Binding and Transcytosis of Glycoalbumin by the Microvascular Endothelium of the Murine Myocardium: Evidence That Glycoalbumin Behaves as a Bifunctional Ligand

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Abstract. The binding and transport of glycoalbumin (gA) by the endothelium of murine myocardial microvessels were studied by perfusing in situ ^{125}I-gA or gA-gold complexes (gA-Au) and examining the specimens by radioassays and EM, respectively. After a 3-min perfusion, the uptake of radioiodinated gA is 2.2-fold higher than that of native albumin; it is partially (~55%) competed by either albumin or D-glucose, and almost completely abolished by the concomitant administration of both competitors or by gA. D-mannose and D-galactose are not effective competitors. Unlike albumin-gold complexes that bind restrictively to plasmalemmal vesicles, gA-Au labels the plasmalemma proper, plasmalemmal vesicles open on the lumen, and most coated pits. Competing albumin pre-

vents gA-Au binding to the membrane of plasmalemmal vesicles, while glucose significantly reduces the ligand binding to plasmalemma proper. Competition with albumin and glucose gives additive effects. Transcytosis of gA-Au, already detected at 3 min, becomes substantial by 30 min. No tracer exit via intercellular junctions was detected. gA-Au progressively accumulates in multivesicular bodies. The results of the binding and competition experiments indicate that the gA behaves as a bifunctional ligand which is recognized by two distinct binding sites: one, located on the plasma membrane, binds as a lectin the glucose residues of gA; whereas the other, confined to plasmalemmal vesicles, recognizes presumably specific domains of the albumin molecule.

IN the general circulation, a small fraction (≤15% [14, 19]) of the albumin is chemically and irreversibly modified by glycation, that is by covalent linkage of glucose primarily to the ε-amino groups of lysine residues (1, 8, 14, 15, 35). Glycated albumin or glycoalbumin (gA)[1] is increased two- to threefold in diabetics and this modification has been connected with the vascular pathology of such patients, especially renal and retinal microangiopathy (4, 7, 9, 18, 21, 24). In diabetics, a high rate of glycation was also detected in hemoglobin (15, 21), low density lipoproteins (18, 51), fibrin (5), membrane proteins of erythrocytes (26, 44), and platelets (52), as well as in tissue and some extracellular matrix proteins such as collagens (4, 7, 34), fibronectin (39, 40), nerve proteins (43), and lens crystallins (27).

Isolated microvascular fragments (including endothelial cells) were shown to take up gA in preference to native albumin (45, 47–49). The process was ascribed to micropinocytosis by plasmalemmal vesicles (45, 47–49) but direct evidence for this assumption was not provided.

Since in our previous studies we have shown that albumin is transcytosed across the endothelium by plasmalemmal vesicles (17, 37), it becomes of interest to find out whether the same mode of transport with an expected increase in rate applies to gA. The work was carried out in the hopes that the results will contribute to our understanding of diabetic microangiopathies.

Materials and Methods

Animals

Reagents

Portions of this work have appeared previously in abstract form (1987. *J. Cell Biol.* 105[No. 4, Pt. 2]:326a).

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1. *Abbreviations used in this paper:* A-Au, albumin–gold; gA, glycoalbumin; gA-Au, glycoalbumin–gold; MVB, multivesicular body.

MO); Pharmalyte 3–10, Sephadex G-25, and IEF marker kit from Pharmacia Fine Chemicals Inc. (Uppsala, Sweden); carrier-free Na[125I] from the Institute of Atomic Energy (Otwock-Swierk, Poland); and Dulbecco's PBS, pH 7.4, from Gibco Laboratories (Grand Island, NY).

Preparation and Characterization of Probes

gA Preparation. 25 mg native albumin dissolved in 1 ml sterile PBS, pH 7.4, was incubated with 90 mM D-glucose for 14 d at 37°C (28, 29). The mixture was repeatedly filtered through 0.22 μm Millipore filters and extensively dialyzed against PBS to remove the unbound glucose. gA was isolated by boronate-affinity chromatography on a minicolumn (1 ml) of Glyco-Gel B previously equilibrated with ammonium acetate solution (0.25 M ammonium acetate, 0.01 M magnesium chloride, pH 8.4) which was used both as sample solvent and washing medium. gA was eluted with 10 ml of 0.2 M Na-citrate, pH 4.5 (23). Albumin in the washed (unbound) and eluted (bound) fractions was determined by UV absorbance at 280 nm or by protein assays according to references 3 and 33. Boronate affinity specifically retains gA and allows nonglycated entities to pass through; as a result, the gA thus prepared is virtually free of significant amounts of native albumin (23).

Aliquots of crystallized albumin dissolved in PBS (at concentrations of 1, 2, or 3 mg/ml), subjected to the same system of boronate-affinity chromatography as above, showed that the native bovine albumin used in our experiments contained <1% gA.

The efficiency of gA separation by boronate-affinity chromatography was assessed by applying to a Glyco-Gel-B column a sample of 1 ml albumin (25 mg) glycated with D-[14C]glucose (750,000 cpm). Protein content and radioactivity were determined in the eluted fractions by spectrophotometry at A = 280 nm and spectrometry in a gamma counter (model 4,000; Beckman Instruments, Inc., Fullerton, CA), respectively. As shown in Fig. 1, gA is eluted as the second proteic peak which contains >85% of the radiolabeled glucose. In the conditions used, 17 ± 4% of native albumin was converted to gA.

The extent of glycation as determined by the thiobarbituric acid method (52) was 2.9 mol glucose/mol albumin, and by using [14C]glucose (28) was 2.8 mol glucose/mol albumin. When incubation was performed at pH 7.8 (2) the level of albumin glycation was increased up to 20%.

