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Apical Membrane Endocytosis via Coated Pits is Stimulated by Removal of Antidiuretic Hormone from Isolated, Perfused Rabbit Cortical Collecting Tubule

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Summary. Antidiuretic hormone increases the water permeability of the cortical collecting tubule and causes the appearance of intramembrane particle aggregates in the apical plasma membrane of principal cells. Particle aggregates are located in apical membrane coated pits during stimulation of collecting ducts with ADH *in situ*. Removal of ADH causes a rapid decline in water permeability. We evaluated apical membrane retrieval associated with removal of ADH by studying the endocytosis of horseradish peroxidase (HRP) from an isotonic solution in the lumen. HRP uptake was quantified enzymatically and its intracellular distribution examined by electron microscopy. When tubules were perfused with HRP for 20 min in the absence of ADH, HRP uptake was 0.5 ± 0.3 pg/min/μm tubule length (n = 6). The uptake of HRP in tubules exposed continuously to ADH during the 20-min HRP perfusion period was 1.3 ± 0.8 pg/min/μm (n = 8). HRP uptake increased markedly to 3.2 ± 1.1 pg/min/μm (n = 14), when the 20-min period of perfusion with HRP began immediately after removal of ADH from the peritubular bath. Endocytosis of HRP occurred in both principal and intercalated cells via apical membrane coated pits. We suggest that the rapid decline in cortical collecting duct water permeability which occurs following removal of ADH is mediated by retrieval of water permeable membrane via coated pits.

Key Words antidiuretic hormone · coated pits · cortical collecting tubule · endocytosis

Introduction

Antidiuretic hormone (ADH) dramatically increases the water permeability of the urinary bladder of certain anurans [11] and mammalian renal collecting tubule [16, 18]. Direct measurements show that ADH increases the water permeability of the apical (urine-facing) plasma membrane of granular cells in the toad bladder [20] and principal and possibly intercalated cells of the rabbit cortical collecting tubule [38]. In both tissues, the increase in apical membrane water permeability is believed to occur through introduction of water-filled pores or channels into the lipid bilayer of the apical plasma membrane which normally has a low permeability to water [8, 17, 24, 25]. Examination of the apical membranes of granular cells of toad bladder [4, 21] and principal cells of the cortical [26], medullary, and papillary [6, 12, 13] collecting ducts by freeze-fracture electron microscopy shows that ADH causes the appearance of distinctive intramembrane particle aggregates. It is generally accepted that particle aggregates are, or are closely related to, water channels in those apical membranes [reviewed in 13, 40].

In the toad bladder, under basal conditions, particle aggregates are located in tubular vesicles called aggrephores which are positioned in the granular cell cytoplasm immediately below the apical plasma membrane [19, 30]. ADH stimulation causes the fusion of aggrephores with the apical plasma membrane which introduces particle aggregates into the apical lipid bilayer [30, 41]. When there is a high rate of osmotic water flow during stimulation with ADH [15] or when ADH is removed [29, 41], water permeability decreases and particle aggregates are retrieved in aggrephore-like vesicles from the apical membrane [5]. There is no evidence suggesting that other endocytic pathways such as coated pits are involved in aggregate retrieval with the exception of a recent report [7] which suggests that aggrephores have a coating at one end which morphologically resembles clathrin.

The ADH-induced apical membrane events in principal cells of the mammalian collecting tubule are less well understood. The mechanism causing the appearance of particle aggregates in the apical

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membrane is unknown. Vesicles analogous to aggrephores have not been identified in collecting tubule cells. In whole animal studies of normal rats and rats with diabetes insipidus, Brown and Orci [1, 2] compared data obtained from freeze-fracture and thin-section electron microscopy and concluded that both coated pits and patches of apical membrane containing particle aggregates were increased in number during ADH stimulation. In addition, both coated pits and membrane patches containing aggregates excluded the antibiotic, filipin, which creates filipin-sterol complexes easily visualized by freeze-fracture electron microscopy. Since both membrane structures excluded filipin, it was concluded that the patches of particle aggregates seen by the freeze-fracture technique were located within coated pits in the principal cell apical plasma membrane.

