it has largely been presumed that these noncoated plasmalemmal vesicles mediated the transport of these select blood-borne macromolecules. Immunogold electron microscopy has shown that endothelial caveolae mediate the transcytosis of albumin (Milici et al., 1987). We recently have developed a model for evaluating receptor-mediated effects on transendothelial transport in vitro and in situ and have shown that antibodies to the albumin binding protein, albondin significantly inhibit the transcytosis of albumin (Schnitzer and Oh, 1994). BLMVEC form a monolayer when grown on filters in culture that exhibit albondin-mediated transendothelial transport of albumin. Therefore, in this study, we attempted to test the effects of filipin using this in vitro transport system and both albumin and insulin as specific probes for caveolae-mediated transcytosis.

Because concentrations as low as 0.5 μg/ml were quite effective in preventing caveolae-mediated intracellular transport of A-Au by BLMVEC, we tested filipin's effects on transcellular transport at levels ≤1 μg/ml. Fig. 10 shows that both BSA and insulin transport was reduced in a manner dependent on filipin concentration. We also used an inert small solute probe, inulin (5,000–5,550 molecular weight), that is transported across endothelium paracellularly via intercellular junction and is not expected to interact with the cell surface. Filipin at 1 μg/ml appears to reduce insulin and BSA transport by about 50–60% while not affecting inulin transport. We have previously shown that BSA transport across these monolayers was 50% dependent on albondin binding and transcytosis whereas the remainder appeared to be inde-

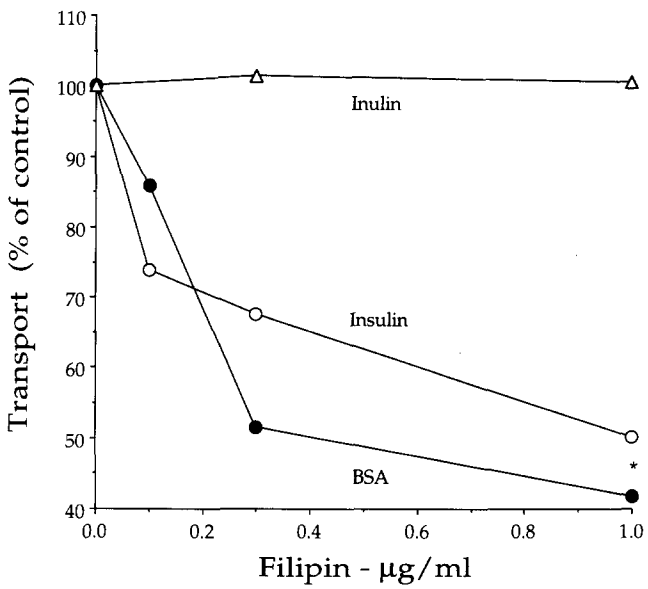

Figure 10. Filipin inhibits the transport of insulin and albumin (but not inulin) across confluent BLMVEC monolayers. BLMVEC grown on Transwell filters were used to examine the effects of filipin at the concentration indicated on the transport of ^{125}I-labeled BSA, ^{125}I-labeled insulin, and [^{14}C]inulin across monolayers in vitro (see Materials and Methods). The results were normalized to the control without filipin. Each point represents the mean of multiple observations ($4 \leq N \leq 18$) with a SD ≤20%. The asterisk shows the mean normalized ^{125}I-labeled BSA transport when assessed in the presence of excess unlabeled BSA (1 mg/ml). The transport of ^{125}I-labeled insulin was reduced to 42% of control in the presence of 1 mg/ml of unlabeled insulin.

pendent of cell surface binding, probably being transported by paracellular and/or fluid phase mechanisms (Schnitzer and Oh, 1994). Others have shown that about 60% of insulin transport across endothelial cell monolayers is inhibited by the interaction of antibodies with the insulin receptor and therefore, is dependent on cell surface binding and receptor-mediated transcytosis. It would appear that ablation of caveolae with filipin can inhibit the specific transcytosis of both albumin and insulin at levels that are quite consistent with past in vitro findings. Filipin does not appear to affect paracellular transport.

Filipin can reduce transport by 60% which is nearly equivalent to the effect elicited by low temperatures which minimize vesicular transport and only allow paracellular transport (Schnitzer and Oh, 1994). When the experiments are performed at 4–8°C, filipin does not further reduce transport in vitro and, as in the case for A–Au discussed above, it does not reduce surface binding of albumin (data not shown). These findings are consistent with filipin inhibiting a temperature-sensitive transcytotic component while not affecting paracellular transport.

