Microvillar channels: a unique plasma membrane compartment for concentrating lipoproteins on the surface of rat adrenal cortical cells

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Abstract Electron microscopic studies of perfused rat adrenals indicate that plasma lipoproteins become concentrated in a specialized cell surface compartment called microvillar channels. Closely associated plasma membranes of sinusoidal microvilli of zona fasciculata cells form channels that normally are filled with electron dense particles the size of high density lipoproteins (HDL). In rats made acutely deficient in plasma lipoproteins (by treatment with 4-aminopyrazolo[3,4-d]pyrimidine (4-APP) for 1 day), particles within the microvillar channels are decreased in number. When adrenal glands of these rats are perfused with media lacking plasma lipoproteins, many but not all of these HDL-like particles are washed out. However, when these adrenals are perfused with large amounts (100–500 μg protein/ml) of HDL, microvillar channels become packed with electron dense particles similar to those found in vivo. These microvillar channels become wider and filled with larger particles when low density lipoproteins (LDL) are perfused through the adrenals. Autoradiograms of ^{125}I-labeled HDL-perfused adrenals show silver grains specifically associated with the cell surface microvillar channels, and confirm the notion that the particles filling the channels are exogenously delivered HDL. Physiologic data from similarly perfused adrenals in a parallel study show that the channel-refilling process is directly related to selective (i.e., nonendocytic) cholesterol uptake and that this cholesterol uptake is associated with corticosterone production. Together, these data suggest the hypothesis that plasma lipoprotein cholesterol utilized for corticosteroid synthesis in rat adrenal fasciculata cells may be derived from lipoproteins trapped in surface-associated microvillar channels. Although the mechanism responsible for the cholesterol transfer is not yet defined, it is clearly distinct from the classical process of receptor-mediated endocytosis and catabolism of lipoprotein particles. —**Reaven, E., M. Spicher, and S. Azhar.** Microvillar channels: a unique plasma membrane compartment for concentrating lipoproteins on the surface of rat adrenal cortical cells. *J. Lipid Res.* 1989. **30:** 1551–1560.

Supplementary key words high density lipoprotein • low density lipoprotein • zona fasciculata cells • 4-aminopyrazolo[3,4-d]pyrimidine • 17α-ethinyl estradiol

Recent evidence from this laboratory indicates that in situ perfused rat adrenals (1), like adrenal glands in vivo (2–4) and adrenocortical cells in culture (5–7), are capable of interiorizing lipoprotein-delivered cholesterol by a nonendocytic or 'alternative' pathway. In the perfused adrenal and in the cultured adrenal cells there is the suggestion of a cell surface lipoprotein binding site of broad specificity. Precisely which components of the lipoprotein and which components of the membrane are involved in this interaction is not clear at present. The data suggest, however, that once lipoprotein interaction with the cell surface occurs, it is followed by differential cell uptake of component parts of the lipoprotein molecule. Thus, unlike the situation with the endocytic pathway where intact lipoprotein is internalized by the cell (8), these studies indicate that adrenal cells can, and do, preferentially take up lipoprotein-supplied cholesterol and cholesteryl esters, relative to the uptake of lipoprotein apoproteins.

In the current report we continue our efforts to understand the nature of this lipoprotein–membrane interaction. It is quite clear that in another steroidogenic tissue, the luteinized ovary (9–12), most supplied HDL and LDL become associated with specialized sites of the cell plasma membrane known as microvillar channels. These are flat, open-ended spaces between closely associated microvilli that appear to trap great numbers of intact lipoprotein particles (both HDL and LDL) during perfusion experiments (11). Our working hypothesis in the luteinized ovary has been that the alternative cholesterol pathway is somehow linked to this channel system (11, 12). However interesting the microvilli channels of this system, we are reminded of the fact that the luteinized ovary is not a normal steroidogenic tissue, but a highly stimulated, rapidly differentiated organ in immature animals which, in view

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; 4-APP, 4-aminopyrazolo[3,4-d]pyrimidine; 17α-E_2, 17α-ethinyl estradiol.

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of its low apoBE receptor content (12), may not function entirely as do other steroidogenic tissues (13). In order to establish a more universal association between the microvillar channel system and the alternative cholesterol pathway, we felt it important to look at a less exotic tissue. Given our favorable results using nonrecycling perfusion techniques to deliver lipoprotein cholesterol directly to adrenal glands of various rat models (1), we felt an in-depth examination of the microvillar surface of the corticosterone-producing cells of this organ was in order.

Accordingly, we examined adrenocortical cells of control and various lipoprotein-deficient rat models. To determine normal lipoprotein–cell surface interactions, we perfused the adrenals in situ with homologous, unmodified lipoproteins whose native electron density permitted direct visualization with the electron microscope. Our studies reveal a complex network of extracellular channels on the sinusoidal face of zona fasciculata cells which contain endogenous HDL-lipoproteins in nonperfused animals, and trap great numbers of HDL or LDL lipoproteins when these lipoproteins are administered by perfusion. These specialized plasma membrane regions may provide the mechanism by which lipoprotein-delivered cholesterol may enter cells by a nonendocytic pathway.

MATERIAL AND METHODS

Materials

^{125}Iodine (sodium iodide, sp act 17.4 Ci/mg) was purchased from E. I. du Pont de Numours & Co. (Inc.), NEN Research Products, Boston, MA. 4-Aminopyrazolo[3,4-d]pyrimidine (4-APP) was the product of Aldrich Chemical Co., Milwaukee, WI. 17α-Ethinyl estradiol, fatty acid-poor bovine serum albumin, cholesterol, and other biochemicals were obtained form Sigma Chemical Company, St. Louis, MO. All other reagents used were of analytical grade.