Isoelectric Focusing. IEF carried out for 90 min at 10°C on IEF-agarose using Pharmalyte 3–10 (at 3,000 V, 14 W) revealed that the gA thus prepared was more anionic (∼pI 4.45) than native (unmodified) albumin (∼pI 5.2). These results are in agreement with data reported in references 8, 16, and 22.
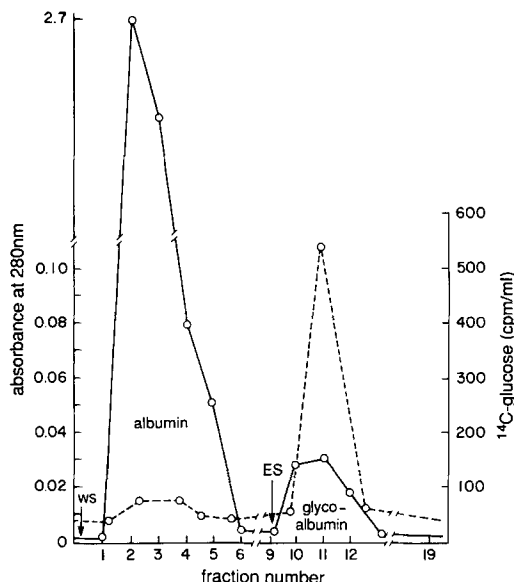

Figure 1. Resolution of an albumin–gA mixture by boronate-affinity chromatography on a Glycogel-B column. (——) O.D. 280 nm (albumin); (---)14C radioactivity (gA) (counts corrected for background). Solutions for washing (*WS*) and elution (*ES*) were applied at the arrows with 0.25 M NH4 acetate–0.01 M MgCl2, and 0.2 M Na-citrate, respectively.

Tryptophan Fluorescence of gA. Fluorescence spectra were comparatively determined for native BSA and gA. The profiles obtained revealed that the tryptophan fluorescence of gA is reduced by ∼30% as compared to nonglycated albumin. In agreement with already recorded data (20, 35) these findings suggest that glycation induces detectable conformational changes in albumin. The effect of these changes on interactions involving gA are still unclear.

Radioiodination of gA and Albumin

Radiolabeled gA was prepared as in (12) with minor modifications. 500 μg gA in 500 ml PBS were reacted with 500 μCi Na[125I] in Iodogen-coated test tubes (70–100 μg Iodogen per tube). To remove unbound 125I, samples were subjected to gel filtration on Sephadex G-25 minicolumns (which were centrifuged at 500 g for 2 min) and then extensively dialyzed against PBS. 125I radioactivity per unit volume of gA solution was determined with a gamma counter (model 4000; Beckman Instruments, Inc.).

By comparison with gA, the fluorescence spectra of radioiodinated gA revealed an additional decrease in tryptophan fluorescence (data not shown).

Radiolabeled albumin was prepared by the same procedure used for 125I-gA; the specific activities of the labeled proteins were ∼1 μCi ± 0.2/ mg protein.

Preparation of gA-gold (gA-Au) Complexes

Colloidal gold suspensions of 5-nm particles were prepared by HAuCl4 reduction with white phosphorus (38). Gold suspension was incubated for 15 min at 24°C with gA at a ratio of 1 ml/80 μg. The mixture was centrifuged at 45,000 g for 60 min at 4°C and the ensuing pellet was resuspended in PBS to a concentration giving an $A_{515}^{1.0} = 1.0$ (17). Before use, the tracer solution was centrifuged for 5 min at 12,000 g to eliminate aggregates.

gA-Au complexes were examined by EM in negatively stained specimens. Measurements made on 120 particles gave a range of 4–7 nm for gold particles (with >80% of 5-nm diameter) and a range of 2–4 nm for the gA coat of gold particles visualized as a negative image. gA-Au complexes vary, therefore, in diameter from 6 to 11 nm. Albumin–gold (A-Au) complexes were prepared as in (17).

Experimental Procedures

General Protocol. All experiments were performed in situ and involved the following steps. (a) General anesthesia by i.p. injection of 5% chloralhydrate (20–30 mg/100 g body weight). (b) Laparotomy followed by catheterization of abdominal aorta (to be used as inlet) and vena cava abdominalis (to be used as outlet) for the perfusion of the heart. (c) Perfusion with oxygenated PBS at 37°C for ∼4 min at a flow rate of 3 ml/min. (d) Perfusion of one of the following probes dissolved in PBS, and warmed up to 37°C: 125I-gA (sp act of 1–1.5 μCi/μg protein) for 3 min; gA-Au at a concentration giving $A_{515}^{1.0} = 1.0$ continuously perfused for 3, 10, or 30 min; 125I-albumin (sp act of 1–1.5 μCi/μg protein) for 3 min; and A-Au, the same amount and conditions as gA-Au. Unbound tracer was washed out by perfusion for 3 min at a flow rate of 3 ml/min with PBS, prewarmed to 37°C.

Tissue Processing. At the end of the procedure described above, heart specimens were processed as follows. For radioassays: fresh tissue fragments were excised and prepared for spectrometry in a gamma counter (model 4,000; Beckman Instruments, Inc.); the data obtained were expressed as cpm/mg wet tissue. For EM: the tissue was fixed by perfusion with 2.5% formaldehyde, 1.5% glutaraldehyde, and 2.5 mM CaCl2 in 0.1 M HCl–Na cacodylate buffer, pH 7.2. The fixative mixture was warmed up to 37°C and administered for 3 min at a flow rate of 3 ml/min. Excised specimens were further fixed by immersion for 60 min at 1°C in a fixative mixture as in (36). Fixation was followed by standard processing for EM. Thin sections, cut on a Reichert ultramicrotome (Reichert Scientific Instruments, Buffalo, NY) or ultracut microtome (American Optical Co., Southbridge, MA), were lightly stained with uranyl acetate and lead citrate and examined with electron microscopes (models 400 HM or 201; Philips Electronic Instruments, Inc., Mahwah, NJ).