Collecting duct apical membrane water permeability decreases following withdrawal of ADH [9]. Although it is well established that the water permeability response of toad bladder is reversed by retrieval of apical membrane when ADH is withdrawn, there is no information as to whether the reversal of the response in the collecting duct is the result of membrane retrieval or inactivation of water-conducting channels that remain in the apical membrane. Since aggrephores have not been identified in collecting duct cells, there is no evidence that the shuttle mechanism which is so well documented in toad bladder, occurs in the mammalian kidney. We reasoned that if water-permeable membrane units (presumably particle aggregates) are retrieved from the apical membrane when ADH is withdrawn, there might be evidence of increased apical membrane endocytosis. We used the fluid phase marker horseradish peroxidase (HRP) to quantify endocytosis and to localize by electron microscopy the apical membrane events that occur in isolated perfused cortical collecting ducts following ADH stimulation and removal. We found stimulation of apical membrane endocytosis during ADH removal. In contrast to observations in toad urinary bladder, HRP endocytosis was via coated pits, which are morphologically distinct from aggrephores.

Materials and Methods

Tubule Perfusion

NIH New Zealand white female rabbits (1.0–1.5 kg) were maintained on Purina rabbit chow and tap water *ad libitum*. Rabbits were sacrificed by decapitation and both kidneys removed and transferred to ice-cold control saline solution containing (in mM): 118 NaCl, 23 $NaHCO_3$, 2.5 K_2HPO_4, 1.2 $MgSO_4$, 2.0 calcium lactate, 5.5 glucose, 10 sodium citrate and 6.0 alanine. Segments of cortical collecting tubules (mean ± SE, length 900.0 ± 50.2 μm, $n = 31$) were dissected from kidney slices in a bath chamber maintained at 15°C and gassed with 95% O_2-5% CO_2.

Tubule perfusion methods were similar to those described previously [4]. Briefly, the tubule was transferred to a laminar flow bath chamber [37, 38] mounted on the stage of a Nikon Diaphot inverted microscope. One end of the tubule was drawn up into a conventional glass holding pipette and the lumen was cannulated and perfused using an inner concentric perfusion pipette. The distal end of the tubule was held in place with another glass holding pipette. Once the tubule was in place, bath perfusion was initiated and maintained at 4–6 ml/min. Bath temperature was maintained at 36–38°C and pH at 7.4 as described previously [37, 38]. Luminal perfusion was maintained at >30 nl/min to facilitate rapid luminal solution switches.

Quantitation of HRP Uptake in Isolated Perfused Tubules

After dissection and setup (usually accomplished in 20 min), all tubules were perfused at the above rates for an initial 50- to 60-min equilibration period. Thereafter, tubules were subjected to one of three protocols. (*1*) *ADH removal* ($n = 14$): Tubules were exposed to peritibular ADH (arginine vasopressin, Grade VIII, Sigma Chemical Co.) for 20–25 min. High concentrations of ADH (500–1000 μU/ml) were used to obtain a maximal response. ADH stimulation was terminated by changing the peritubular bath to hormone-free solution. Simultaneously, the luminal solution was switched so that the tubule lumen was perfused with control saline solution containing 10 mg/ml of HRP (Type II, Sigma Chemical Co.) and 1 mM mannose (Polysciences Inc.). This luminal perfusate solution had been dialyzed for 10–12 hr against a 500-fold excess of control saline with 1 mM mannose. Mannose was added to reduce potential binding of HRP by any mannose receptor proteins exposed on the apical surface of the epithelium [35, 39]. After 20 min of perfusion with the HRP solution, the luminal solution was rapidly switched to control saline containing 1 mM mannose (Polysciences Inc.) and the lumen was perfused for an additional 10 min to remove luminal HRP which was not internalized by cells of the collecting tubule.

(*2*) *Continuous ADH exposure* ($n = 8$): Tubules were stimulated with ADH for 40–60 min. During the last 20 min of the ADH stimulation period, the tubules were luminally perfused with HRP followed by a 10-min HRP washout as described above.