Preparation of lipoproteins

Rat serum-derived lipoproteins (rHDL, rLDL) were prepared and characterized as previously described from this laboratory (10–12). Rat HDL was isolated between densities of 1.080 and 1.210 g/ml, rLDL was isolated between 1.030 and 1.055 g/ml, and both ligands were used directly. Rat HDL contained apoA-I, apoA-IV, and apoE as major apoproteins with smaller amounts of apoCs (11); rLDL contained apoB and apoE (11). Human HDL_3 used in autoradiographic studies was isolated between densities 1.125 and 1.21 g/ml (9, 12) and contained only apoA-I; trace amounts of apoE present in $hHDL_3$ preparations were removed by heparin-agarose affinity chromatography (14). Iodination of the lipoproteins for autoradiography was carried out as previously reported (9–12).

Adrenal perfusion

Animals. Male Sprague-Dawley rats (VAF-viral free, Charles River Co., Portage, MI) weighing 180–200 g were used for all studies. The rats were maintained in filtered cages (Maxi-Miser Positive Individual Ventilated System, Thoren Caging System, Hazleton, PA), two rats per cage at 72°C on a 12-h dark/light cycle for approximately 1 week before use. The rats were used as untreated controls or given 4-aminopyrazolo[3,4-d]pyrimidine (4-APP (10) 20 mg/kg body wt) for 1 or 3 days, or 17α-ethinyl estradiol (17α-E_2 (10) 5 mg/kg body wt) for 5 days to reduce circulating levels of lipoproteins.

Perfusion techniques. For perfusion of the adrenal, the rats were anesthetized with sodium thiamylal (Surital, 4.5 mg/100 g body wt) and prepared for closed circuit perfusion via retrograde cannulation of the abdominal aorta at the level of the right renal artery. For this purpose, the aorta was clamped immediately below the diaphragm, and the coeliac and superior mesenteric arteries were tied off. Both kidneys were tied at the hilus and effluent perfusate was collected from the left renal vein. Initially, the adrenals were flushed with McCoy's 5a medium (2.2 ml/min, Harvard perfusion pump model 9-75, Harvard Apparatus Co., South Natick, MA) until the effluent ran clear (~5 min). McCoy's 5a medium was freshly prepared each day, saturated with oxygen, enriched with 0.5% fatty acid-poor bovine serum albumin (BSA), 15 mM HEPES, buffer pH 7.4, 100 μg/ml streptomycin, and 100 U/ml penicillin. The buffer flush period was directly followed by perfusion of lipoproteins (2.2 ml/min) for 60–90 min. After ligand perfusion, the adrenals were washed for 2 min and either excised for biochemical processing or perfusion-fixed with 2.5% glutaraldehyde (in 0.1 M cacodylate buffer at pH 7.3–7.4, 22°C). Perfusion studies carried out under these conditions result in good ligand uptake, and a strong corticosterone response.

To observe removal or turnover of the perfused lipoproteins, some perfused adrenals were washed for up to 90 min with buffer following ligand perfusion experiments.

Morphological studies

Perfusion-fixed adrenals were excised, cleaned of adherent fat, blotted, and weighed. Each adrenal was thinly sliced and each slice was cut into pie-shaped wedges permitting subsequent identification of all cortical layers during microscopy. Samples were further fixed in glutaraldehyde for ~18 h, post-fixed in 2% osmium tetroxide in water for 2.5 h, and subsequently stained en bloc in 2.5% uranyl acetate (in water) for 3 h before dehydration and embedment in epon-araldite plastic (9–12). Thick (1 μm) sections of the embedded adrenal slices were examined at the light microscope level for identification of specific adrenal cortical zones. For electron microscopy, the tissue blocks were trimmed further and primarily

zona fasciculata cells were thin-sectioned, stained with saturated solution of uranyl acetate (15 min) and Reynolds lead citrate (8 min), and examined.

Electron microscopic autoradiography was carried out as previously described from this laboratory (9–12).

Miscellaneous procedures

Cholesterol in plasma and lipoprotein fractions was determined enzymatically according to the procedure of Nöel, Dupras, and Filion (15). The protein content of lipoproteins was determined by a modification of the procedure of Lowry et al. (16) as described by Markwell et al. (17).

RESULTS

General information regarding animal models

A preliminary examination of adrenals from control, 1–3-day 4-APP-treated, and 5-day 17α-E_2-treated rats was carried out to determine an appropriate model in which to examine the in situ interactions of adrenocortical cells with exogenously supplied lipoproteins. Overall, the animals given either 4-APP for 3 days or 17α-E_2 for 5 days had greatly reduced circulating levels of cholesterol (1, 10). However, adrenocortical cells from these animals showed patho-morphological changes in surface mem-