Filipin Inhibits Caveolae-mediated but Not Paracellular Transport In Situ

So far, all our studies have focused on cultured endothelial cell monolayers in vitro. Therefore, we extended the testing of filipin by evaluating its effect on transport in situ.

Inhibition of Capillary Permeability of Albumin in the Rat Lung In Situ. First, we tested for possible effects of filipin in situ by examining albumin's permeability in the rat lung vasculature by pulmonary artery perfusion as determined in our past work (Schnitzer and Oh, 1994). Fig. 11 shows that filipin was very effective in a dose-dependent manner in reducing the transcapillary transport of albumin and the calculated capillary permeability-surface area (PS) product for albumin. The PS product for albumin attains a maximum reduction of 50–60% at concentrations greater than or equal to 0.3 μg/ml. It should be noted that with filipin treatment, we did not observe any changes in hemodynamic parameters such as vascular resistance, perfusion pressures and flows nor any evidence for edema formation as indicated by water content per gram of tissue. It was not surprising under the harsher conditions inherent to the transport assays in situ with convective hydrodynamic flow and pressure gradients that the transport observed was more sensitive to filipin than in the studies in vitro. The maximal effect was achieved with much shorter exposure times and about two-fold lower concentrations. At least part of the explanation for this increased sensitivity in situ probably relates to the inherent diffusive nature of the system in vitro versus the higher pressure and convective nature of vascular perfusions in situ.

Selective Inhibition of Tissue Uptake in the Rat Lung In Situ. The tissue uptake after vascular perfusion of various probes was also examined in the rat lung in order to test various endothelial cell transport pathways in situ. Inulin is small enough to be transported paracellularly across the endothelial barrier via intercellular junctions while A–Au was used to assess caveolae-mediated endocytosis. Fig. 12 shows that filipin inhibited the tissue accumulation of A–Au, BSA (both ^{125}I-labeled and ^{14}C-labeled) but not inulin in the rat lung after perfusion via the pulmonary artery. A–Au transport was reduced by 60%. In agreement with the studies performed in vitro, filipin effectively inhibits the caveolae-mediated pathways in situ including both transcytosis and endocytosis without affecting paracellular transport via intercellular junctions.

Membrane Permeabilization at Higher Filipin Concentrations. At sufficiently high concentrations and long enough

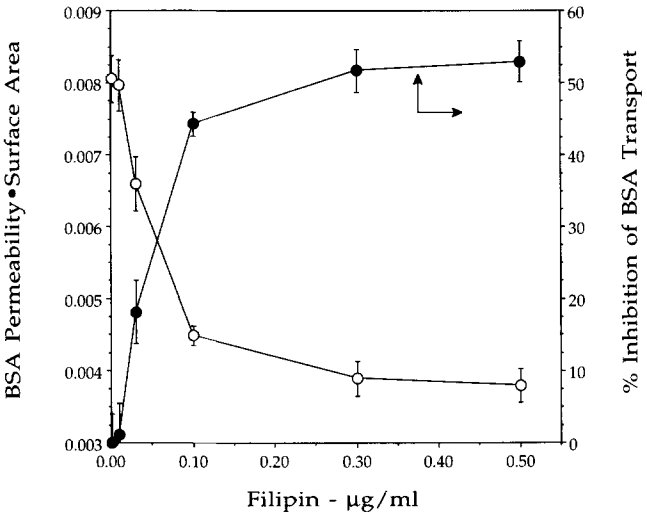

Figure 11. Filipin lowers capillary permeability to albumin in the rat lung. The capillary permeability of ^{125}I-labeled BSA was measured in situ in rat lungs treated with filipin at the indicated concentration (see Materials and Methods). The permeability-surface area product (*left ordinate* and *open circles*) and the percent inhibition of ^{125}I-labeled BSA transport (*right ordinate* and *solid circles*) are presented. Each point with SD given as error bars represents the mean of multiple observations from 28 or more tissue samples ($28 \leq N \leq 42$) from at least two rats.