(*3*) *Control perfusion* ($n = 6$): Control (unstimulated) tubules received the treatment detailed in #1 but ADH was omitted and the tubules were perfused for 90 min without ADH prior to HRP exposure.

For quantitation of cellular HRP activity in isolated collecting ducts, we adapted a sensitive colorimetric assay of peroxidase activity originally designed for studies of endocytosis in cultured cells [34, 36]. Single tubules were transferred from the bath chamber to a single well in a 96-well tissue culture plate (Costar Inc.). Any saline transferred to the well with the tubule was removed with a micropipette and 50 μl of 50 mM sodium phosphate buffer solution (pH 5.0) containing 1% Triton X-100 (wt/vol) (Biorad Labs) was added. The tubule was incubated for a minimum of 30 min to ensure cellular lysis. Duplicate 20 μl aliquots were assayed for peroxidase activity by measuring the rate of development of a colored reaction product of O-diansi-

dine at 460 nm. HRP uptake is expressed as pg HRP/min/μm of tubule-length. These rates of HRP uptake were determined over a 20-min HRP perfusion period and are not meant to reflect true initial rates of HRP endocytosis.

Ultrastructural Localization of HRP In Cortical Collecting Tubles

When control and ADH-stimulated tubules were initially treated as described above, intracellular HRP activity was predominantly located in lysosomes. To examine the events immediately after HRP internalization, tubules were perfused with the 10 mg/ml HRP solution for 1–5 or 20-min intervals immediately followed by perfusion for 1 min with control perfusion saline containing 1 mM mannose. Tubules were then rapidly fixed by flushing 2.5% glutaraldehyde (Polysciences) buffered with 0.1 M sodium cacodylate (pH 7.4) at 23°C through the bath chamber. After fixation for 3–4 min, each tubule was gently removed from the perfusion pipette and transferred to a 35-mm plastic tissue culture dish which had been coated with polylysine and filled with saline. Polylysine coating was accomplished by incubating the interior surface of the dish with a 2–4 mg/ml solution of polylysine hydrobromide (Sigma Chemical Co. average mol. weight = 175,000) in phosphate buffered saline pH = 7.4 (PBS) for 30 min followed by several rinses with PBS. After the tubule settled and adhered to the bottom of the polylysine-coated dish, the saline solution was removed and replaced with 2.5% glutaraldehyde fixation solution followed by incubation for 30–40 min at 23°C. The dish was then rinsed several times with 0.1 M sodium cacodylate buffer and stored in that buffer at 4°C until further processing.

Glutaraldehyde-fixed tubules were first incubated for 10 min with a solution containing 4 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in 10 ml of PBS buffer to which were added 3 μl of 30% H_2O_2. After development of HRP reaction product, the specimens were rinsed in PBS, fixed in 1% osmium tetroxide for 30 min, washed in distilled water and dehydrated through a series of ethanol solutions. The tissue was embedded in Epon 812 and sections cut with a diamond knife. These were counter-stained with lead citrate and examined under 40 kV with a 400T Phillips electron microscope.

Results

Spectrophotometric Quantitation of HRP Uptake

To estimate apical membrane endocytosis, we measured the uptake of HRP from the luminal solution in single isolated perfused cortical collecting tubules (Table). Preliminary experiments showed that control (no ADH stimulation) and ADH-stimulated tubules exhibited no endogenous peroxidase activity (*data not shown*). As is characteristic in studies of physiological properties of this nephron segment [31, 32], there was considerable variability between individual tubules despite efforts to standardize the preparation. The mean HRP uptake in control tubules (protocol #3) perfused with HRP for 20 min was 0.5 ± 0.3 pg/min/μm tubule length (n = 6). Tubules exposed to continuous ADH stimulation (protocol #2) and perfused with HRP for 20 min had a mean HRP uptake of 1.3 ± 0.8 pg/min/μm (n = 8) which was not significantly different from control ($P > 0.4$).

Table.