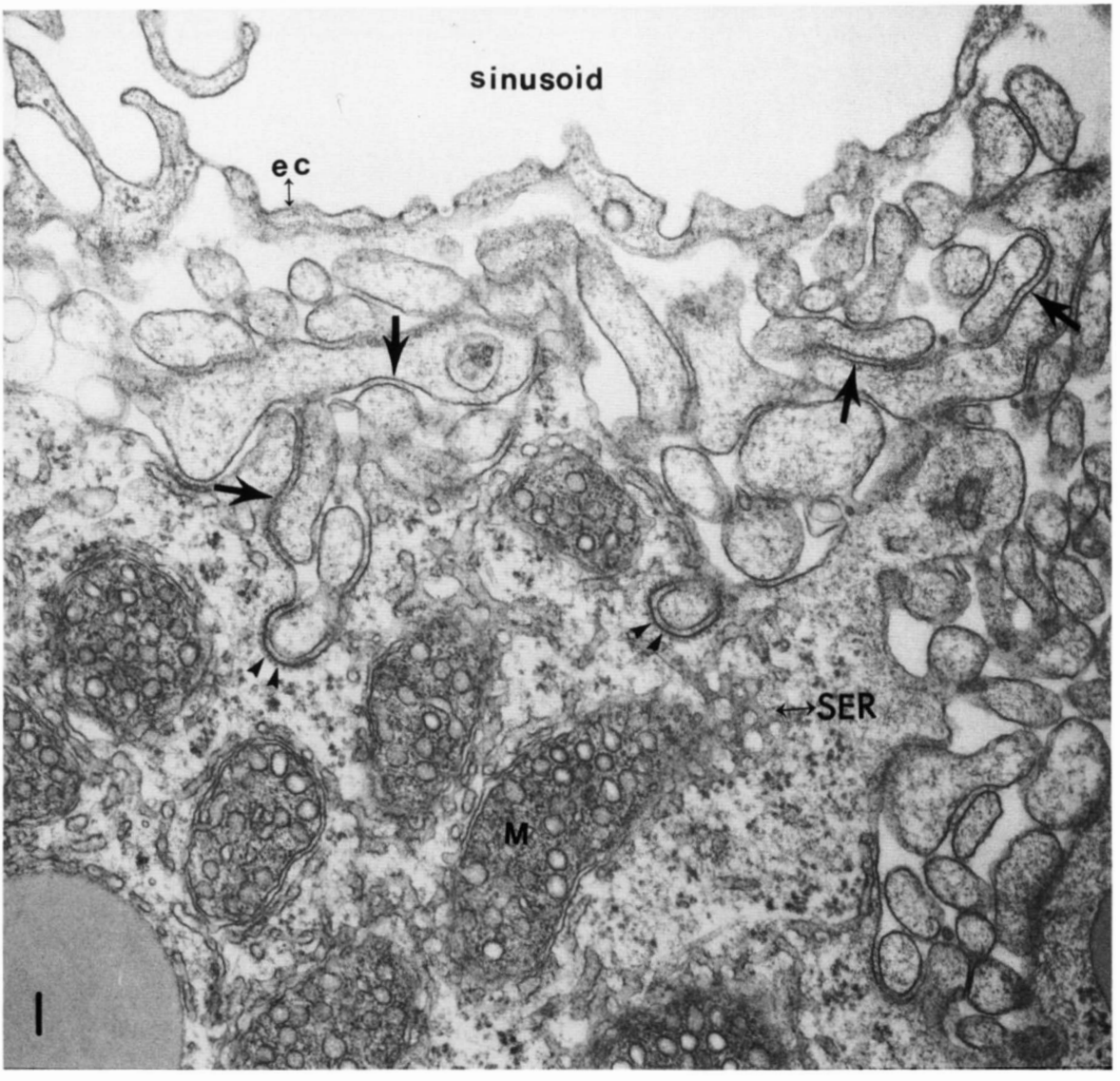

Fig. 1. Representative view of the sinusoidal face of a zona fasciculata cell from the adrenal of a young control rat. The adrenal tissue was perfusion-fixed after a short (<5 min) washout period with buffer. Starting with the sinusoid, the structures of interest are the endothelial cell (ec), a complex of apparently randomly oriented microvilli and microvillar channels (arrows), cytoplasm-embedded regions of microvilli showing microvillar channels with clathrin-like coated cytoplasmic membranes (arrowheads), mitochondria (M), and lipid droplets (L) surrounded by layers of SER. × 42,000.

brane structures which left them poor candidates for the intended study of the cell surface. For example, z. fasciculata cells from such treated rats showed varying amounts of membrane debris in subendothelial spaces (not seen in cells of perfused control rats) and the microvilli of the cells often appeared partially disintegrated with some unidentifiable electron-dense material binding the microvilli into clumps. Indeed, in some 5-day 17α-E_2-treated rats, all free plasma membrane surfaces of the z. fasciculata cells were totally covered with abnormal-appearing endogenous spherical particles, larger in average diameter (20.8 mμ) than the rat HDL or LDL (see Table 1) used in perfusion studies.

On the other hand, microvillar regions of the acutely treated (1 day) 4-APP rats showed none of these pathological changes although serum cholesterol levels were reduced by ~50% (1). Parallel studies also showed that adrenals of 1 day 4-APP rats take up perfused ^{125}I-labeled HDL avidly, internalize HDL-supplied cholesterol, and , when supplied with HDL (500 μg protein/ml) for 90 min, the adrenals secrete 5–7 times the level of corticosterone secreted by adrenals perfused with buffer alone (1). Overall, these characteristics showed that the 1 day 4-APP-treated rat was a reasonable model of early lipoprotein deficiency without any of the untoward pathological problems associated with the more chronically treated rats. This animal model was used for all subsequent experiments involving perfused lipoproteins.

Morphological observations regarding adrenal microvilli and microvillar channels in non-perfused, control and 24-h 4-APP-treated rats

In both control and 1-day 4-APP-treated rats, the surface of the hormone-secreting cells of the adrenal cortex (notably, z. fasciculata cells) displays prominent sinusoidal microvilli that extend into the subendothelial cell space (ec) (**Fig. 1**). The adrenocortical cell microvilli share a number of features with those previously described in the luteinized ovary (11), and this includes the presence of channels between closely apposed microvilli or between microvilli and nonmicrovillar regions of the cell surface (Fig. 1, arrows). One major difference between the two organs is the clear presence of endogenous lipoproteins in the microvillar channels of all adrenal models (including those from lipoprotein-deficient rats), whereas the channels of nonperfused luteal cells are either empty or contain disc-like profiles believed to be lipoprotein remnant particles (9). Instead, in adrenals of control, untreated rats, the microvillar channels of z. fasciculata cells show endogenous spherical particles presumed (on the basis of size and appearance) to be HDL (**Fig. 2**). Fewer endogenous particles are seen in the microvillar channels of the 1-day 4-APP-treated rats and, inasmuch as many of these particles are removed by continuous perfusion with media as shown in **Fig. 3**, it is possible to use this model for subsequent lipoprotein binding studies.