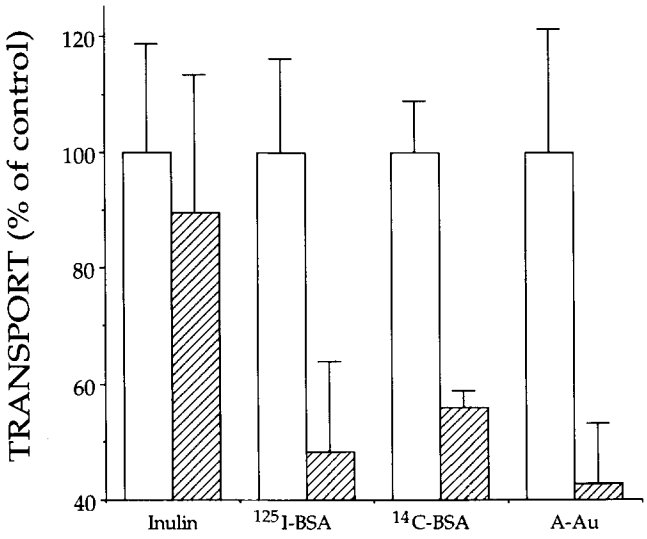

Figure 12. Selective inhibition of capillary transport in rat lung in situ. The endothelial transport of [^{14}C]inulin, ^{125}I-labeled BSA, ^{14}C-labeled BSA, and ^{125}I-labeled A–Au were assessed in rat lungs either untreated (*blank bars*) or treated with 0.3 μg/ml of filipin (*hatched bars*). The results were normalized to the control without filipin. Each bar represents the mean of multiple observations ($6 \leq N \leq 28$) with the SD given as an error bar.

exposure times, cholesterol binding agents such as filipin and nystatin are well-known for their ability to permeabilize cells by disrupting the plasmalemma. When we increased by several-fold the exposure times and concentrations of filipin used in our in situ and in vitro assays, we also found evidence for sufficient cell membrane permeabilization that the overall transport observed was greatly increased. With 10-fold increases over the optimum conditions described above, both inulin and albumin transport increased many times over the control. In situ perfusions at higher concentrations also resulted in significant edema with large increases in lung weight and percent water weight (data not shown).

Discussion

Specific vesicular transport of macromolecules is a fundamental cellular process that is well documented in a variety of cells for intracellular trafficking, receptor-mediated endocytosis, and to a lesser extent transcytosis. At least two morphologically distinct types of vesicles exist on the cell surface of many cells, albeit in disparate proportions ranging from cells such as hepatocytes with a plethora of coated vesicles/pits and few noncoated ones to many endothelia with an abundance of noncoated plasmalemma vesicles and few coated vesicles. Even endothelia in different tissues have a wide spectrum of distributions of these vesicles with most endothelia of the continuous type having many caveolae and few coated vesicles (except for brain where both types are underrepresented) whereas sinusoidal endothelia have many coated vesicles with noticeably fewer noncoated ones. Noncoated plasmalemmal vesicles and caveolae are interchangeable terms for the same structure. By electron microscopy, they appear in many cells types as invaginated pits or vesicles that do not have a thick electron-dense fuzzy coat (distinct for coated vesicles) but have a uniform diameter of about 800–1,000 Å. In some cells, much larger noncoated plasmalemma vesicles have been described (Oliver, 1982). In other cells, especially endothelium, many individual noncoated plasmalemma vesicles exist but also several vesicles can be connected to each other in a stringlike fashion forming a racemose, treelike vesicular structure.

Caveolae-mediated Scavenger Endocytosis

When ligands such as LDL and albumin are modified to certain nonnative states, they interact selectively with a new set of cell surface proteins called scavenger receptors that mediate endocytosis of these aberrant proteins (Brown and Goldstein, 1983; Sparrow et al., 1989; Schnitzer et al., 1992; Schnitzer and Bravo, 1993). As a group, scavenger receptors bind proteins that have been modified appropriately by a variety of means such as oxidation, nonenzymatic glycation, acetylation, maleylation, or even surface adsorption. Scavenger endocytosis of modified but not native proteins appear to serve a protective function necessary to maintain local tissue integrity by removing old, damaged, or possibly deleterious proteins for selective degradation or catabolism (Steinberg et al., 1989). Such scavenging may remove proteins that become damaged within tissues by either normal aging or protective or pathological responses such as oxidation during inflammation or hyperglycation in diabetes. Binding to scavenger receptors is classically thought to initiate ligand internalization via endocytotic mechanisms involving clathrin-coated plasmalemmal vesicles (Pitas et al., 1985; Mommaas-Kienhuis et al., 1985). In this study, we demonstrate a novel filipin-sensitive pathway for the scavenger endocytosis of modified protein involving caveolae. Our electron microscopy studies show that modified albumins preferentially interact with endothelial caveolae. They are internalized, not by coated vesicles, but almost exclusively by caveolae, and are then shuttled through a series of early to late endosomes to multivesicular bodies and lysosomes.