Perfusion protocol[a]	HRP uptake (pg/min/μm tubule length)	n
Control (no ADH)	0.5 ± 0.3	6
Continuous ADH	1.3 ± 0.8	8
ADH removal	3.2 ± 1.1	14

[a] The details of the perfusion protocol for individual isolated perfused rabbit cortical collecting tubules are described in Materials and Methods. The lumen of each tubule was perfused with 10 mg/ml of horseradish peroxidase (HRP) for 20 min followed by a 10-min washout period of luminal perfusion with a HRP-free solution. The tubule was then transferred from the perfusion apparatus to a small plastic well where it was lysed by addition of buffer containing 1% Triton X-100. Tubule peroxidase assays were performed in duplicate using the method of Steinman and Cohn [36] and the average peroxidase activity was compared to a known amount of HRP from the same perfusion solution. The values represent the mean ± SEM. n = number of tubules.

Perfusion of ADH-stimulated tubules with HRP for a 20-min interval immediately following the removal of ADH from the bath solution (ADH removal, protocol #1) resulted in a marked increase in HRP uptake (Table). The value of 3.2 ± 1.1 pg/min/μm (n = 14) is significantly greater than that observed in control tubules ($P > 0.005$) or tubules exposed to continuous ADH stimulation ($P > 0.025$).

Electron-Microscopic Localization of HRP Uptake

The rabbit cortical collecting tubule is composed of principal and intercalated cells present in a ratio of approximately 2 : 1 [22]. The two cell types are easily discernible on the basis of their ultrastructural characteristics. Examination of tubules subjected to protocol #3 demonstrated that there was no HRP adhering to the membranes of cells facing either the luminal or bath solution (Fig. 1). This indicates that the colorimetric measurements of HRP (Table) represent HRP which was endocytosed via the apical membrane of cells during the interval of luminal HRP perfusion.

In tubules perfused with HRP for 20 min in the absence of ADH stimulation (protocol #3) and subjected to only 1 min of washout with luminal HRP-free saline perfusion, there was uptake of HRP by

