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Transcytosis in the continuous endothelium of the myocardial microvasculature is inhibited by *N*-ethylmaleimide

D. PREDESCU*, R. HORVAT, S. PREDESCU, AND G. E. PALADE

Division of Cellular and Molecular Medicine, Gilman Drive, University of California, San Diego, La Jolla, CA 92093-0651

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ABSTRACT **In a murine heart perfusion system, we were able to "turn off" the transport of derivatized albumin [dinitrophenylated albumin (DNP-albumin)] from the perfusate to the tissue, by preperfusing the system with 1 mM *N*-ethylmaleimide (NEM) for 5 min at 37°C, followed by a 5-min perfusion of DNP-albumin in the presence of NEM. Using a postembedding immunocytochemical procedure, we showed that (*i*) a 30-sec to 1-min treatment of heart vasculature with 1 mM NEM reduces the transendothelial transport of DNP-albumin and nearly stops it after 5 min, and (*ii*) DNP-albumin is detected exclusively in plasmalemmal vesicles (PVs) while in transit across endothelial cells. Perfusion with 10 mM dithiothreitol for 1 min before NEM prevents the inhibition of vesicular transport. To quantitate the NEM effect on vesicular transport inhibition, we developed an ELISA and a dot-blot assay for measuring DNP-albumin in supernatants of perfused whole-heart homogenates. The results obtained indicate that the treatment of the heart vasculature with 1 mM NEM decreases the vesicular transport of DNP-albumin by 78–80%. Since NEM is known to inhibit the fusion of different types of vesicular carriers with their target membranes in other cell types and in *in vitro* reconstituted cellular systems, by alkylating a NEM-sensitive factor, we assume that the same mechanism applies in our *in situ* system. The decrease of vesicular transport can be explained by NEM preventing the fusion of recycling vesicles with their targets—i.e., the abluminal and luminal domains of the plasmalemma. The results open to question previous interpretations from other laboratories according to which plasmalemmal vesicles are sessile, immobile structures.**

The role of plasmalemmal vesicles (PVs) in the transcytosis of particles and macromolecules in continuous microvascular endothelia has been extensively documented (1, 2). More recently, the evidence in case has been strengthened by the results of experiments that have used dinitrophenyl (DNP)-derivatized albumin (DNP-albumin) as tracer (3) and have localized it by immunocytochemical procedures compatible with adequate sampling and satisfactory preservation of relevant endothelial structures.

PV function in endothelial transcytosis has been contested, however, on a number of grounds by capillary physiologists that have postulated that exchanges between the plasma and the interstitial fluid occur through pores located along intercellular junctions (4); more recently, however, vesicular transport has met with increasing acceptance (5). Tridimensional reconstruction from thin serial sections of chemically fixed endothelial cells has revealed that in such specimens PVs are organized in sessile dendritic structures with no continuity across the endothelium and only a few free vesicles scattered in the cytoplasm (6, 7). More recently PVs (in fibroblasts) have been ascribed to an entirely different function, called "potocytosis" (8, 9), assumed to involve uptake of substrate followed by digestion and transport to the cytosol. Explicitly or implicitly, the conclusions reached in refs. 6 and 8 are not compatible with PV transcytosis in vascular endothelia.

Transcytosis implies repeated PV fission from one domain of the plasmalemma coupled with repeated PV fusion to the opposite domain, in a process generally comparable to that at work in transport by vesicular carriers along the intracellular exocytic and endocytic pathways. Work on reconstituted vesicular carrier transport in cell-free systems (10, 11) has identified an *N*-ethylmaleimide (NEM)-sensitive factor (NSF) as an essential component of a "membrane fusion machine" required for vesicular transport along the pathways mentioned above (12, 13). Although the PVs belong to a different type of vesicular carriers than those so far studied (14, 15), we surmised that their fusion to the target membrane may depend on NSF (or related proteins) and, hence, be sensitive to NEM. To test this assumption, we have studied quantitatively the transcytosis of DNP-albumin perfused through the microvasculature of murine heart in the presence or absence of NEM in the perfusate. The results presented in this paper indicate that NEM severely inhibits transcytosis of DNP-albumin by PVs.