It is now becoming clear that modified albumins such as A–Au can be internalized via either coated or noncoated vesicles depending on the cell type. Very recently Faustmann et al. (1992) showed that subarachnoid macrophages endocytose A–Au via coated vesicles. In situ studies in liver, bone marrow, and adrenal tissue, which have microvascular beds lined with a noncontinuous type of endothelium with few caveolae, showed that coated vesicles mediate A–Au endocytosis (Geoffrey and Becker, 1984; De Bruyn et al., 1985; Bumbasirevic et al., 1990). However, in other vascular beds with a continuous endothelium (lung, heart, and diaphragm), modified albumins such as glycated A–Au can bind within caveolae and in some cases, accumulation within lysosomes has been noted (Villaschi et al., 1986; Predescu et al., 1988). Like coated vesicles (Bretscher et al., 1980), noncoated vesicles appear to have the ability to act as a molecular filter that screens the endocytosis of select molecular cargo.

Albumin Receptors and Caveolae-mediated Transcytosis vs. Endocytosis

Three cell membrane-associated albumin binding proteins called albondin, gp30 and gp18 have been identified (Schnitzer et al., 1988*b*; Ghinea et al., 1988; Schnitzer and Oh, 1994); however, more recent studies indicate that two of these proteins, gp30 and gp18, actually interact much more avidly with modified albumins including A8-Au than with native albumin (Schnitzer et al., 1992). Because albumin's native conformation is modified when it is absorbed to surfaces such as colloidal gold particles (Schnitzer et al., 1992), A–Au is not an equivalent probe for native albumin. The binding behavior of gp30 and gp18 closely resembles that of other known scavenger receptors (Schnitzer et al., 1992). Conversely, albondin, formerly named gp60, interacts with native albumin (Schnitzer and Oh, 1994) and appears to share a common albumin binding domain with another known albumin binding protein, SPARC (Schnitzer and Oh, 1992). Antibodies specific for albondin inhibit cell surface binding and internalization of native but not modified albumins by BLMVEC. Moreover, anti-albondin antibodies along with unlabeled native but not modified albumins significantly inhibit radiolabeled albumin transport across lung microvascular endothelium both in situ and in culture (Schnitzer and Oh, 1994).

Although both native and modified albumins can be internalized by caveolae, only modified albumins are directed primarily to endosomes and lysosomes for degradation (Schnitzer and Bravo, 1993; Schnitzer and Oh, 1994). Studies done in situ show that continuous endothelium transport "native" monomeric albumin across the cell via caveolae without evi-

dence of accumulation in endosomes or lysosomes (Milici et al., 1987; Ghitescu and Bendayan, 1992). The extent of specific transcytosis of modified albumins such as A–Au remains controversial. In the murine lung, Ghitescu et al. (1986) have observed transcytosis of A–Au with some accumulation in endosomes. But in the rat lung, Villaschi et al. (1986) found little transport across the endothelium of surface bound A–Au with most of it eventually being found in endosomes and lysosomes. Herein, our electron microscopy studies found caveolae-mediated endocytosis of A–Au directed to endosomes and lysosomes. Furthermore, our recent studies (Schnitzer et al., 1992; Schnitzer and Bravo, 1993; Schnitzer and Oh, 1994) have shown that: (*a*) greater than 90% of the modified albumin internalized by cultured endothelium is exocytosed degraded whereas greater than 90% of internalized native albumin is exocytosed in non-degraded form; (*b*) unlike their endocytosis and degradation which is strongly dependent on cell surface binding, modified albumins such as Mal–BSA cross BLMVEC monolayers in a manner independent of cell surface binding by fluid-phase and/or paracellular transport; and (*c*) native albumin can be transported transcellularly in a facilitative manner via its specific interaction with the cell surface protein, albondin.