Competition Experiments. Considering the special chemical nature of gA, the binding and uptake of either the radiolabeled or gold-conjugated probe was determined in various competition conditions. The binding of 125I-gA, perfused for 3 min in situ, was competed with either (a) native albumin at 5, 20, 40, and 200 μg/ml; (b) D-glucose at 45 and 90 mM; (c) 200 μg/ml native albumin together with 90 mM glucose; (d) 100 μg/ml gA; (e) D-mannose at 45 mM; or (f) D-galactose at 45 mM (Fig. 2).
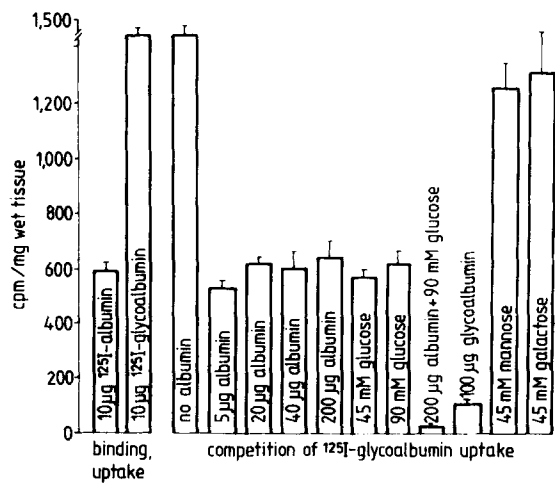
Figure 2. Quantitation of heart-associated radiolabeled tracers and the effect of the competitors used. First two columns, association (presumably binding and uptake) of ^{125}I-albumin and ^{125}I-glyco-albumin (gA) with murine hearts after 3-min perfusion in situ. The concentrations of the tracers in the perfusate at the beginning of the experiments are indicated in each column. Next eleven columns, the effect of competition with albumin, glucose, gA, mannose, and galactose on ^{125}I-gA binding and uptake by perfused murine hearts. Competitor concentration is given in each column.

The interaction of capillary endothelium with gA-Au, perfused for 3 min in situ, was competed by either (a) native albumin at 20 and 40 µg/ml; (b) D-glucose at 45 and 90 mM; (c) 20 µg/ml albumin together with 45 mM glucose; or (d) gA at 20 and 40 µg/ml. Competition with glucose was also carried out in experiments in which gA-Au was perfused in situ for 30 min.

All solutions were prewarmed at 37°C and the experiment's protocol was the same as described above, except that, before and during perfusion of the probes, the competitors were added to the perfusate. The preparation for radioassays and for EM was the same as indicated above.

Morphometry. For each experimental condition involving perfusion with gold-conjugated probes (A-Au and gA-Au) with or without competitors, six specimens were randomly collected from the left ventricle. Each specimen was processed for transmission electron microscopy; from each specimen 35-55 sections were obtained; and in each section capillary profiles were photographed and printed to final enlargements of 45,000 or 90,000×. Each microvascular profile on these micrographs was used to measure the length of plasma membrane proper, coated pits, and plasmalemmal vesicles open

to the luminal front, count gold particles associated with each of these domains, and count plasmalemmal vesicles and coated pits. Data were expressed as numerical densities of gold particles either per various endothelial cell structures or per unit length of luminal microdomain profile. For special measurements, additional sampling data are given in the footnotes to Tables I-III. The differences between means were compared by the *t* test.

Results

Our experimental results indicated that gA and albumin bound primarily to the luminal plasmalemma of the endothelium of capillaries and postcapillary venules. The binding is extensive and characteristic, yet different for the two tracers. The binding to the endothelium of arterioles and muscular venules is limited in extent and does not exhibit a characteristic pattern.

Uptake of Monomeric Radioiodinated gA and Albumin

Perfusion with the same amount (10 µg) of ^{125}I-albumin or ^{125}I-gA followed by a PBS flash established that the amount of ^{125}I-gA (expressed per mg wet tissues) retained in the tissue is 2.2-fold higher than the amount of ^{125}I-albumin (Fig. 2).

gA-Au and A-Au Have Different and Characteristic Binding Patterns

In experiments with A-Au, the tracer marked the vast majority (~97%) of plasmalemmal vesicles open to or closely associated with the luminal plasmalemma from the earliest time points examined in the endothelium of capillary and postcapillary venules. A-Au complexes appeared bound primarily to the vesicular membrane as already established in our previous publications (17). Binding to plasmalemma proper and coated pits was limited in both extent (Table I) and linear density (Table II).

In experiments with gA-Au, the binding pattern on capillary and postcapillary venule endothelia was clearly distinct. Although the labeling of plasmalemmal vesicles was just as extensive (~96% of total vesicles open on the luminal front) and slightly more pronounced (Table II), the plasmalemma

Table I. Comparison of A-Au to gA-Au Binding to Endothelial Luminal Plasmalemmal Microdomains in Murine Myocardial Capillaries. Effects of Competition with Albumin (40 µg/ml) or/and Glucose (90 mM), and gA (100 µg/ml)

Experimental conditions*	Plasma membrane proper (% of length)	% Plasmalemma vesicles	% Coated pits§
A-Au	18 ± 4.5	97 ± 2.9	3 ± 2.0
gA-Au	71 ± 11.2	96 ± 5.1	13 ± 3.1
gA-Au competed by albumin‡	74 ± 9.1	20 ± 4.7	10 ± 3.3
gA-Au competed by glucose‡	23 ± 6.1	91 ± 6.3	2 ± 1.0
gA-Au competed by gA‡	11 ± 2.1	23 ± 5.1	4 ± 1.5
gA-Au competed by albumin + glucose‡	13 ± 3.2	14 ± 3.7	3 ± 1.5

For each experimental condition, ~160 µm plasmalemma proper (luminal front), 410 plasmalemmal vesicles open to the lumen, and 30 coated pits were examined (for sampling, see Morphometry). All values are mean ± SD.
* Specimens perfused for 3 min with each tracer.
‡ The competitors were used before and during gA-Au perfusion.
§ All areas free of coated pits were discarded.

Experimental conditions*	Gold particles/μm membrane length		
	Plasmalemma proper	Plasmalemma vesicles	Coated pits
A-Au	9 ± 3.2	67 ± 8.2	0.3 ± 0.1
gA-Au	53 ± 6.1	86 ± 9.1	2.0 ± 0.7
gA-Au competed by albumin	45 ± 3.6	24 ± 4.2	1.5 ± 1.0
gA-Au competed by glucose	18 ± 2.1	79 ± 10.2	0.5 ± 0.3
gA-Au competed by gA	10 ± 3.2	20 ± 3.8	2.5 ± 1.0
gA-Au competed by albumin + glucose	7 ± 2.1	17 ± 4.4	2.0 ± 0.5

For the sampling and administration of competitors, see Table I and Morphometry (under Materials and Methods). All values are mean ± SD.
* Specimens perfused for 3 min with each tracer.
‡ Concentrations given in Table I.

proper was also heavily labeled. Approximately 70% of its profiles were quasicontinuously covered by gA-Au particles either as a monolayer or as small clusters (Fig. 3). The labeling of coated pits was also more extensive (~13%) and heavier than that obtained by A-Au (Tables I and II). As in the case of A-Au (17, 37), gA-Au binding is particularly extensive on the endothelium of capillaries and postcapillary venules. By comparison, the binding to the endothelium of arterioles and muscular venules is almost negligible.