MATERIALS AND METHODS

Animals. The studies were performed on 15- to 20-g BALB/c male mice kept under standard housing conditions and anesthetized intraperitoneally as described (16).

Antibodies and Reagents. Rabbit anti-DNP and the horseradish peroxidase (HRP)-conjugated anti-DNP antibodies were from Dako; reporter antibodies—i.e., goat anti-rabbit IgG, alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG, and HRP-conjugated goat anti-rabbit IgG—were from Cappel and Kirkegaard and Perry Laboratories. All other chemicals were of reagent grade.

Preparation of Probes. DNP-albumin prepared as described (3) was separated from free DNP by gel filtration over PD-10 columns from Pharmacia. The reporter antibody, 9-nm-gold-tagged goat anti-rabbit IgG, was prepared and stabilized as described (17), stored at 4°C in phosphate-buffered saline (PBS) containing 200 μg of polyglutamic acid per ml, and diluted with PBS before use to a gold OD at 520 nm = 0.1 (OD_{520}^{Au} = 0.1).

Perfusion Protocol. The perfusions were carried through as described (16) with glucose-supplemented, oxygenated, PBS (sPBS) prewarmed to 37°C. In NEM experiments, a 5-min perfusion with 1 mM NEM in sPBS preceded a 5-min perfusion with DNP-albumin at 30 mg/ml in sPBS containing 1 mM NEM. In control experiments, NEM was omitted at any perfusion step. Additional experiments were carried through with the same perfusion protocol except that 9-nm albumin–gold complexes were used as tracers (instead of

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Abbreviations: PV, plasmalemmal vesicle; DNP, dinitrophenol; NEM, *N*-ethylmaleimide; NSF, NEM-sensitive factor.

*To whom reprint requests should be addressed.

DNP-albumin) or that 10 mM dithiothreitol was perfused for 1 min before NEM and was included in the next two perfusion steps—i.e., NEM and NEM/DNP-albumin.

Specimen Processing for EM. The hearts were fixed *in situ* by a 20-min perfusion with a mixture of 4% (vol/vol) formaldehyde (freshly prepared from paraformaldehyde) and 2.5% glutaraldehyde in 0.1 M sodium cacodylate·hydrochloride buffer (pH 7.4). Specimens, removed from fixed hearts, were cut in small blocks, fixed for 1 hr in the same aldehyde mixture, postfixed in 1% OsO_4 in the same buffer, dehydrated, and finally embedded in Epon 812 by standard procedures. Thin sections, cut from blocks cured for 72 hr at 60°C and mounted on nickel grids were quenched with 0.1 M glycine in PBS (10 min), blocked with 1% albumin in PBS (30 min), and incubated (1–2 hr) with the first antibody (rabbit anti-DNP diluted 1:2000 in PBS containing 0.1% albumin). The grids were washed three times for 15 min each in PBS, incubated 1 hr with the reporter antibody diluted in PBS to an $OD_{520}^{Au} = 0.2$, washed again three times for 15 min each in PBS with 0.1% albumin, stained with uranyl acetate and lead citrate, and finally examined and micrographed in a Philips CM-10 transmission EM. We were able to use this new procedure since the DNP antigen is not affected by either aldehyde–OsO_4 fixation or Epon embedding; thereby, we obtained satisfactory structural preservation and adequate sampling.