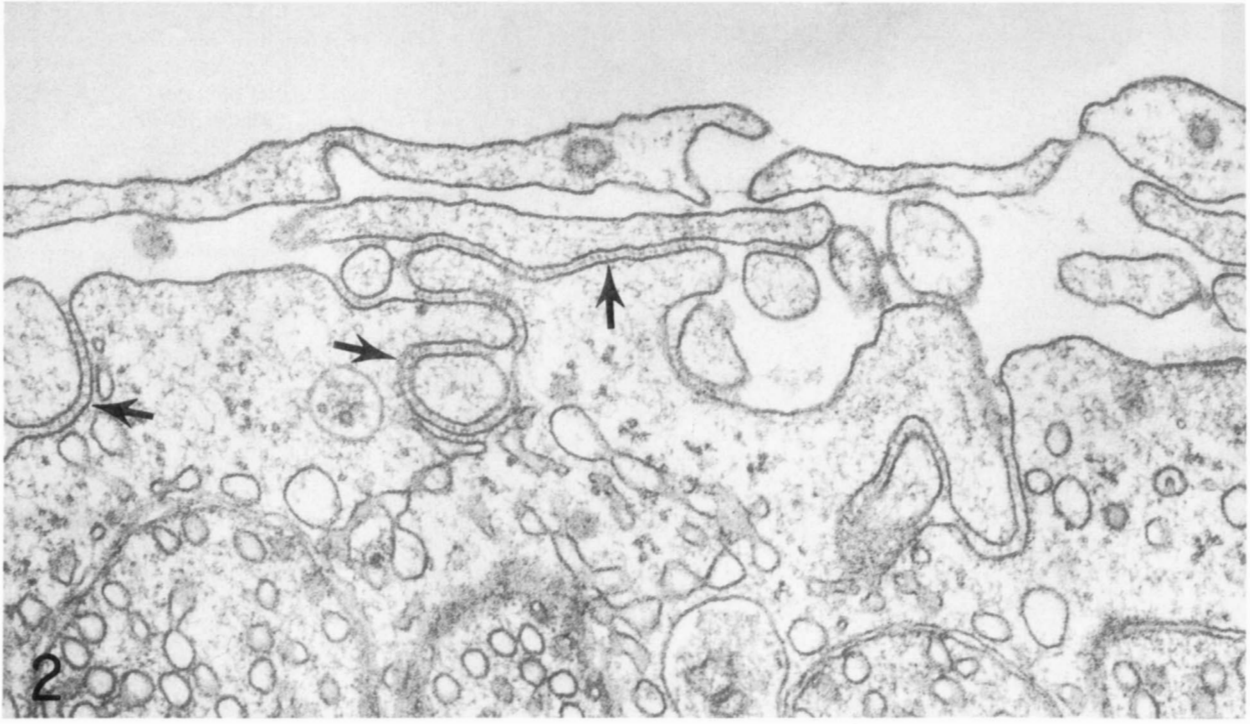

Fig. 2. Slightly enlarged view of the surface of a zona fasciculata cell of a nonligand-perfused control rat showing large numbers of spherical particles (believed to be endogenous HDL) in all microvillar channels (arrows). × 80,000.

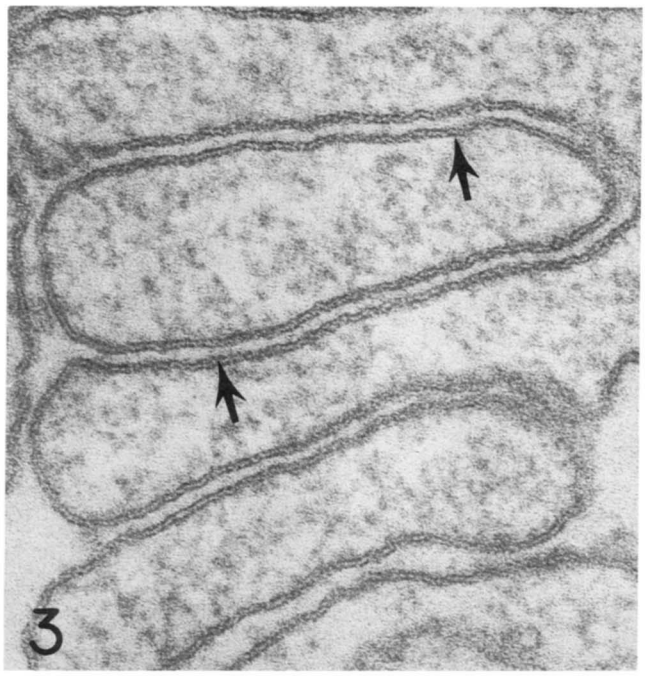

Fig. 3. Enlarged view of several microvillar channels (arrows) from a buffer-perfused z. fasciculata cell of a 1-day 4-APP-treated rat. These acute lipoprotein-deficient rats have fewer endogenous particles associated with z. fasciculata microvillar channels than do control rats, and when perfused with buffer for 40 min, as in this case, many channels appear empty (arrows). × 216,000.