Although at this time, it is unclear if the control of caveolae-mediated transport is dependent on ligand binding to its receptor, our work shows that caveolae, like coated vesicles, can mediate scavenger endocytosis of conformationally modified proteins for lysosomal degradation. Modified albumins may bind via their receptors, gp30 and gp18, to a select subpopulation of noncoated pits/vesicles that are involved in receptor-mediated endocytosis and degradation whereas native albumin may bind another set of vesicles mediating transcytosis. Alternatively, modified albumin binding could cause vesicles to follow a degradative pathway whereas the binding of native albumin to albondin results in transcytosis. Selective binding to each set of receptors may result in the clustering of ligand–receptor complexes and the formation of distinct vesicles destined for different sites in the cell.

Selective Inhibition of Caveolae-mediated Transport

The effects of exposing endothelium to cholesterol-binding agents are quite significant and include ablation of endothelial caveolae and reduction of endothelial transcytosis, scavenger endocytosis and capillary permeability in a manner that appears to be quite selective for the noncoated vesicular-mediated pathways. Filipin treatment of endothelial cells both in vitro and in situ inhibits the intracellular and transcellular transport of select macromolecules. The transport of two important blood proteins, namely insulin and albumin, are inhibited greatly. Native albumin is transcytosed via caveolae from the blood to the interstitium in many endothelia of the continuous type (Milici et al., 1987; Ghitescu and Bendayan, 1992), apparently through its interactions with albondin (Schnitzer and Oh, 1994). Filipin significantly reduces albumin's transendothelial transport both in situ and in vitro. Albumin's permeability in the rat lung vasculature is reduced significantly. Conversely, the paracellular transport of the small solute inulin is not affected in situ or in vitro. These results indicate that caveolae play an important role in the transport of macromolecules circulating in the blood and that filipin can prevent caveolae-mediated transcellular transport without significantly disrupting paracellular transport.

Intracellular transport mediated by caveolae can also be inhibited by filipin both in culture and in situ. Direct A–Au degradation studies performed in vitro show strong inhibition with filipin, nystatin and digitonin. Filipin inhibits A–Au uptake and degradation in a dose-dependent manner. Many different experimental approaches show that filipin reversibly prevents A–Au internalization and delivery to endosomal and lysosomal compartments. Conversely, both morphological and functional studies indicate that the clathrin-coated vesicular pathway is not disrupted significantly by the treatment of the endothelial cells. Here, we show that the degradation of ligands of the coated vesicular endocytic pathway, insulin and activated α_2-macroglobulin, is not affected by filipin treatment. Moreover, subcellular localization of transferrin receptors (known endogenous resident markers of coated pits, vesicles, and early endosomes) is not altered in BLMVEC by filipin (our unpublished observations). Rothberg et al. (1992) also provide evidence that cholesterol binding agents disrupt noncoated vesicles but not coated vesicles. Just a 5–10 min treatment of fibroblasts causes almost total loss of noncoated vesicles on the cell surface with little effect on other membrane structures such as clathrin-coated pits. Sterol binding agents remove cholesterol from cell membranes and cause vesicle flattening from disassembly of the infrastructure of the caveolae (Rothberg et al., 1990, 1992). Using electron microscopy, we also describe a large decrease in the number of caveolae on the endothelial cell surface when BLMVEC are treated with filipin. The time course for the effect of filipin on endothelial cell caveolae is slightly longer (about 15–30 min) than observed for fibroblasts probably because of the much greater density of caveolae in endothelium (at least 10-fold). Furthermore, our studies using fluorescence and electron microscopy indicate that after filipin treatment, A–Au is located diffusely over the BLMVEC surface with little, if any, evidence of its internalization by caveolae and delivery to endosomes and lysosomes (see Figs. 3 and 4). Together, our studies provide evidence that the coated vesicle-mediated degradation pathway, including coated pits/vesicles, endosomes and lysosomes, remains sufficiently unaltered by filipin treatment to remain structurally and functionally intact. Conversely, our studies with A–Au and those of Rothberg et al. (1990) examining the 5-methyltetrahydrofolate receptor show that filipin treatment permits ligand binding at the cell surface but effectively prevents receptor clustering within caveolae. Filipin also inhibits A–Au degradation by selectively preventing endocytosis through the noncoated vesicular pathway.