Biochemical Studies. Hearts perfused with the tracer and then flushed for 5 min with PBS were minced by hand and homogenized in PBS at a ratio of 1:10 (wt/vol) by using first a Tekmar Tissumizer at maximum speed for 1 min at 4°C and then a Microson ultrasonic cell disrupter at 80% of output three times for 30 sec each at 4°C. The ensuing homogenate was centrifuged at 4°C for 5 min at 14,000 rpm in a 4515C Eppendorf Microfuge (with standard rotor); the resulting supernate was recentrifuged at 4°C and 42,000 rpm in a Beckman TL-100 ultracentrifuge with a TLC-45 rotor. The final supernate, which is expected to contain DNP-albumin that had undergone transendothelial transport, was used for (*i*) protein determination by the bicinchoninic acid (BCA) micromethod (using a Pierce kit), (*ii*) ELISA assays, and (*iii*) dot-blot assays.

Immunoblotting. To test the specificity of the DNP antibody, samples from final supernates prepared as indicated above were subject to SDS/PAGE with 4–20% Novex precast minigels, and the resolved proteins were transferred to nitrocellulose membranes by using an Idea Scientific transfer apparatus for 2 hr at 24 V as described (18, 19). The transferred electrophoretograms were blocked with 5% nonfat dry milk in PBS for 14–16 hr and incubated 1 hr with either anti-DNP or HRP-tagged anti-DNP antibodies at a dilution of 1:1000 in PBS containing 0.05% Tween 20. Antibody–antigen complexes were detected either directly or with a goat anti-rabbit IgG coupled to HRP by using tetramethylbenzidine as a substrate.

ELISA and Dot-Blot Assays. For ELISA, 100-μl aliquots from increasing dilutions of 1 ml of final supernate (that originally contained 1 mg of protein) were used to coat the wells. After a 1-hr incubation at 37°C and four washes with TTBS (20 mM Tris·HCl, pH 7.4/145 mM NaCl/0.05% Tween 20), the bound DNP-albumin was revealed by applying anti-DNP antibody diluted 1:2000 in TTBS for 1 hr at 37°C followed by four washes in TTBS and a goat anti-rabbit IgG coupled to alkaline phosphatase. The color reaction was developed using *p*-nitrophenyl phosphate as a substrate, and the plates were read at 405 nm in an ELISA reader. Dot-blots were performed with a Bio-Rad slot-blotting apparatus using 250 μl of sequentially diluted supernate per slot. The Magna Graph membranes (nylon transfer membranes, 0.45 μm) were treated with the HRP-conjugated anti-DNP antibody diluted 1:2000 in PBS, developed with the electrochemiluminescent (ECL) substrate kit from Kirkegaard and Perry Laboratories, and exposed for 1 min, in the dark to hyperfilm-ECL (Amersham). The films were read with an UltroScan laser densitometer LKB–Pharmacia. A dose–response curve was constructed for each assay, and the DNP-albumin concentration was determined for multiple protein concentrations in the linear part of the curve.

RESULTS

Protocol. Protocol parameters were either taken from the literature or determined experimentally by surveying by EM the number of tracer molecules transported to the pericapillary spaces. This number began to decrease after 30 sec or 1 min of NEM perfusion, and the reduction became obvious by 5 min. The amount of DNP-albumin in the NEM-containing perfusate was increased from 1 to 30 mg/ml (which is the physiological concentration of albumin in the plasma), and the perfusion time was varied from 30 sec to 1, 2, 5, 10, and 30 min. After 10 min there was structural damage to both endothelia and myocytes. At 5 min of tracer perfusion, the endothelium appeared to be intact, the only detectable morphological change being a condensation of the mitochondrial matrix in all cell types. Accordingly, the final protocol included a 5-min preperfusion with 1 mM NEM followed by a 5-min perfusion of DNP-albumin at 30 mg/ml in the presence of 1 mM NEM.

Antibody Validation. DNP-albumin has been extensively characterized (20) and appears to be an acceptable substitute for albumin. The anti-DNP antibodies used in this work recognized only DNP-albumin and no other proteins, endogenous albumin included (Fig. 1). However, albumin antibodies recognize DNP-albumin (results not shown).