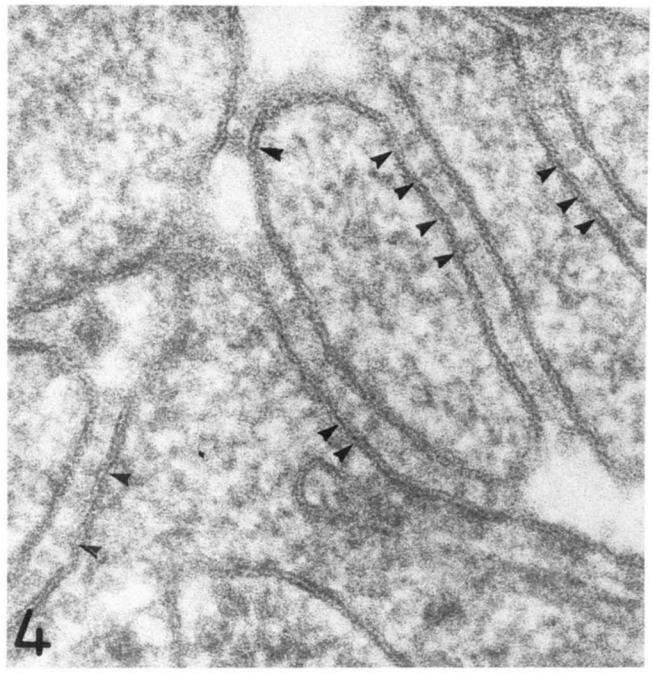

Fig. 4. A similar view of microvillar channels from cells of the 1-day 4-APP-treated rat shown in Fig. 3, except in this case the adrenal was perfused with rHDL (200 μg protein/ml) for 40 min. All channel areas are crowded with spherical particles ~12 nm in diameter (arrowheads). Note that channel width is ~double that of channels of the non-ligand-perfused adrenal of Fig. 3. × 216,000.

Another feature of the microvillar channels which differs between luteal cells and adrenocortical cells has to do with the appearance of microvilli that are embedded in the cytoplasm of the endocrine cells. In adrenocortical cells, many such inverted microvilli (and the associated channel formed by the two adjacent plasma membranes) show coated borders on their cytoplasmic surfaces. These coated regions differ from plasma membrane coated pits in other tissues (18) in several respects: *1*)a large segment of the plasma membrane may be involved; *2*) a double membrane is present (Fig. 1, arrowheads); and *3*) lipoprotein particles (presumed to be endogenously derived HDL) often fill the channels formed by the adjacent membranes.

Refilling of microvillar channels with newly perfused lipoproteins

In 1-day 4-APP-treated rats perfused with rHDL for 1 h (100–500 μg protein/ml), most of the microvillar channels become packed with HDL (**Fig. 4**). Some particles bind to the free surfaces of the microvilli, but the great majority of particles are trapped in the channel-like spaces found between adjacent plasma membranes. As this occurs, the channels enlarge to accommodate the 11.9-nm diameter particles (**Table 1**). Perfusion with rat LDL (200 μg protein/ml) instead of rHDL, results in microvillar channels being filled with larger (17.4 nm diameter) spheres (**Fig. 5**) and in this case, the average width of the channels enlarges still further (Table 1). [Autoradiograms at the electron microscope level show that $hHDL_3$ also enter the channels in great numbers but, due to their small size and lack of electron density, these particles are not clearly seen by standard microscopic techniques (11).]

That the particles filling the microvillar channels are newly bound lipoproteins (and not endogenous HDL) is shown by the fact that the channels can be filled with the large rat LDL (Fig. 5 and Table 1), and also by autoradiographs such as **Fig. 6** which indicate that the newly bound ^{125}I-labeled rHDL (identified by the exposed grains) are also associated with the microvillar surface of the z. fasciculata cells.

TABLE 1. Microvillar channel width of zona fasciculata cells of adrenal glands from rats treated with 4-APP for 1 day

Perfused Particle	Mean Particle Size	Channel Width
	nm	*nm*
None		7.9 ± 0.5
rHDL	11.9 ± 0.6	15.2 ± 0.4
rLDL	17.4 ± 0.6	25.8 ± 1.4

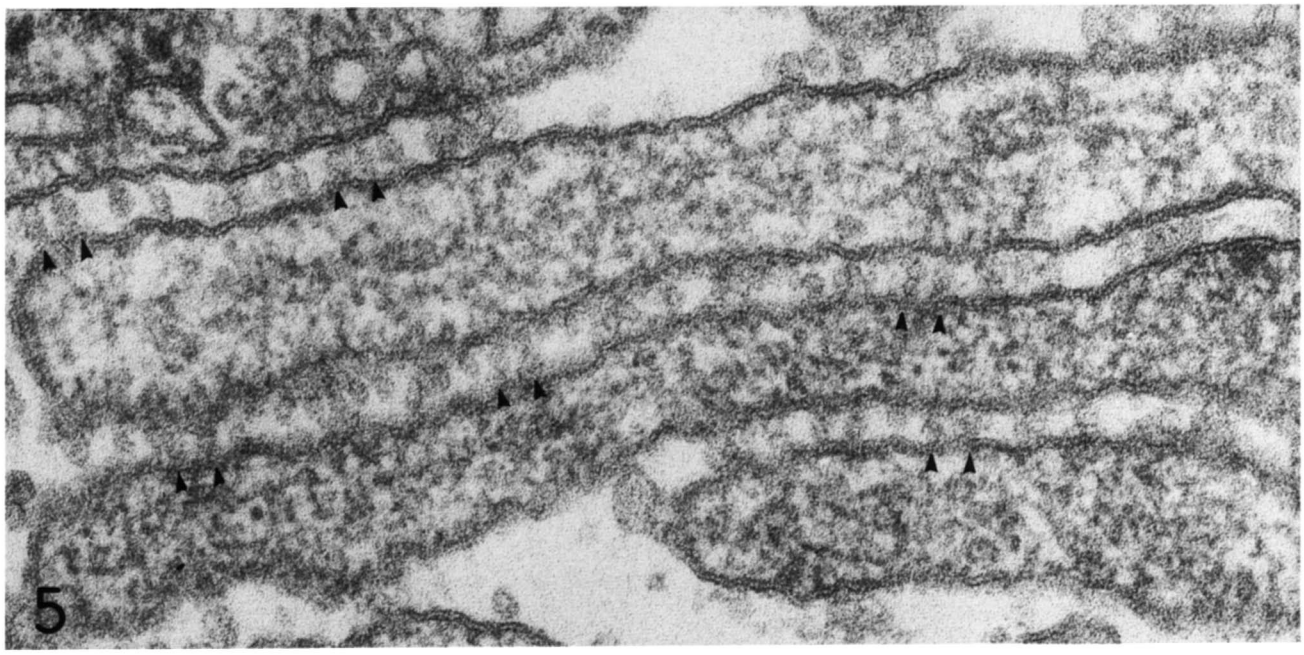

Fig. 5. Similar view of zona fasciculata microvillar channels from the 1-day 4-APP-treated rat perfused with rLDL (200 μg protein/ml) for 40 min. Again, channel spaces are filled with spherical particles (arrowheads), but in this case the channel width is wider than seen in Fig. 4 to accommodate the larger size of the perfused (17 nm) rLDL particle. × 216,000.

