BIDIRECTIONAL TRANSPORT OF HORSERADISH PEROXIDASE IN PROXIMAL TUBULE OF NECTURUS KIDNEY

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INTRODUCTION

Protein transport directed across the luminal membrane has been studied by histologic techniques in the rat proximal tubule (9, 10). Protein movement in the opposite direction, that is, from capillary across basement, basal and/or lateral cell membranes, and into the epithelial cell has been studied in gut (7) and in isolated renal collecting tubules (5) where separate access to luminal (mucosal) and basal (serosal) compartments is possible.

Localization of transepithelial transport paths of protein molecules may provide information regarding relative permeability characteristics of individual membranes which comprise the proximal tubular epithelial membrane barrier. This study demonstrates rapid penetration of a protein molecule,
horseradish peroxidase, from the capillary circulation through the tubular basement membrane into intercellular spaces and suggests that the basal membrane complex including the peritubular capillary is highly permeable.

METHODS

Adult Necturus were anesthetized with tricaine methanesulfonate (2.0 g/liter). With micropuncture techniques, horseradish peroxidase (HRP, Sigma Type II, Sigma Chemical Co., St. Louis, Mo.) dissolved in amphibian bicarbonate Ringer solution (0.1–5.0 mg/ml) was slowly microperfused into the proximal tubular lumen.

In experiments where blood-to-lumen transport was studied, kidneys were perfused through the aorta and renal portal system with oxygenated amphibian Ringer bicarbonate solution as previously described (1). In these experiments, the inferior vena cava was cut to allow drainage of renal portal flow and prevent access of perfusate into the systemic circulation. HRP (15–25 mg in 1 ml Ringer solution) was infused for 15–25 min into the catheter perfusing the renal portal system. Perfused kidneys were also used in some experiments in which HRP was injected intraluminally.

Fixation of the perfused kidney was accomplished by replacing Ringer solution with 1.25% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3–7.5, 210 milliosmols). Simultaneously, fixative was dripped onto the kidney surface. In experiments with non-perfused kidneys, fixation was accomplished by dripping 2% buffered glutaraldehyde onto the kidney surface. After about 10 min of in situ fixation, the tissue was cut out and put in 6% buffered glutaraldehyde containing 7.50% sucrose for an additional 2 hr. 40-µ sections were cut with a Smith and Farquhar tissue chopper and incubated in 3, 3'-diaminobenzidine and 0.01% H2O2 according to the method described by Graham and Karnovsky (4). The tissue was postfixed in 1 % osmium tetroxide buffered in 0.1 M phosphate, dehydrated in graded concentrations of ethanol, and embedded in Epon or an Araldite-Epon mixture. Thin sections were cut on an MT-1 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), placed on grids, and stained with uranyl acetate or with uranyl acetate and lead citrate. A Siemens 1A electron microscope was used. For light microscopy, frozen (– 5 s) sections were cut, incubated with 3, 3'-diaminobenzidine and H2O2, and then stained with 1% osmium tetroxide in 0.1 M phosphate buffer.

Preparation of Antibody and Immunofluorescence

Monospecific anti-horseradish peroxidase serum was prepared by immunizing rabbits with purified HRP (Sigma type VII). This antiseraum was labeled with fluorescein isothiocyanate according to the methods described by Beutner et al. (3). The direct fluorescent antibody technique (11) was employed to demonstrate tissue localization of HRP in treated and nontreated Necturus.

Effect of HRP on Solute Transport

HRP (5 mg/ml or 10 mg/ml) was prepared in Ringer solution and tested against pure Ringer solution in the same proximal tubule by using the split drop technique for measuring solute coupled net volume flux. The split drop method adapted for Necturus proximal tubule has been previously described (1).

RESULTS

Intraluminal Microperfusion

In proximal tubules fixed during the first 2 min after single tubule microperfusion of HRP, typical darkly staining reaction product was noted in the brush border and in small apical vesicles which appear to have invaginated from the luminal membrane. Tissue fixed from 5 to 30 min after microperfusion of HRP demonstrated diffuse staining throughout the cytoplasm as viewed in the low-power, frozen section photograph (Fig. 1 a). No staining was observed in adjacent interstitial tissue beyond that expected from nonspecific peroxidase activity of interstitial cells. This nonspecific stain was also found in interstitial cells of control kidney tissue not exposed to HRP. Tissue stained for HRP by the immunofluorescent technique yielded similar results.

On higher resolution all cellular HRP appeared confined to membrane-enclosed cytoplasmic vacuoles (Fig. 1 b), and there was no evidence of basement membrane staining (Fig. 1 c) even after 30 min when HRP-filled vacuoles were distributed throughout the cell. HRP was not observed in intercellular spaces either in tissue fixed early after intraluminal microperfusion or after cytoplasmic vacuoles were heavily stained (Fig. 1 b).