NEM Does Not Affect Albumin–Gold Complex Binding. Since reduced transcytosis could be the secondary effect of NEM inhibition of albumin binding to the plasmalemma and of albumin access to PVs, the albumin–gold complex was used as a tracer because it is a better detector of albumin binding to the endothelium than either monomeric albumin (21) or DNP-albumin (3). The results (Fig. 2) showed that NEM does not preclude either albumin–gold binding to the luminal plasmalemma or albumin–gold labeling of PVs. Occasionally heavily labeled PVs were found next to the abluminal plasmalemma but not yet open to the subendothelial

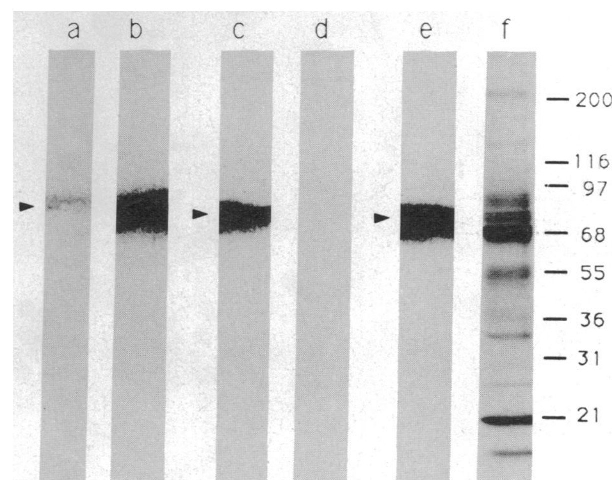

FIG. 1. Anti-DNP antibodies recognize DNP-albumin (lanes c and e) but not native albumin (lane d) by immunoblot analysis of the transfer of an SDS/8% polyacrylamide gel electrophoretogram of the two proteins. The load per lane was 10 μg. In the final supernate of DNP-albumin-perfused murine hearts, the anti-DNP antibody shows a much weaker signal in NEM-perfused specimens (lane a) than in controls (no NEM) (lane b). A stained transferred electrophoretogram of the final supernate is shown in lane f; the position of albumin is marked by arrowheads. Sizes are shown in kDa.

spaces (Fig. 2 *Inset*). Albumin–gold labeling of the pericapillary spaces was drastically reduced by comparison with controls (not shown).

NEM Inhibits Transcytosis. Finally a series of experiments were carried out with DNP-albumin as tracer, and the results were followed in parallel by immunocytochemistry and by quantitative measurements of the amount of tracer transported to the tissue. In controls, DNP-albumin was detected in PVs within the endothelium, including vesicles discharging on the abluminal front (Fig. 3*A*). Tracer molecules were also detected in relatively large numbers in the pericapillary spaces. In NEM-treated specimens, the structural integrity of the endothelium was preserved, but there was limited labeling of PVs and drastic reduction of tracer molecules in the pericapillary spaces (Fig. 3*B*). In both cases, the intercellular junctions were closed with no evidence of DNP-albumin leaving the lumina via the paracellular pathway. Preperfusion with dithiothreitol prevented the effect of NEM on DNP-albumin transcytosis as assessed by immunocytochemistry (results not shown). Different gold particles from 5 to 10 nm were used for reporter antibodies, and in all cases a striking difference was detected between control and NEM-perfused specimens.

The graphs in Fig. 4 give the results of five consecutive experiments and compare the concentration of DNP-albumin in controls and in NEM-treated specimens by two different procedures—ELISA assay (Fig. 4*A*) and a dot-blot assay (Fig. 4*B*). The final supernates of perfused-heart homogenates were used for these determinations, since they are the tissue fractions expected to include interstitial fluid and hence tracer transported by endothelial transcytosis. The measurements were made at a series of protein concentrations in the linear part of the DNP-albumin signal versus supernate protein curves. Both assays revealed that transcytosis is 75–80% inhibited by NEM at all points tested. Preliminary experiments with an anti-NSF antibody (provided by J. Rothman, Sloan–Kettering Institute) show that NSF can be detected by immunoblotting in homogenates of heart, lung, and cultured microvascular endothelial cells.