It is of interest, too, that microvillar channel-trapped particles in rHDL-perfused adrenals are not easily removed. Washout experiments with perfused buffer indicate that only 50% of the newly bound adrenal radioactivity can be released after 90 min of wash and this correlates well with parallel autoradiographs of the washed adrenal showing many exposed grains still associated with the surface of the endocrine cells (data not shown).

Special morphological features associated with the microvillar channels

Although most of the microvilli (and therefore microvillar channels) are located within subendothelial cell spaces, the sinusoidal surfaces of the endocrine cells display numerous inverted microvilli embedded in the cytoplasm of the cells. Channels formed around these structures are also filled with lipoproteins in control rats as well as after perfusion experiments (Figs. 1, 2, 6, and **Fig. 7**) and quite often the blinded ends of these microvilli show clathrin-like coats on their cytoplasmic surfaces (Fig. 7A, 7B, arrows). Such invaginated microvilli containing newly bound (i.e., radiolabeled) ligand can be seen also in autoradiograms such as in Fig. 6 (arrows).

It is of interest, too, that mitochondria of the endocrine cells are often closely associated with the sinusoidal plasma membrane surfaces of the cell. The smooth endoplasmic reticulum (SER) surrounding such mitochondria may be in very close proximity to microvillar channels containing both clathrin-like coats (**Fig. 8**, arrowheads) and lipoprotein particles (Fig. 8, arrow).

DISCUSSION

Overall, we have demonstrated that the corticosterone-producing cells of the rat adrenal share certain structural features of the cell surface with another steroidogenic tissue, the luteinized rat ovary (11). Thus, we see that on the sinusoidal face of zona fasciculata cells of the adrenal cortex, the large majority of administered lipoprotein particles (both HDL and LDL) find their way into a complex of cell surface microvillar channels. Once trapped in the microvillar channels, turnover of the lipoproteins is quite slow. This fact complicates the interpretation of cholesterol uptake experiments since it is not immediately clear which of the retained tissue-associated particles are, in fact, trapped extracellularly and which are truly internalized by the cells. In other work, quantitative autoradiography done at the electron microscope level suggests that as much as 82–88% of z. fasciculata cell-bound HDL is bound to the plasma membrane during the 90-min perfusion experiments (1), and in the autoradiograph shown in this report, we see that the cell surface grains are closely associated with the microvillar channels themselves.

It was of interest to discover that adrenal microvillar channels in control rats contain large numbers of endo-