Peritubular Capillary Perfusion

When HRP was infused into peritubular capillaries, the basement membranes were darkly stained and HRP could be seen extending through into intercellular spaces (Fig. 2). After 15 min of peritubular capillary perfusion, cytoplasmic staining of proximal tubules was evident. No luminal staining was observed (Fig. 2). Glomeruli were not
FIGURE 1a  Surface proximal tubule perfused by micropuncture with Ringer solution containing 5 mg/ml HRP and fixed 30 min after intraluminal micropерfusion. The cytoplasm of most cells comprising the microperfused tubule is heavily stained for HRP as contrasted with adjacent tubules. Interstitial cells (IC) containing an endogenous peroxidase demonstrate nonspecific staining reaction. (L) is the lumen of the perfused tubule. Light micrograph of frozen section (~5 µ). X 630.

FIGURE 1b  Electron micrograph of adjacent apical portions of proximal tubular cells fixed 30 min after intraluminal perfusion of Ringer solution containing 5 mg/ml HRP. The darkly staining reaction product is confined to membrane-enclosed vacuoles scattered throughout cytoplasm (presumably lysosomes). Intercellular spaces (IS) are free of HRP. The tight junction (TJ), brush border (BB), and lumen (L) are labeled. Stained with uranyl acetate. Marker, 1 µ. X 6450.

FIGURE 1c  Electron micrograph of the base of one of the proximal tubular cells shown in Fig. 1b. Note that the basement membrane (BM) is free of dark staining. Stained with uranyl acetate. Marker, 1 µ. X 21,600.

FIGURE 2  Light micrograph of frozen section of Necturus kidney perfused through the renal portal system with Ringer solution containing 20 mg/ml HRP for 20 min prior to fixation. The glomerulus (G) demonstrates no peroxidase staining. Intercellular spaces (IS), tubular basement membranes, and interstitial cells are heavily stained. X 1200.
FIGURE 3a Electron micrograph of the basal aspect of a proximal tubule perfused through the renal portal system with Ringer solution containing 20 mg/ml HRP for 15 min prior to fixation. The basement membrane (BM) and intercellular spaces (IS) are heavily stained. HRP is seen in cytoplasmic vacuoles. Stained with uranyl acetate and lead citrate. Marker, 1 μ. X 5200.

FIGURE 3b Electron micrograph showing HRP confined to the intercellular space (IS) between two proximal tubular cells after renal portal perfusion of Ringer solution containing 20 mg/ml HRP for 15 min prior to fixation. The dark vacuoles in the cell to the right are naturally occurring and are occasionally seen in some proximal tubular cells. Stained with uranyl acetate and lead citrate. Marker, 1 μ. X 12,000.

FIGURE 3c Electron micrograph of the apical portion of adjacent proximal tubular cells fixed after 15 min of renal portal perfusion of Ringer solution containing HRP, 20 mg/ml. The HRP reaction product in the intercellular space extends from basement membrane up to the tight junction (TJ). Stained with uranyl acetate and lead citrate. Marker, 1 μ. X 34,500.

FIGURE 4. Light micrograph of frozen section (≈5 μ) of kidney tissue perfused through the renal portal system with Ringer solution containing 15 mg/ml HRP for 90 min prior to fixation. The inhomogeneity of cytoplasmic staining is apparent (arrows) in spite of uniform staining of the tubular basement membranes (BM). X 1000.
stained, indicating that HRP had no access into the lumen from glomerulus (Fig. 2). In color photomicrographs, specific fluorescence was observed in basement membranes, intercellular spaces, and to a lesser extent, in cell cytoplasm.

On higher resolution, penetration of HRP into and past the tubular basement membrane and into intercellular spaces was striking (Fig. 3 a, 3 b). Accumulation of HRP reaction product in cytoplasmic vacuoles (Fig. 3 a) is indicative of transfer across lateral and/or basal cell membranes. Electron-micrographs of control tubules not injected with HRP but treated with H2O2 and 3,3'-diaminobenzidine were, on occasion, difficult to interpret because of darkly stained cytoplasmic bodies, as has been previously observed by Himmelsch and Karnovsky (6). However, no electron-opaque material was seen in intercellular spaces or basement membranes. We have thus interpreted the cytoplasmic vacuoles in Fig. 3 a as being HRP-positive because the electron opacity is intense and is of the same magnitude as that of the intercellular space and basement membrane staining. On the other hand, in Fig. 3 b the intercellular space density is greater than that of the cytoplasmic body, and the latter is interpreted as negative for HRP. No penetration through the tight junction (Fig. 3 c) was observed. In some experiments, small, dark, globular indentations suggest that HRP enters the cell across the lateral or basal cell membrane by a mechanism similar to that observed at the luminal membrane. On the other hand, our data do not exclude the possibility that the molecule first traverses the lateral and basal cell membranes by diffusion and then is accumulated in vacuoles.

Heterogeneity with respect to uptake of HRP by proximal tubular cells was apparent from the lack of staining of cells adjacent to heavily stained cells particularly when HRP was introduced into the peritubular side (Fig. 4).