DISCUSSION

We have already mentioned in the introduction the published evidence that documents the transcytosis of particles and macromolecules by PVs in the continuous type of microvascular endothelium found in the myocardium, lung, and diaphragm. This documentation has been extended in recent studies to macromolecules of smaller dimensions, such as orosomucoid, myoglobin, and myoglobin fragments (22). The findings presented in this paper further document, this time by quantitative assays, the role of PVs in transcytosis of albumin (more precisely, of DNP-albumin). Moreover, they demonstrate that vesicular transport across the endothelium can be severely inhibited by NEM, a general alkylating reagent (23) known to inhibit exocytic (24) and endocytic (25) vesicular transport by inactivating a critical protein component, NSF, required for the fusion of vesicular carriers with target membranes (25, 26). Our new findings bring the PV

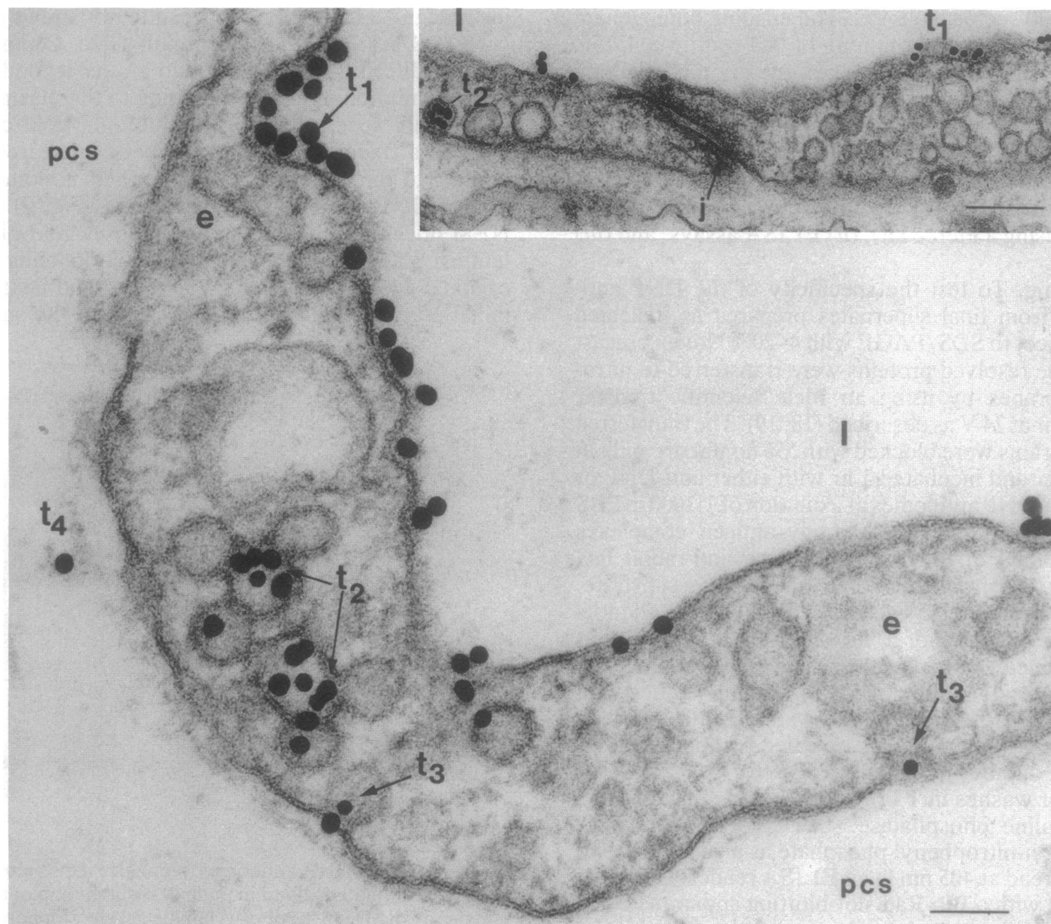

FIG. 2. Segment of a capillary from a specimen preperfused for 5 min with 1 mM NEM followed by perfusion for 5 min with the albumin–gold complex in the presence of 1 mM NEM. The tracer is detected (*i*) on the luminal plasmalemma (t_1), (*ii*) in the PVs within the endothelium (t_2), (*iii*) in discharging PVs on the abluminal plasmalemma (t_3), and (*iv*) in the pericapillary spaces (t_4). An intercellular junction (j) unmarked by the tracer and a heavily loaded PV close to (but not open on) the abluminal plasmalemma (t_2) are seen in *Inset*. l = Lumen; e = endothelium; pcs = pericapillary spaces. (Bar = 0.089 μm; *Inset* bar = 0.228 μm.)