Caveolae as Transport Structures

Our knowledge about the standard mechanisms for the transport of molecular cargo from one compartment to another in the cell has evolved largely from studies examining the clathrin-coated vesicular pathways. This transport generally is thought to involve the dynamic "shuttling" of vesicles from one membrane surface to another. Vesicles form through the indentation of the membrane to form a "pit" which invaginates further to create a "flask-shaped" vesicle that even-

tually "buds off" from the membrane to become a free vesicle in the cytoplasm. Such vesicles can be delivered to a specific destination within the cell for membrane fusion and release of their contents by a specific set of targeting receptors and fusion proteins (Sollner et al., 1993). For endocytosis and protein trafficking, it is clear that vesicles can deliver their discrete packages through a "shuttling" process. This is also probably true for at least some caveolae which appear to be able to deliver their cargo either to endosomes or across the cell to the opposite cell membrane surface. Because this "fission–fusion" model of vesicular transport has held true for many different types of vesicles in many different cellular processes including endocytosis, exocytosis, protein trafficking, and even the very specialized case of synaptic transmission, it appears logical that caveolae are not an exception to the rule but adhere to this general mechanism.

However, some investigators believe that caveolae are all static or permanent structures that can not function in endocytosis or transcytosis because they can neither arise de novo by invagination of the cell membrane, detach from the cell membrane to form free cytoplasmic vesicles, nor fuse with other cellular components (Frokjaer-Jensen, 1980; Bundgaard, 1983). Serial sectioning of endothelium indicates that very few vesicles can be found free in the cytoplasm and unattached to other vesicles which is interpreted as evidence against the existence of vesicular budding (Frokjaer-Jensen, 1980). An alternate interpretation might be that in the time frame of specimen processing, few free vesicles can be found because the budding and fusion process once initiated is very rapid and the thermodynamic tendency favors fusion with cell membranes. In this study, it appears that vesicles containing A–Au can be found on the cell surface in various stages of invagination from noncoated pits to fully formed vesicles, suggesting a more dynamic role for caveolae. The A–Au preferentially accumulated within caveolae is internalized to endosomes. Favorable sections show single vesicles attached to, or apparently docking with, spherical endosomes several times the diameter of caveolae. Moreover, Ghitescu et al. (1986) have noted that A–Au may be transported across endothelium by caveolae. Once inside a vesicle, A–Au are discrete large particles that cannot directly permeate the cell membrane and therefore, can only be delivered to intracellular endosomes or even across the cell by the movement and/or fusion of hte vesicles with the targeted cellular components. It seems unlikely that static vesicles without any ability to bud from, and/or fuse to, membrane could accomplish endocytosis and transcytosis. It is still conceivable that caveolae could remain attached to the cell surface and also deliver their contents to nearby endosomes by intermittent fusions without budding of the vesicles from the surface. However, Hansen et al. (1991) used serial thin sectioning and rapid labeling of vesicles with Con A to establish that coated and noncoated vesicles both can endocytose by pinching off completely from the plasmalemma. We do not wish to imply that all noncoated plasmalemmal vesicles must be dynamic; it is quite possible that not all caveolae are equivalent and a subpopulation may be less dynamic, which may be necessary for a different function, such as potocytosis (Anderson et al., 1992). Our observation that only a subset of the vesicles (~40%, see Table I) interact with the A–Au also provides evidence that subpopulations of caveolae may indeed exist.

The static view for caveola seems quite incongruous with the observations that discrete large protein–gold complexes found preferentially within caveolae are ultimately delivered to intracellular organelles. Furthermore, if caveolae are indeed static without a role in transcytosis or endocytosis, then the ablation of caveolae should have little, if any, affect on cellular transport. To the contrary, we show here that disassembly of caveolae with sterol binding agents is associated with not only a very significant loss in the transport into and across endothelial cells but also reduced capillary permeability. Although at this time we can not rule out the possibility of confounding multiplicity of effects elicited by filipin resulting in altered transport by other as yet unknown mechanisms, it seems most logical to accept the simplest apparent explanation based on the current available data. Filipin reduces the caveolae density in endothelium which inhibits caveolae-mediated endocytosis, transcytosis and capillary permeability of select macromolecules.