Internalization of gA-Au

As a function of perfusion time, labeled plasmalemmal vesicles appeared throughout the endothelium from the luminal to the abluminal plasmalemma. The pattern of labeling varied considerably from a single to a few gA-Au particles per vesicle especially at early time points (3-5 min). The finding could be explained by early internalization during initial

labeling of the luminal plasmalemmal vesicles or by partial transfer of the gA-Au load from luminal vesicles to vesicles located at some depth in the cytoplasm.

By comparison with gA-Au findings, A-Au internalization appeared to be less extensive, but a morphometric analysis was not carried out because in sections it is not always possible to distinguish reliably surface binding from true internalization of markers.

Transcytosis of gA-Au

In some capillaries at 3-min perfusion, some plasmalemmal vesicles labeled by gA-Au were found attached to or open on the abluminal front of the endothelium (Fig. 4). When perfusion was extended to 10-30 min, gA-Au complexes were found on vesicles discharging on the abluminal front as well as in the pericapillary spaces (Fig. 4, A-D; Fig. 5 A). As during internalization, the number of gA-Au complexes was
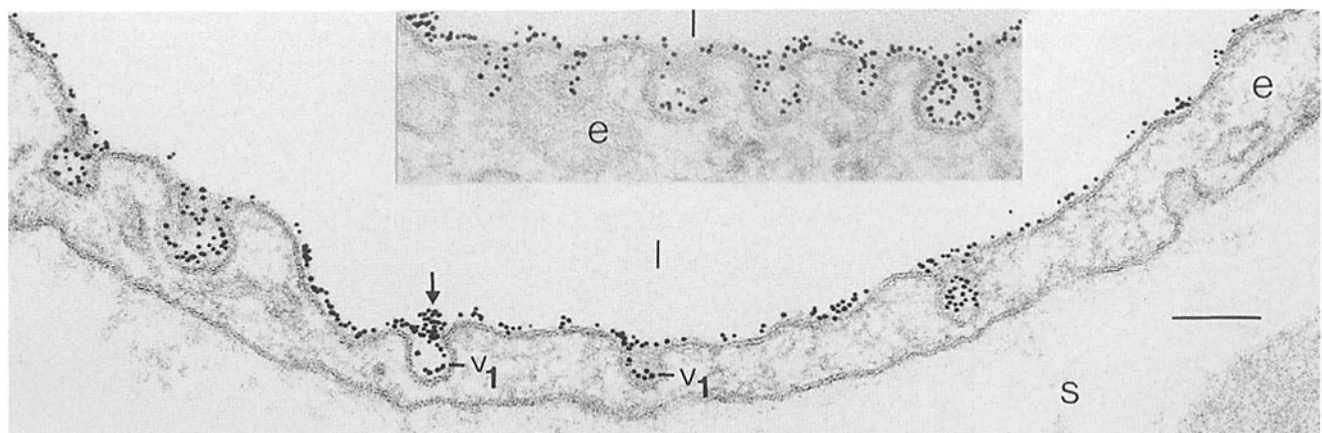