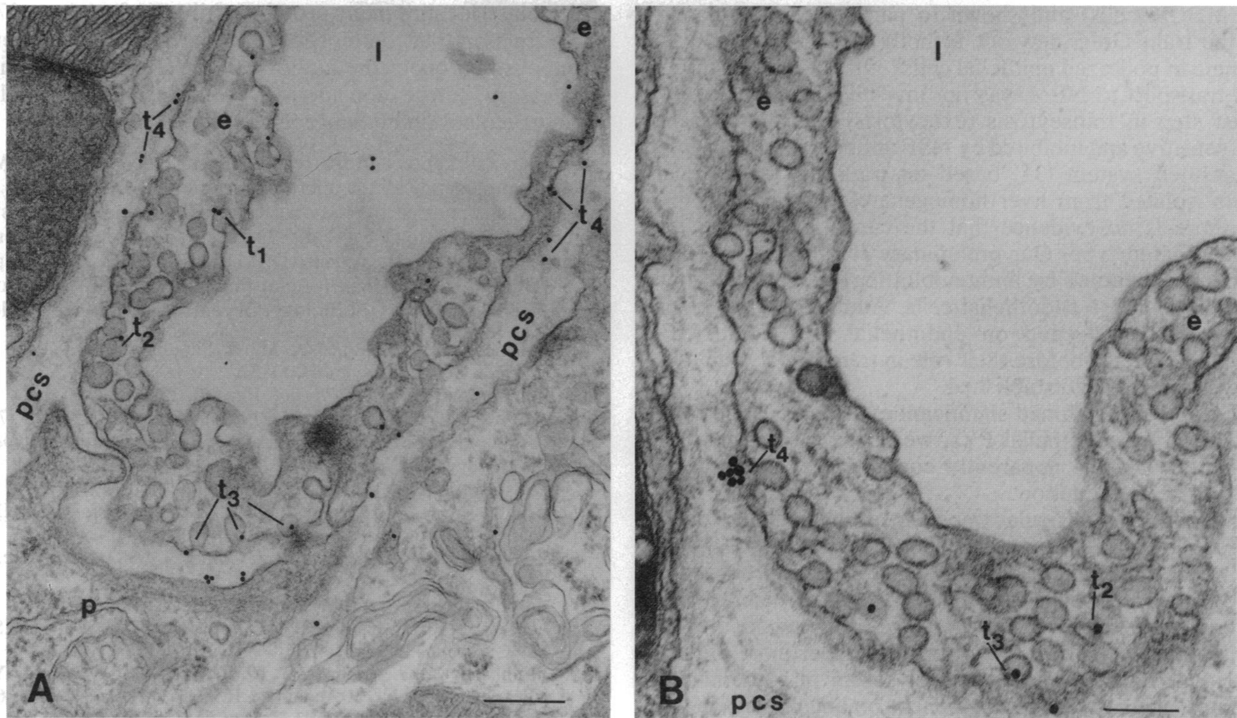

FIG. 3. (*A*) Segment of a myocardial capillary from a specimen perfused with DNP-albumin for 5 min. The tracer was detected by anti-DNP antibody followed by a 9-nm-gold-conjugated goat anti-rabbit IgG reporter antibody, which detects DNP-albumin in the lumen (arrow) on the luminal plasmalemma (t_1), in PVs within the endothelium (t_2), in abluminally discharging PVs (t_3), and in significant numbers in the pericapillary spaces (t_4). p, Pericyte. Other abbreviations are as in Fig. 2. (Bar = 0.288 μm.) (*B*) Segment of a myocardial capillary preperfused for 5 min with 1 mM NEM followed by a 5-min perfusion with DNP-albumin in the presence of 1 mM NEM. Detection of the tracer and indications as to its localization are the same as in *A*. Tracer labeling of PVs and the pericapillary spaces is considerably reduced by comparison with *A*. The cluster of gold particles at t_4 may be the result of the discharge of an overloaded PV as in Fig. 2 *Inset*. Other abbreviations are as in Fig. 2. (Bar = 0.235 μm.)