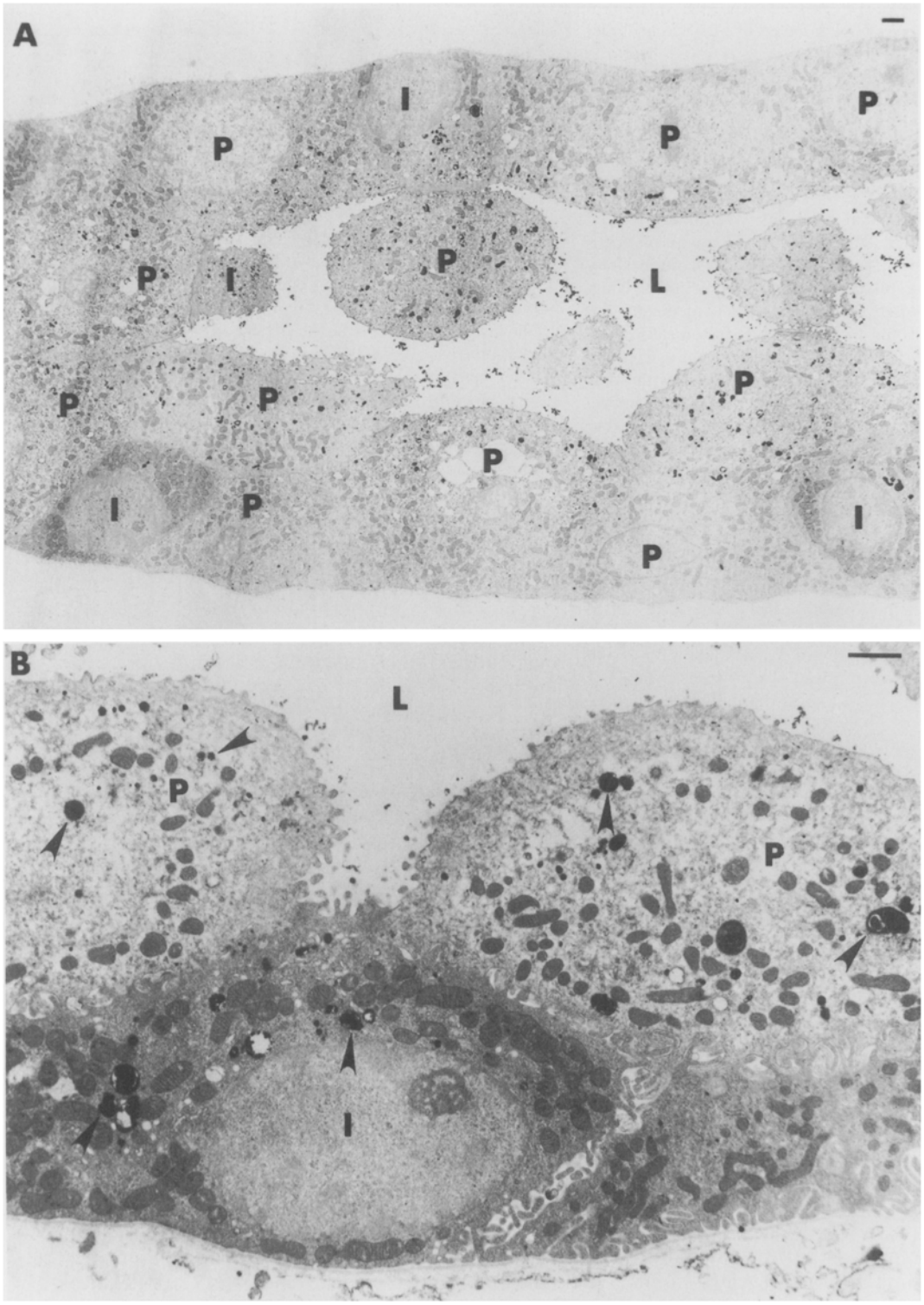

Fig. 1. The distribution of horseradish peroxidase (HRP) in perfused tubules. Both panels (*A*) and (*B*) show portions of a tubule subjected to protocol #3. Note the presence of the dark HRP reaction product in many intracellular vesicles as indicated by the arrows, in contrast to the minimal adherent HRP on the luminal and basolateral surfaces of principal (*P*) and intercalated (*I*) cells. The lumen of the tubule is indicated as (*L*). Magnifications: (*A*) 3250×, (*B*) 9000× with each bar in the upper right corner measuring 1 μm