Figure 3. Endothelial segment from a capillary perfused with gA-Au complexes. All plasmalemmal vesicles open on the luminal front are marked by the tracer which in many cases appears to be closely associated with the vesicle membrane (V₁). In contradistinction with A-Au, gA-Au decorates the luminal plasmalemma with a quasicontinuous monolayer of particles. Clusters of particles are found at the introit of some plasmalemmal vesicles (arrow). The inset illustrates the extensive character of plasmalemmal vesicle labeling: all vesicles in this row are labeled; variations in number of gA-Au particles per vesicle can be ascribed to the level of sectioning which is grazing in all cases except for the far right vesicle. e, endothelial cells; l, lumen; s, subendothelial space. Bar, 0.1 μm.
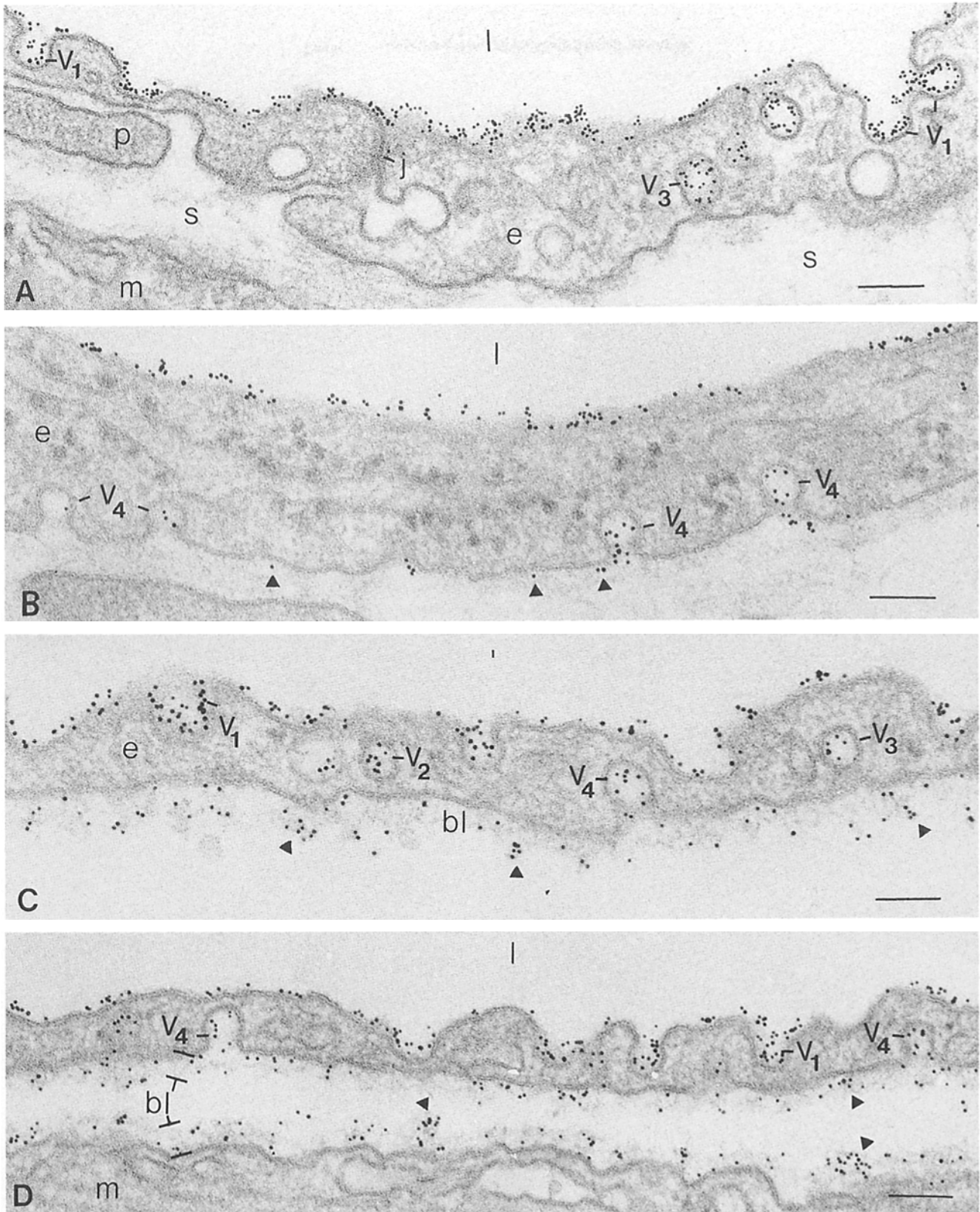
Figure 4. Time series of the tracer binding and transport in myocardial capillaries perfused in situ with gA–Au. (*A*) At 3 min, the label marks extensively vesicles open to the luminal front (V_1) and occasionally vesicles apparently ready to open on the abluminal front of the endothelium (V_3). There is no labeling of the intercellular space abluminal to the junction (*j*) and no tracer is detected in the pericapillary space (*s*). *l*, lumen; *e*, endothelial cells; *p*, pericyte; *m*, muscle cells. (*B* and *C*) After 10 min, gA–Au complexes are found in variable number (1–9) in the profiles of vesicles apparently discharging on the abluminal front (V_4). The amount of tracer (*arrowhead*) already transported to the pericapillary spaces varies from capillary to capillary from low (*B*) to relatively high (*C*). In the pericapillary space, the tracer appears to be associated primarily with the basal lamina (*bl*). (*D*) After 30 min, the tracer (*arrowheads*) is found in variable but generally large amounts in the pericapillary spaces and interstitia, and appears to be preferentially associated with the basal laminae (*bl*). In the endothelium itself, essentially all plasmalemmal vesicles irrespective of their location are labeled. *l*, lumen; *m*, muscle cells. Bars, 0.1 μm.
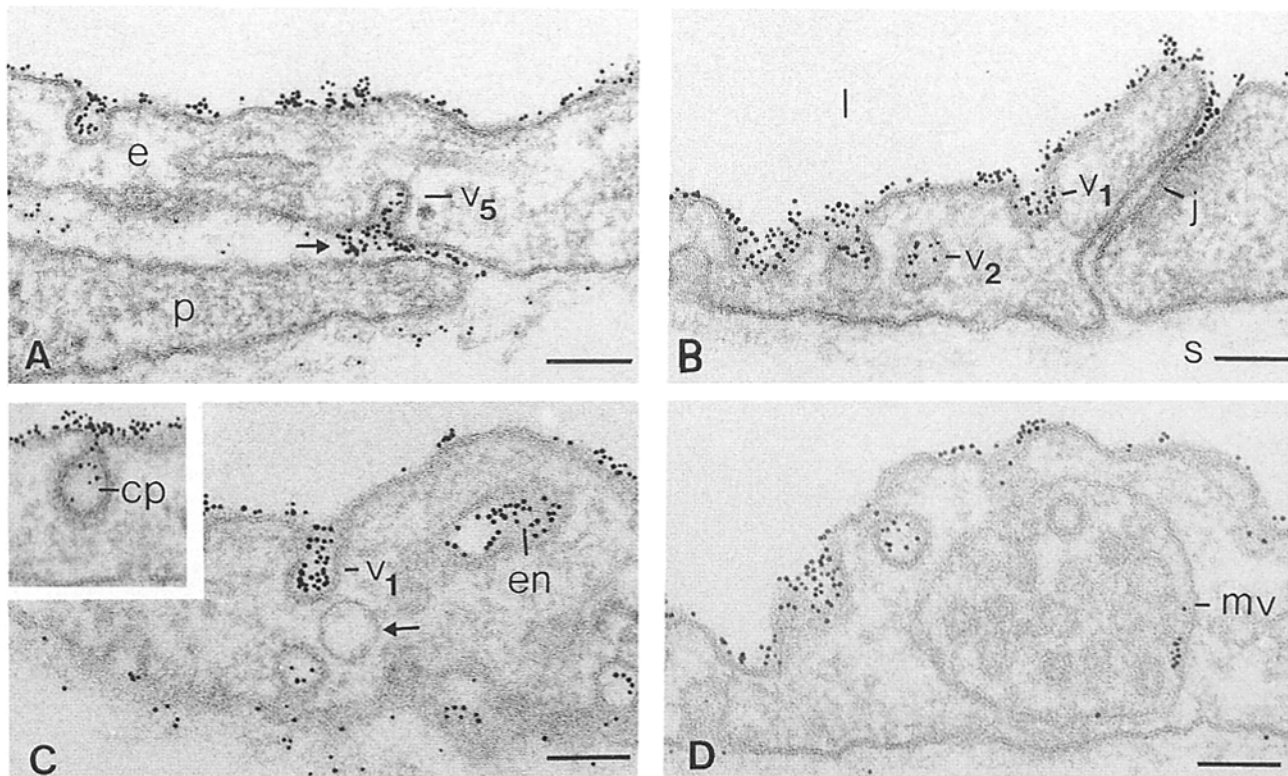
Figure 5. (*A*) Endothelial segment from a specimen perfused with gA-Au for 10 min. The vesicular profile (V_5) seen discharging gA-Au particles in a narrow interstitium between the endothelium (*e*) and a pericyte (*p*) probably represents the exit from a transendothelial channel. That would explain the large amount of particles (*arrow*) associated with this element. (*B*) Endothelial segment from a specimen perfused for 10 min. The labeling pattern of plasmalemmal vesicles is as described for the previous figures. The field also includes a junction (*j*) and associated intercellular spaces (*s*). The luminal infundibulum is marked by the tracer but the intercellular space abluminal to the junction is not. There is no tracer found in the pericapillary space in this field. *l*, lumen; V_1, vesicle open to the lumen; V_2, vesicle apparently internalized. (*C* and *D*) Endothelial segments from specimens perfused with gA-Au for 10 min. The figures illustrate the presence of the tracer in a large relatively pleomorphic vesicle assumed to be an endosome (*en*) and in a multivesicular body (*mv*). Labeling pattern of plasmalemmal vesicles and pericapillary space is as already described for the previous figures. *C* also illustrates the difference in labeling between luminal and abluminal plasmalemmal vesicles and the existence of an unlabeled internalized vesicle (*arrow*). The inset shows a labeled coated pit (*cp*) in the process of becoming a coated vesicle. Bars, 0.1 μm.