It is interesting to note that unlike coated vesicles, noncoated plasmalemmal vesicles, especially in the endothelium, can join together to form strings of vesicles in a racemose, treelike structure. In a logical permutation of the classic "ferryboat" or "shuttling" hypothesis for vesicular transcytosis, Michel (1992) has proposed that the racemose caveolae may indeed be more permanent structures with only the terminally attached vesicles being involved in actual transport or transcytosis. This so-called "fission–fusion" hypothesis maintains that vesicles do not form de novo nor move from one side of the cell all the way across to the other side of the cell but merely far enough to fuse with the most immediate, neighboring caveolar tree so as to allow release of their contents back to the outside milieu on the other side of the cell not directly but by diffusion through the "pores" of the other linked vesicles of the caveolar tree. This mechanism represents a refined permutation describing transcytosis and appears to involve "shuttling" in a more limited fashion between the terminal ends of neighboring caveolar trees located on opposite sides of the cell. Lastly, vesicles may participate in transport across the cell by forming patent transendothelial channels through the fusion of interconnecting luminal and abluminal caveolae (Wagner and Chen, 1991). Although this latter mechanism may provide passage for some molecules, it seems an unlikely pathway for providing direct specific transport of select molecules into or across the cell.

The controversy over transport via discrete vesicular carriers is an old one that has existed not only in the area of microvascular transport and capillary permeability but also in many other fields such as coated vesicular endocytosis and protein trafficking and secretion in general. In these latter cases, the vesicular carrier hypothesis has held true and is now widely accepted. It may now be time to accept caveolae as another type of transport vesicle which like clathrin-coated vesicles can function in both intracellular and transcellular transport. Furthermore, caveolae may resemble other vesicles involved in subcellular trafficking in other ways. Our recent studies indicate that caveolae-mediated endocytosis and transcytosis described in this study can also be inhibited by N-ethylmaleimide, suggesting as previously described for other vesicular processes that this transport pathway requires membrane fusion dependent on a NEM-sensitive factor(s) (Schnitzer et al., 1995). Lastly, purifica-

tion of caveolae from the luminal endothelial cell membrane derived directly from rat lungs shows that they have the appropriate molecular machinery for the specific transport of albumin via binding to albondin (Schnitzer, J. E., P. Oh, B. S. Jacobson, and A. M. Dvorak. 1994. *Mol. Biol. Cell.* 5:75A).

In summary, modified albumins such as A-Au bind the endothelial cell surface with high affinity, preferentially within caveolae to initiate their internalization to endosomes and lysosomes for degradation. Caveolae provide a novel mechanism for scavenger endocytosis of nonnative proteins such as modified albumins apparently via selective interactions with gp30 and gp18. The selective disassembly of caveolae with cholesterol binding agents such as filipin significantly reduces caveolae-mediated scavenger endocytosis and degradation without functionally disrupting the coated vesicular degradative pathway. Likewise, caveolae-mediated transcytosis of albumin and insulin is significantly inhibited whereas paracellular transport through the intercellular junctions is not affected by filipin treatment. Albumin's capillary permeability in the rat lung is reduced significantly. Although this study does not discern between the various models of vesicular transcytosis, it does show that ablation of caveolae with filipin treatment is associated with inhibition of the transport of select macromolecules not only into the cell (endocytosis) but also across the cell (transcytosis). Furthermore, filipin's selectivity in the disruption of caveolae may provide a useful tool for separating not only intracellular transport mediated by coated vs. noncoated vesicles but also transvascular transport in endothelium occurring via paracellular or transcellular pathways. Caveolae not only transport many molecules by fluid-phase uptake as originally proposed at their discovery in endothelium but now it is clear that they can also provide an independently regulated, receptor-mediated pathway for specific intracellular and transcellular processing of a variety of select ligands.

This work was presented in part at the 1992 ASCB meeting in Denver (Schnitzer, J. E., J. Bravo, A. Sung, and E. Pinney. 1992. *Mol. Biol. Cell.* 3:59a) and the 1993 and 1994 Federation of American Societies for Experimental Biologists meetings (Schnitzer, J. E., and E. Pinney. 1993. FASEB *[Fed. Am. Soc. Exp. Biol.] J.* 7:A639; Schnitzer, J. E., J. Allard, and P. Oh. 1994. FASEB *[Fed. Am. Soc. Exp. Biol.] J.* 8:A1057). It was supported in part by National Institutes of Health grants HL43278 and HL52766 and a Grant-In-Aid from the American Heart Association, National and California affiliates. Much of this work was done during the tenure of an Established Investigator Award from the American Heart Association and Genentech.

Received for publication 2 September 1994.

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