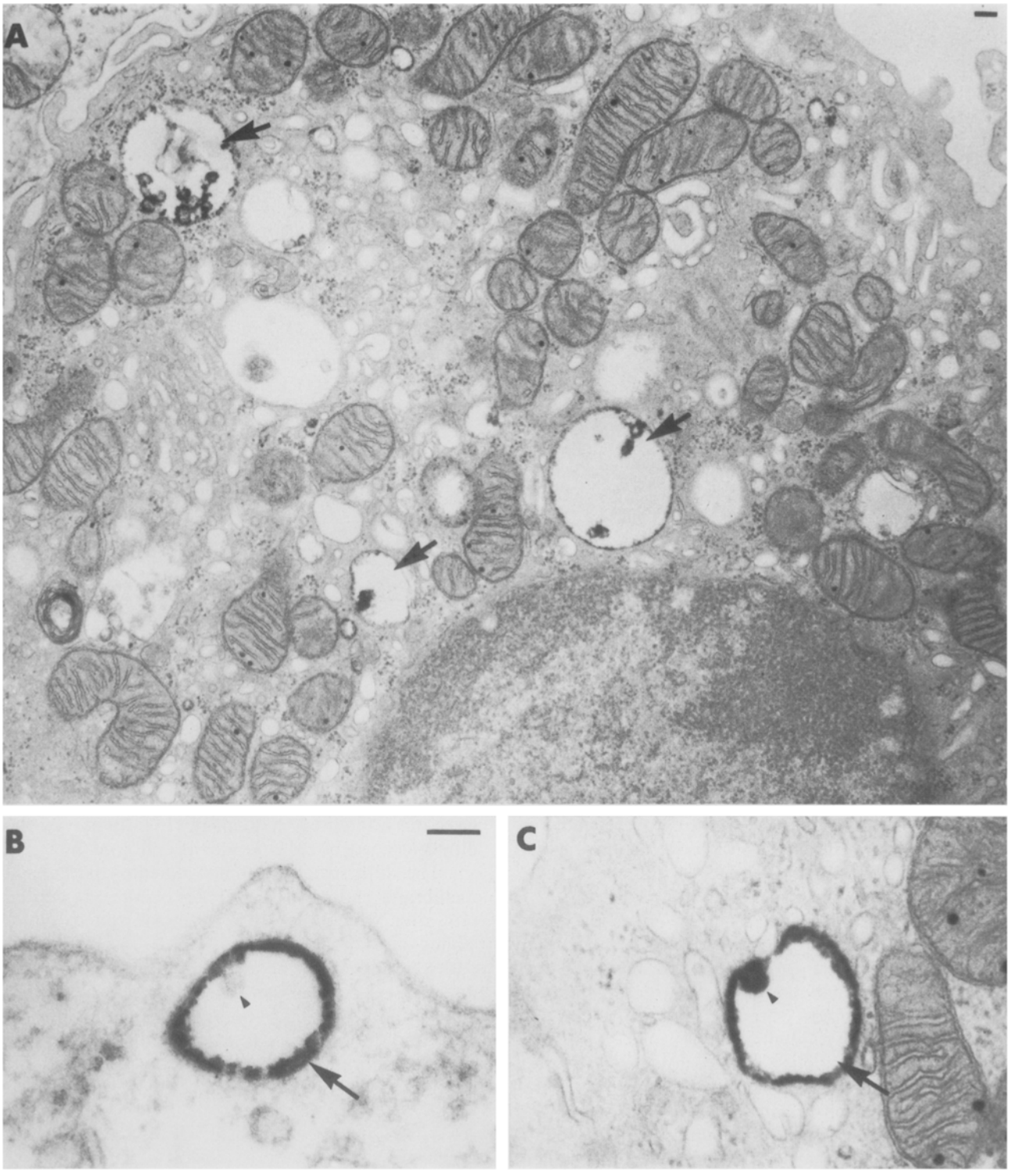

Fig. 2. Intracellular localization of HRP after HRP perfusion in the absence of ADH stimulation. Tubules were perfused with HRP-free saline for only 1 min after a 20-min HRP exposure. In both intercalated (*A,C*) and principal (*B*) cells HRP was predominantly localized to lysosomes and endosomes. The larger arrows localize endosomes labeled with HRP. The smaller arrowheads point to small circular membranous structures located within the endosomes. Magnifications (*A*) 33,000×, (*B*) and (*C*) 90,000× with the bars in the upper right corner of (*A*) and (*B*) measuring 0.1 μm