lower in plasmalemmal vesicles discharging on, or closely associated with, the abluminal front but the particle density per vesicle appeared to increase with time.

The distribution patterns of A-Au complexes described in previous papers (17, 37) and reexamined as controls in the present experiments were generally the same as those described above for transcytosis except that the amount of gA-Au moved by transcytosis was higher. A morphometric analysis of transcytosis was not performed because the data were not considered sufficiently reliable since the label is expected to be continuously drained from pericapillary space to the lymphatics. Yet, the increase mentioned above was in agreement with the results obtained by radioassay (Table I).

Endocytosis of gA-Au

Coated pits and coated vesicles were often found labeled by gA-Au complexes at all perfusion times (Fig. 5 *C, inset*). Already at 3 min gA-Au appeared in irregular relatively large pleomorphic structures with a light content tentatively identified as endosomes (Fig. 5 *C*). Complexes were also found in multivesicular bodies (MVB), structures which can be

identified reliably as endosomes (Fig. 5 *D*). The fraction of MVB labeled and the density therein increased with time (Table III). The data in Table III indicate that, depending on the perfusion time, about twice as many MVB were labeled by gA-Au than by A-Au.

In none of the micrographs examined was the tracer found in elements of the endoplasmic reticulum or Golgi complex.

Intercellular Junctions

gA-Au complexes were found only within the luminal introits of the 200 intercellular junctions examined, whereas the junctions proper and the intercellular spaces abluminal to the junction were unlabeled (Fig. 5 *B*). In a smaller number of the junctions, the tracer was found within the intercellular space abluminal to the junction, in considerably lower concentration than within the adluminal introit. After extended perfusion (30 min), extensive labeling of the basement membrane and pericapillary spaces was localized primarily in phase with endothelial segments provided with many plasmalemmal vesicles. High concentration of the tracer was not found preferentially associated with the exit from intercellular junctions.

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Table III. Comparison of MVB Labeling by A-Au and gA-Au in Murine Myocardial Capillary Endothelia. Effects of Competition with Albumin, Glucose, or gA[‡]

Experimental conditions*	Percentage of MVB labeled as a function of perfusion time		
	3 min	10 min	30 min
A-Au	12 ± 3	36 ± 4	39 ± 3
gA-Au	34 ± 4	62 ± 7	60 ± 11
gA-Au competed by albumin	21 ± 3	ND	ND
gA-Au competed by glucose	16 ± 4	ND	ND
gA-Au competed by gA	26 ± 4.4	ND	20 ± 4.0
gA-Au competed by albumin + glucose	21 ± 3.5	ND	18 ± 4.5

For each experimental condition, 22-26 multivesicular bodies were examined (for the sampling, see Table II and Morphometry (under Materials and Methods). All values are mean ± SD.
‡ Concentrations given in Table I.

Competition Studies

Competition of Monomeric gA Uptake. The binding and uptake of [^{125}I]gA were competed in parallel studies with native albumin and a number of hexoses. Native albumin at concentrations ranging from 5 to 200 μg competed to the extent of ~60% the [^{125}I]gA uptake. D-glucose alone at concentrations of 45 and 90 mM was equally effective. The competitive effects of albumin and glucose appeared to be additive since their concomitant administration virtually abolished the binding of radioiodinated gA. A similar strong inhibition was obtained with gA. The effect of either mannose or galactose was negligible (Fig. 2).

Competition of gA-Au Binding and Transport. The uptake of gA-Au was competed with concentrations of albumin (20 or 40 μg), glucose (45 or 90 mM), or gA (100 μg) expected to be optimally efficient on the basis of the radioassays already described. Albumin competed primarily for gA-Au labeling of plasmalemmal vesicles (Fig. 6). Both the number of labeled vesicles and the linear density of label showed an approximate fourfold reduction (Tables I and II). In contrast,

albumin did not affect significantly the labeling density of plasmalemma proper and coated pits (Tables I and II).

Competition with glucose gave essentially complementary results; the labeling of the plasmalemma proper was substantially reduced (by 60% of control) whereas the gA-Au binding to vesicles was only marginally affected (Fig. 7, A and B; and Tables I and II).

When either gA or albumin and glucose administered concomitantly were used as competitors, the labeling of all plasmalemmal microdomains (vesicles included) was drastically diminished up to abolition (Tables I and II; data not shown).

Labeling of MVB measured at 30 min of gA-Au perfusion showed that endocytosis of this ligand showed an approximately threefold reduction by competition with either gA or albumin and glucose concomitantly administered (Table III).