Effect of HRP on Net NaCl Transport

Ringer solution with added HRP demonstrated no significant difference in split drop half time (τ_{1/2}) when compared to plain Ringer solution tested in the same tubule. The average τ_{1/2} (Ringer) was 28.3 min compared to 27.2 min for Ringer solution containing HRP, n = 5. The ratio of the τ_{1/2} (HRP) to the τ_{1/2} (Ringer) was 0.93 ± 0.12 sd.

DISCUSSION

These studies demonstrate that a relatively large molecule (mol wt 40,000) injected into peritubular capillaries can diffuse across capillary endothelium through basement membrane of Necturus proximal tubule into intercellular spaces, and that penetration into the cell from the basal direction is readily accomplished. Similar pathways for cellular accumulation of HRP have been observed in mouse duodenum (7), and the highly permeable nature of basement membrane to HRP and consequent intercellular space staining up to the tight junction have also been demonstrated in isolated renal collecting ducts of rabbit (5).

The movement of protein molecules from lumen across the luminal membrane of proximal tubule of the mammal has been studied extensively by Maunsbach (9, 10), and the present studies are confirmatory. Maack and Kinter (8) demonstrated significant transepithelial transport of a small protein molecule, lysozyme (mol wt 14,000), after initial luminal accumulation in flounder tubules. Although, we found no histologic evidence for the complete passage of the intact HRP molecule across the Necturus proximal tubular epithelial barrier in either direction, the technique used was not quantitative and, furthermore, transport may vary depending on species and protein structure or size.

A consideration of importance is whether epithelial cell penetration might not be secondary to a nonspecific toxic effect of the HRP molecule on proximal tubule. This is unlikely because mice have shown no ill effect from large doses of HRP given intravenously (4), and intraluminal HRP has no effect on net NaCl transport or on osmotic water permeability of the Necturus proximal tubule (2).

The passage of protein molecules across the epithelial basement membrane into intercellular spaces and the cell is similar to the route postulated for transport of secretory gamma A immunoglobulin in various human epithelial tissues, including kidney (11). Thus, "retrograde" protein transport across proximal kidney tubule may be of biologic significance.

SUMMARY

Horseradish peroxidase microperfused into the proximal tubular lumen of Necturus penetrated the luminal membrane by pinocytosis and was concentrated in cytoplasmic vacuoles. No horseradish
peroxidase was seen in the intercellular space or the tubular basement membrane. Horseradish peroxidase administered via the renal portal system into peritubular capillaries readily diffused across the tubular basement membrane and entered intercellular channels up to the tight junction. Accumulation in cytoplasmic vacuoles was observed in some but not all tubular cells, but the exact mechanism for passage across lateral and/or basal membranes is unknown.

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REFERENCES

1. Bentzel, C. J., T. Anagnostopoulos, and H. Pandit. 1970. Necturus kidney: its response to effects of isotonic volume expansion. Amer. J. Physiol. 218:205.

2. Bentzel, C. J., B. Parsa, and D. K. Hare. 1969. Osmotic flow across proximal tubule of Necturus: correlation of physiologic and anatomic studies. Amer. J. Physiol. 217:570.

3. Beutner, E. H., M. R. Sepulveda, and E. V. Barnett. 1968. Quantitative studies of immunofluorescent staining. Bull. World Health Organ. 39:587.

4. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291.

5. Grantham, J. J., C. E. Ganote, M. B. Burg, and J. Orloff. 1969. Paths of transtubular water flow in isolated renal collecting tubules. J. Cell Biol. 41:562.

6. Himmelhoech, S. R., and M. J. Karnovsky. 1961. Oxidative and hydrolytic enzymes in the nephron of Necturus maculosus. J. Biophys. Biochem. Cytol. 9:893.

7. Hugon, J. I., and M. Borgers. 1968. Absorption of horseradish peroxidase by the mucosal cells of the duodenum of mouse. J. Histochem. Cytochem. 16:223.

8. Maack, T., and W. B. Kinter. 1969. Transport of protein by flounder kidney tubules during long-term incubation. Amer. J. Physiol. 216:1034.

9. Maunsbach, A. B. 1966. Absorption of 125I-labeled homologous albumin by rat kidney proximal tubule cells. A study of microperfused single proximal tubules by electron microscopic autoradiography and histochemistry J. Ultrastruct. Res. 15:197.

10. Maunsbach, A. B. 1966. Absorption of ferritin by rat kidney proximal tubule cells. Electron microscopic observations of the initial uptake phase in cells of microperfused single proximal tubules. J. Ultrastruct. Res. 16:11.

11. Tourville, D. R., R. H. Adler, J. Bienenstock, and T. B. Toms, Jr. 1969. The human secretory immunoglobulin system: immunohistological localization of γA, secretory "piece," and lactoferrin in normal human tissues. J. Exp. Med. 129:411.