transcytosis operating in the continuous microvascular endothelium in line with current work on vesicular carrier transport in other cell types, an area of intensive and rapidly advancing research in cellular and molecular biology.

Endothelial PVs have a caveolin-containing coat on their cytoplasmic aspect and, thereby, belong to a structurally and chemically different class of vesicular carriers than those so far studied (27). Caveolin-† or vesicular integral protein (VIP) 21-coated vesicles have been identified in cultured epithelial

†PVs were originally found in vascular endothelia (28) and subsequently described in epithelia by Yamada (29) under the name of caveolae intracelulares. Caveolin is a recent derivation (9) from Yamada's caveolae.

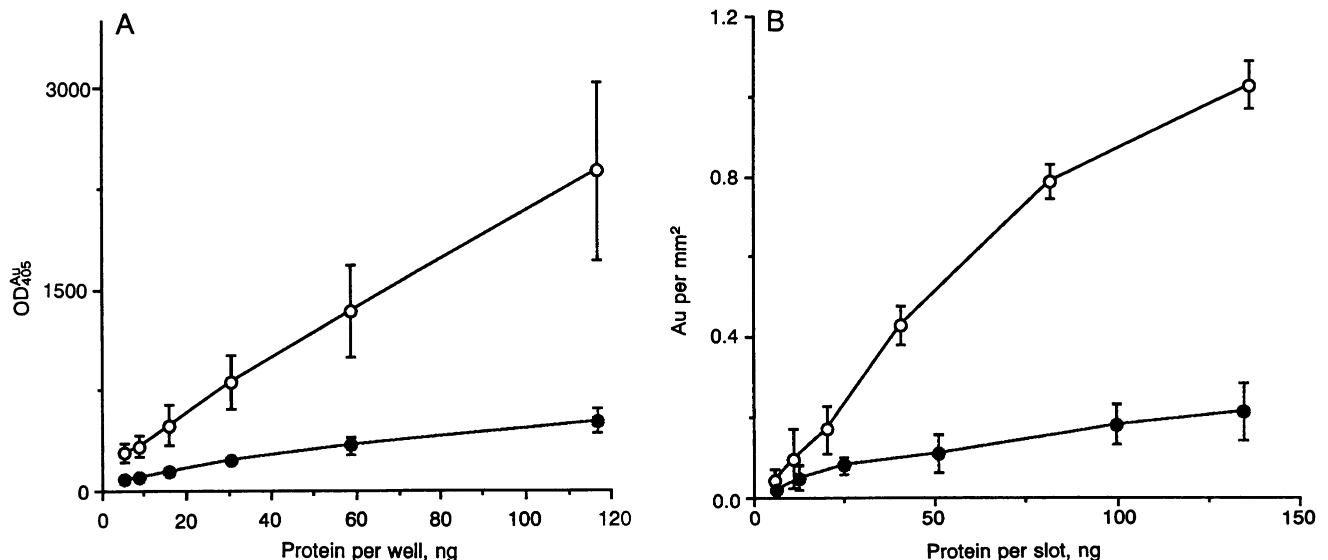

FIG. 4. Comparison of DNP-albumin concentration in the final supernates of myocardial homogenates in control (○) versus NEM-perfused (●) whole-heart specimens. Tracer amounts were determined by ELISA (*A*) and by dot-blot assays (*B*). DNP-albumin concentrations were assayed at a series of supernate protein concentrations on the linear part of the dose–response curve in five consecutive experiments.