Transcytosis of gA-Au in the presence of albumin or gA is largely reduced; after 30-min tracer perfusion, only rare particles were seen in abluminal plasmalemmal vesicles (apparently in fluid phase) and in the subendothelial space. The transendothelial transport of the ligand seemed insignificantly affected by glucose (findings not illustrated).
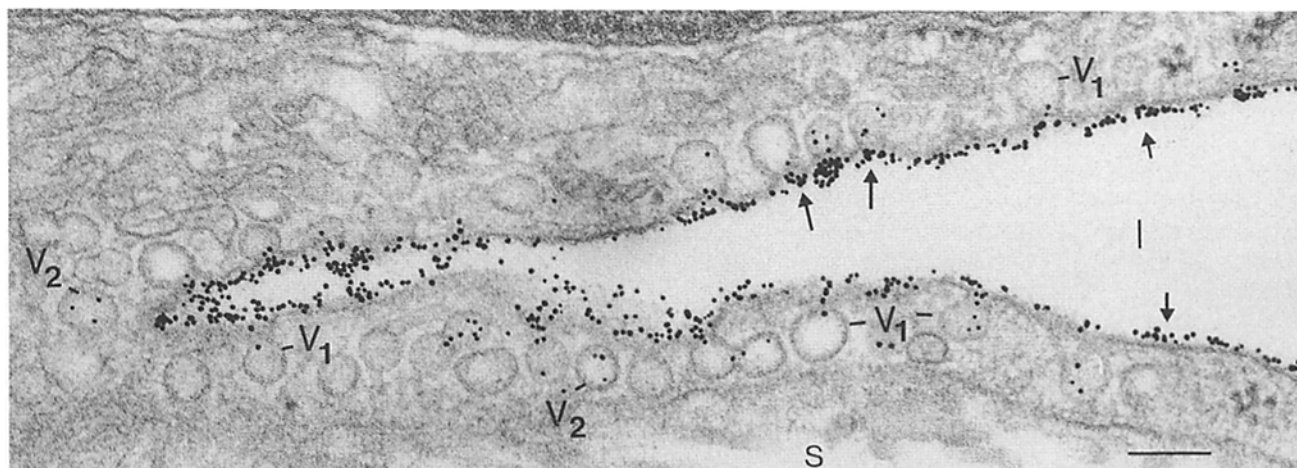

Figure 6. The effects on the binding of gA-Au of competition with albumin (40 μg/ml). Competition does not affect the binding of the tracer to the plasmalemma proper (*short arrows*) and the infundibula leading to the vesicles (*long arrows*), but reduces considerably the labeling of plasmalemmal vesicles (V_1, V_2). *s*, subendothelial space; *l*, lumen. Bar, 0.1 μm.
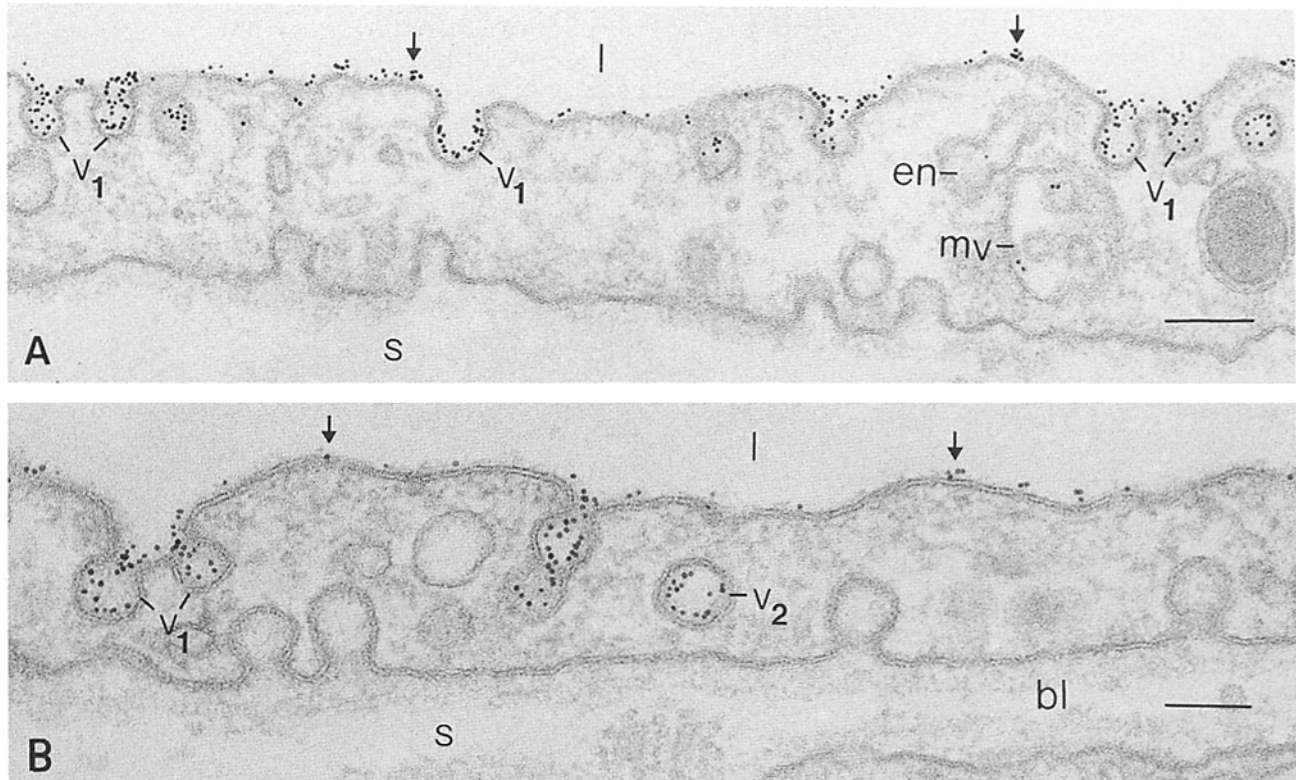
Figure 7. The effects on the binding of gA-Au of competition with D-glucose. (45 mM, *A* and 90 mM, *B*) Competition markedly reduces the gA-Au binding to plasmalemma proper (*short arrows*) without affecting significantly the extent and linear density of labeling of plasmalemmal vesicles (*V₁*, *V₂*). In *A* an endosome (*en*) and an MVB (*mv*) are already marked by gA-Au complexes at this early time point (3 min). *s*, subendothelial space; *l*, lumen; *bl*, basal lamina. Bars, 0.1 μm.