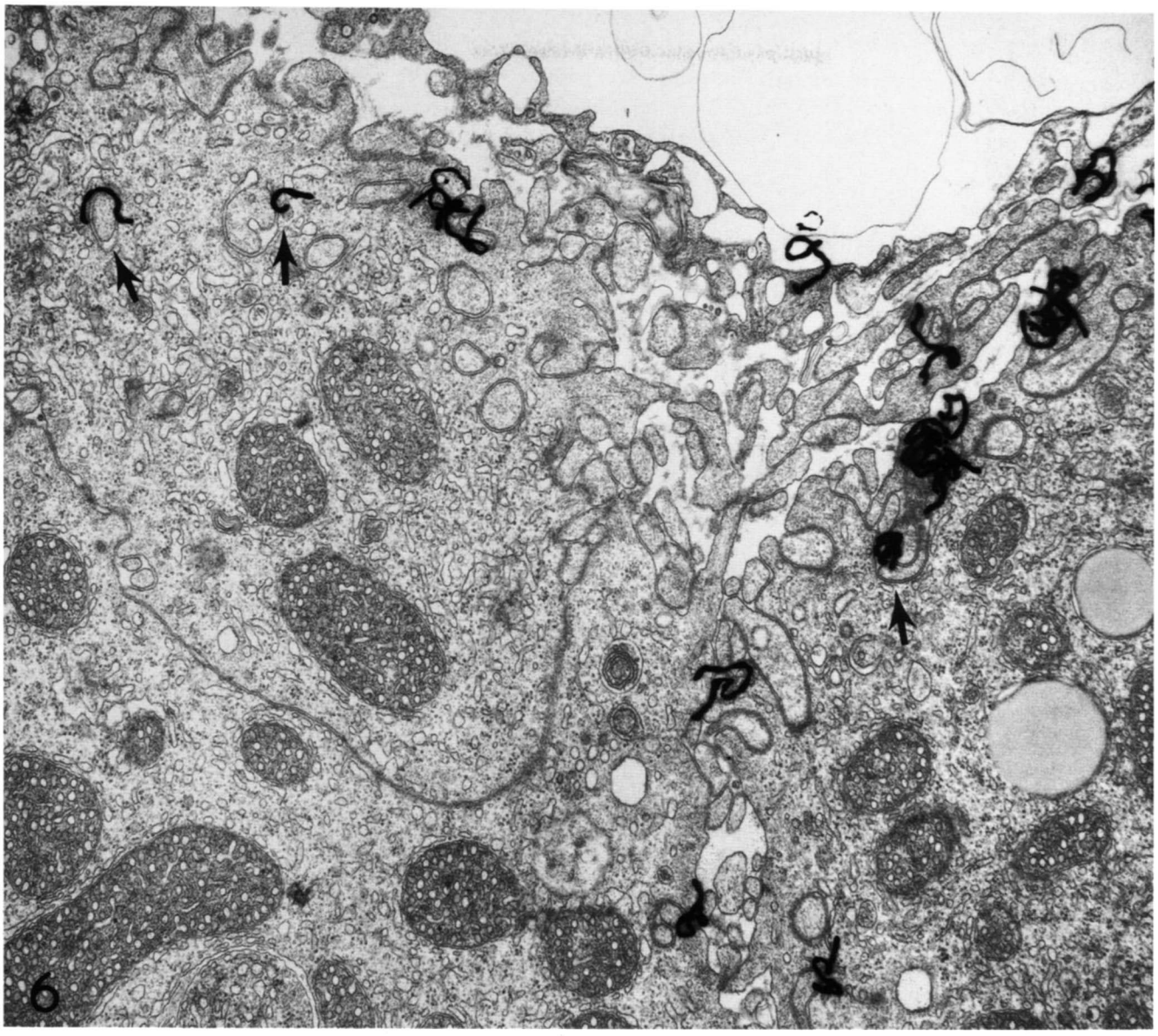

Fig. 6. Autoradiogram of the sinusoidal surface of zona fasciculata cells of a 1-day 4-APP-treated rat perfused with rHDL for 90 min. The exposed grains representing the distribution of ^{125}I-labeled rHDL apoprotein are generally associated with microvilli, and show particular preference for microvillar channels even when these regions are embedded in the cell cytoplasm (arrows). × 29,000.

genous HDL-like particles. This observation has been confirmed in Sprague-Dawley rats of both sexes and of all ages up to 18 months (E. Reaven, personal observation). Similar images described as "extensive cell contacts with intercellular particles" were also seen by Friend and Gilula many years ago in the rat adrenal cortex (19), though these authors believed the structures to be similar to the septate junctional complexes found in invertebrates. The situation in the adrenal is quite different from that of the luteinized ovary where the microvillar channels of the non-ligand-perfused rat contain only filamentous material, possibly HDL remnants (9). Although we do not understand the reason for this difference between the ovary and adrenal tissues, it reminds us that there are also functional differences between these tissues that relate to their lipoprotein-induced responsiveness, i.e., untreated luteal cells are immediately responsive to exogenously provided lipoproteins (20), whereas untreated adrenocortical cells in situ or in vitro will respond only after a lag period of many hours (21). Perhaps when lipoproteins are ever-present in the adrenal microvillar channels, intracellular storehouses are kept saturated with cholesterol, decreasing the possibility of an induced response with newly provided lipoproteins.

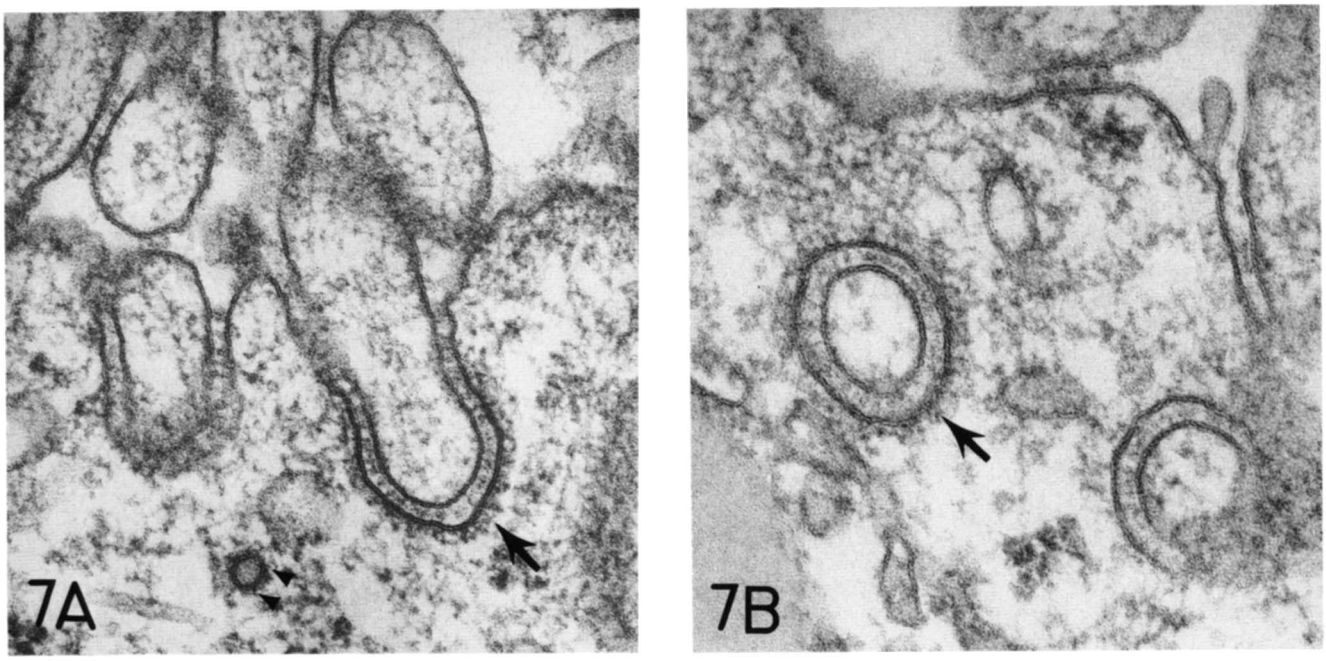

Fig. 7. High magnification view of coated microvillar channels of rHDL-perfused 1-day 4-APP-treated rat. Fig. 7A shows a blinded end of microvillar channel embedded in cell cytoplasm in which the channel is filled with rHDL and the cytoplasmic face of the channel shows a typical clathrin-like coat (arrow); note the size difference of a typical coated vesicle in the same micrograph (arrowheads). Fig. 7B shows a similar area in which the plane of section caught the end of an inverted microvillus (as seen in 7A) so that the double-membraned stucture containing rHDL and showing an external clathrin-like coat appears to be physically separated from the cell surface (arrow). × 100,000.