cells (MDCK cells) and shown to participate in transport from the trans-Golgi network to both domains of the plasmalemma in polarized epithelial cells (30), but the sensitivity of the transport to NEM was not investigated. Conversely, the last step in transcytosis (exocytosis) was found to be NEM-sensitive and inhibited by NSF antibodies in an *in vitro* reconstituted system (31) based on transcytotic vesicular carriers isolated from liver homogenates (32). But, in this case, there is no evidence that the carriers belong to the caveolin-coated type. Our preliminary studies indicate that NSF can be detected by immunoblotting in lysates of lung, heart, and cultured endothelial cells. Admittedly, however, further work is needed on endothelial NSF and NSF-associated proteins before their role in transcytosis could be considered as firmly established.

Since we have obtained significant evidence in support of transcytosis by endothelial PVs, we next consider possible explanations for the apparently conflicting evidence mentioned in the introduction.

For a long time, vesicular transport has been denied physiological significance by capillary physiologists because their experimental data indicated the existence of a large convective component in albumin transport (33) that could be explained by efflux through pores and not by vesicular transport. Recent experiments have shown, however, that under more physiological conditions (i.e., presence of serum in experimental perfusates) the convective component practically disappears (5), which leaves vesicular transport as a reasonable explanation of the data. However, the structural basis for the increase in convective efflux of albumin in the absence of plasma components in the perfusate remains to be elucidated.

The work of Bundgaard *et al.* (6) and Frokjaer-Jensen (7) relied on tridimensional reconstruction of segments of microvascular endothelium from exquisitely thin sections and arrived at the conclusion that the vast majority of the vesicles was organized in sessile branched structures with essentially no free vesicles left in the cytoplasm to act either as shuttles or connectors between the vesicular chains anchored on the two sides of the endothelial plasmalemma. Hence, they claimed that, on morphological grounds, transcytosis by PVs is not possible. The specimens examined represent, per force, a very small sample; moreover (and more importantly), the specimens were chemically fixed and the fixative procedure used has been shown to take 9 or more seconds until vesicular movement is brought to a complete halt (34). In the twilight period between the beginning and the end of fixation (viewed as progressive crosslinking of proteins), vesicular distribution and interactions can be altered. Fusing with one another may be for PVs the equivalent of a minimum free-energy condition. In any case, physiological criteria, like following the pathway of a tracer as done in this and previous studies, provide more reliable evidence because they are, at least in part, independent of structural rearrangements that may occur as chemical fixation progresses to its end point.

A recently advanced hypothesis postulates that caveolin-coated vesicles, studied in other cells than endothelial, function in a novel process called potocytosis (8), which implies that the PVs are sessile structures that do not detach from the plasmalemma and accordingly cannot function in transport. Our results and those already documented in the literature indicate that this conclusion cannot apply to the microvascular endothelium whose activity in transcytosis cannot be ignored. However, potocytosis or a variant thereof could be considered for other cell types that have caveolae but do not appear to use them for transport operations.

A necessary step towards the elucidation of the function of endothelial PVs would be their isolation and chemical characterization. Previous immunocytochemical work (9, 35, 36) and recent cell fractionation work on caveolae isolated (as a detergent-insoluble residue) from other cell types (MDCK, fibroblast) localize many proteins of diverse functions (37, 38) in a fraction that, on morphological grounds, is still heterogeneous. To quote the authors (38): "further proof will be necessary before considering Triton-insoluble complexes and caveolae" interchangeable entities.

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