Discussion

Our previous findings (17, 37) and the comparative data reported in this paper indicate that the interaction of gA with the microvascular endothelium is quantitatively and qualitatively different from that of native, nonglycated albumin. The difference applies to both the uptake of monomeric radioiodinated ligands and to the binding and transport of the polymeric gold-conjugated albumin or gA. Despite the differences between the monomeric albumins (native albumin or gA) and their polymeric gold complexes, their binding and transport by endothelium is influenced in the same sense by the competitors used.

The uptake of radioiodinated gA perfused for 3 min was 2.4-fold higher than that of radiolabeled albumin (Fig. 2). Morphometric data show that, after 3-min perfusion, while A-Au complexes bind almost exclusively to plasmalemmal vesicles, gA-Au particles appear firmly and extensively attached both to the luminal plasmalemma proper and the membrane of plasmalemmal vesicles open on the luminal front. In contrast to A-Au, gA-Au also decorates a substantial fraction of coated pits.

Uptake of ^{125}I-gA is reduced by ∼60% by either albumin or glucose but is almost completely abolished by albumin and glucose (perfused concomitantly), and to a nearly similar extent, by gA. The results obtained in the dual competitor experiments suggest that gA behaves as a bifunctional ligand which interacts with two separate binding sites on the endothelial cell surface. These findings are supported by the

gA-Au observations which reveal that different microdomains of endothelial membrane have distinct sensitivities to the competitors used in our experiments: while albumin reduces substantially the labeling of plasmalemmal vesicles without affecting the decoration of plasmalemma proper, glucose largely prevents the gA-Au binding only to plasmalemma proper and coated pits, and not to vesicles. The almost complete inhibition of labeling when both competitors are simultaneously used shows that albumin and glucose have additive effects. These competition experiments suggest that the endothelial cell membrane has two distinct classes of binding sites: one, on the plasmalemmal vesicles, recognizes certain domain(s) of the albumin molecule, whereas the other, on the plasmalemma proper, interacts like a lectin with the hexose residues of gA. It appears that conformational changes introduced in the gA molecule by either radioiodination or adsorption on gold particles preclude neither the ligand interaction(s) with its cognate binding sites nor its displacement by competitors.

gA-Au Transcytosis

Our findings indicate that the ligand gA-Au is transported in time to the pericapillary spaces and is restricted to plasmalemmal vesicle profiles while in transit through the endothelium. These profiles may represent either isolated vesicles or sections through transendothelial channels. The interpretation is in keeping with variations in particle density per discharging vesicular profile; i.e., a high density (Fig.

5 A) could imply a channel. There is no indication of gA–Au exit through the intercellular junctions. The tracer is detected in the pericapillary spaces beginning with 3 min and increases progressively in amounts up to 30 min. We believe that our findings establish convincingly the involvement of plasmalemmal vesicles in the transport of the tracer, but we recognize that our data do not allow a reliable assessment of the amount and rate of gA–Au transport. By comparison, both parameters appear to be greater than in the case of A–Au complexes.

Transcytosis in our experiments can be affected by a number of factors including low concentration of albumin in the perfusate, number of albumin molecules per gold particle and the size of the gA–Au complex. These parameters are expected either to accelerate or slow down transcytosis to an unknown extent. Transport of monomeric albumin in perfused myocardial capillaries appears to be considerably faster (25). According to its dimensions, which range from 6 to 11 nm, the gA–Au complexes qualify as probes for both the small and large pore systems postulated in the capillary physiology literature (for review see reference 32). Our findings implicate the plasmalemmal vesicles of the endothelium as the structural equivalent of both pore systems. In the pericapillary spaces, the tracer appears concentrated in the basal lamina in areas in phase with high concentration of abluminal plasmalemmal vesicles. The results of our competition experiments suggest that albumin binding sites rather than glucose binding sites are primarily involved in transcytosis.

Endocytosis

gA–Au complexes were found in coated pits, polymorphic uncoated vesicles, presumably endosomes, and MVB assumed to be either endosomes or lysosomes. The number of labeled MVB and the gA–Au concentration therein increased with time. Our data do not permit a reliable quantitation of transcytosed vs. endocytosed tracer, but they strongly suggest that transcytosis is considerably more active than endocytosis.

Integration with the Literature

Uptake and discharge of albumin vs. gA was studied on isolated microvessel fragments by Williams et al. (47–49). The two operations (uptake–discharge) were interpreted as vesicular transport, but the involvement of plasmalemmal vesicles in the process studied was not directly proved. A–Au and gA–Au were used in perfused lung preparations by Villaski et al. (42) who reported limited transcytosis and extensive endocytosis by the capillary endothelium for both tracers. The partial lack of agreement between their results and ours probably reflects differences in protocol, especially concerning the amount of tracer used.

We believe that our findings presented in this and previous papers (17, 25, 37) establish convincingly the participation of vesicles in the transport of albumin, A–Au, and gA–Au across the endothelium. In this respect they are not in agreement with the conclusions Bundgaard et al. (6) and Frojkaer-Jensen (13) have drawn from their three-dimensional reconstruction of plasmalemmal vesicles. These authors have found that the great majority of plasmalemmal vesicles are part of sessile structures associated with one or the other side of the endothelium with practically no vesicle left free in the cytoplasm. Since such free vesicles were assumed to be a prerequisite for transport, the authors claimed that vesicular transport cannot exist. Our studies illustrate the advantage of integrated functional and morphological approaches which monitor the pathway followed by recognizable particulate tracers during transport across the endothelium. We consider that this approach provides more reliable data than a strictly morphological analysis of the vesicular system which can be affected by the inherent small size of the sampling examined and by vesicle redistribution during the slow process of chemical fixation.

There are numerous reports in the literature concerning the increased microvascular permeability (including permeability for macromolecules) in diabetes (10, 11, 16, 30, 41). There is also an extensive literature bearing on pathological changes of the vasculature in diabetes. The large fraction of gA found in diabetes may play a role in these physiopathologic changes (5, 24, 46, 50). Our findings support in a qualitative way the assumption that endothelial permeability for gA is increased. Yet it is difficult to extrapolate from these short-range experimental findings to the vascular pathology of diabetes which is most probably the result of long term chronic alterations in the vessel wall functions, permeability included.

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