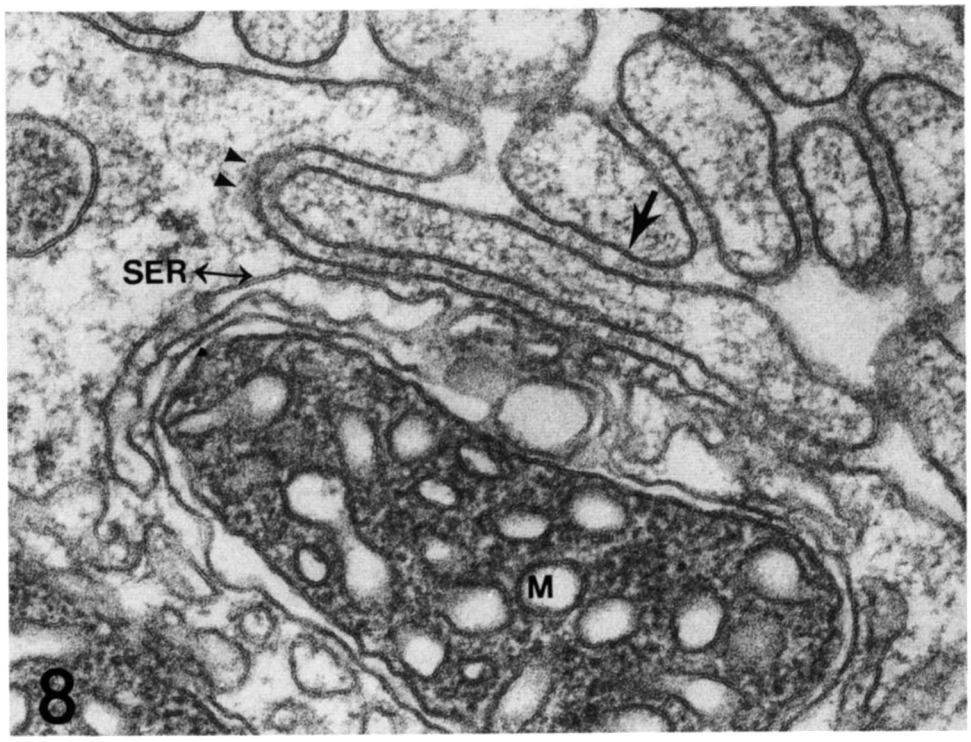

Fig. 8. Micrograph of zona fasciculata cell surface showing intimate relationship between microvillar channel [with entrapped lipoproteins (arrow)] and smooth endoplasmic reticulum (SER) surrounding a mitochondrion (M). Arrowheads point out clathrin-like coat on the blinded end of a microvillar channel. × 108,000.

Microvillar channels of the adrenal of 1-day 4-APP-treated (lipoprotein-deficient) animals contain fewer endogenous particles than do those of control rats and it is possible to reduce this particle number still further by prolonged periods of washout. However, when the adrenals of such animals are provided with either isotope-labeled or unlabeled exogenous lipoproteins, the channels refill with great numbers of the various HDL and LDL particles. Evidence that these channel particles are not endogenous lipoproteins comes from the observation that LDL [identified by their large size (see Fig. 5)] and isotope-labeled HDL [identified in autoradiograms (see Fig. 6)] can also be shown to be within the channels. It is important to note that the refilling and/or trapping of the lipoproteins in the channels is a rather specific process in control or 1-day 4-APP-treated rats, since in virtually every section viewed, the number of particles within the channels is in great excess of that seen on the free plasma membrane surfaces of the same cells.

The important aspect of this refilling process of the microvillar channels is that it is associated precisely with the period in which corticosterone production is dramatically increased in similar lipoprotein-perfused rats (1). The fact that accumulated HDL-derived cholesteryl esters are also preferentially accumulated during the perfusion period [as compared to accumulated HDL-apoproteins (1)] reinforces the idea that all these factors are interrelated; i.e., that the presence of the lipoproteins in the microvillar channel system of z. fasciculata cells is in some way responsible for the associated increase in corticosterone production, and that this increase in hormone production is a result of directly internalized lipoprotein cholesteryl esters.

Such similar findings in both the adrenal and the luteinized ovary lead us to speculate on the mechanism by which the microvillar channels in steroidogenic tissues may assist in the transfer of lipoprotein cholesterol to the interior of the cell. We do not imagine that the channels serve merely as a conduit for the endocytic pathway, though there is no reason to believe that apoB,E receptor-induced endocytosis could not begin there also (see also Fig. 9 in ref. 11). Indeed, the coated regions of the channel membranes may function in this capacity. However, if a more direct cholesterol transfer is to proceed from the lipoproteins in the microvillar channels to the interior of the cell, the challenge is to understand how this could occur.

Two general possibilities exist. The first and most acceptable view might be that cholesterol and/or cholesteryl ester transport is achieved via sterol carrier-like proteins that directly shuttle cholesterol molecules to their specific intracellular destinations (22). A second, and to our minds, intriguing possibility is that various cholesterol-rich lipoproteins directly transfer cholesteryl esters to plasma membranes, and that these cholesterol-enriched plasma membranes, by selective interaction with endomembranes of the cell (e.g., the endoplasmic reticulum of Fig. 8), may transfer cholesteryl ester (or esterase-derived free cholesterol) to the desired intracellular sites. This transfer within the plane of the endoplasmic reticulum might occur with, or without, the assistance of membrane-associated lipid transfer proteins (23, 24). Whether cholesterol transport through such novel membrane-membrane transfer mechanisms could take place in vivo is not known, although preliminary studies in our laboratory using isolated microvillar membranes (25) suggests that it occurs in vitro. Current studies are exploring this process further.

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