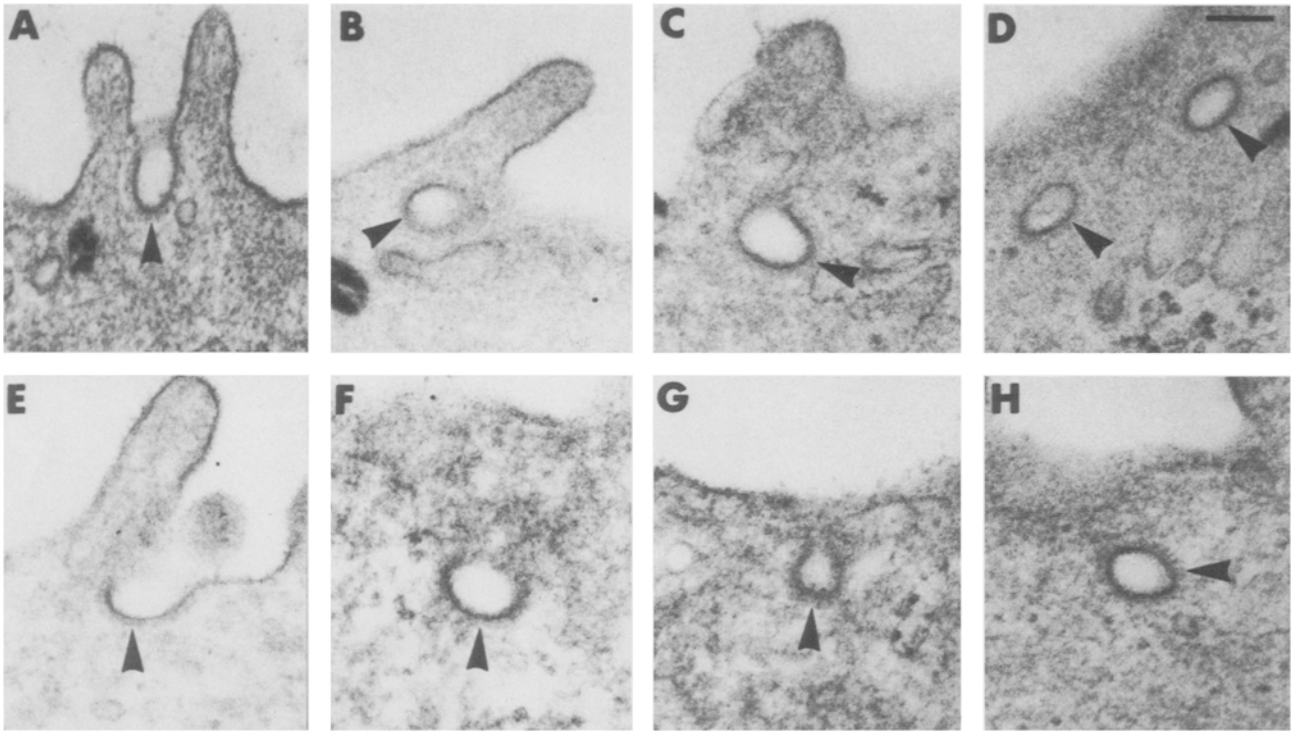

Fig. 3. Coated pits are numerous on the luminal membranes of both cell types. The arrows indicate coated pits on the luminal membrane surface of intercalated (*A–D*) or principal cells (*E–H*). All magnifications are 90,000× with the bar at the upper right measuring 0.1 μm

both principal and intercalated cells (Fig. 2). The distribution of HRP uptake was the same in ADH-treated tubules (protocol #1) perfused for 20 min with HRP (*data not shown*). In both protocols, the bulk of the HRP was localized in intracellular vesicles whose morphologic features were indistinguishable from endosomes (or receptosomes) which have been described in a wide variety of cell types [reviewed in references 33, 42, 43].

Apical membrane coated pits were easily identified and frequently observed in both principal and intercalated cells (Fig. 3). We found, however, that significant HRP labeling of these structures did not occur when tubules were perfused with HRP-free saline for 10 min following a 20-min HRP exposure. This is most likely due to the removal of HRP from apical surface coated pits by the rapid and prolonged luminal perfusion with HRP-free saline and by the rapid cycling of endocytosed HRP to endosomes and lysosomes. To circumvent this problem we reduced the HRP perfusion period to 1–5 min and the HRP washout period to less than 1 min. Under these conditions numerous HRP-filled coated pits were observed in both cell types (Fig. 4).

When tubules were stimulated with ADH and then perfused with HRP immediately after hormone withdrawal (protocol #1), an increase in HRP uptake was observed as compared to protocols #2 and #3 (Table). Both intercalated and principal cell types were labeled with HRP in protocols #1, #2 and #3 (Figs. 5 and 6). In all these protocols, all of the HRP was present in intracellular vesicles. We have not attempted to quantify HRP uptake by the different cortical collecting duct cell types.

The ultrastructural data indicate that apical membrane endocytosis of HRP occurs via coated pits after which the HRP is transferred to endosomes and then lysosomes. This internalization pathway appears to be active in both principal and intercalated cells under baseline and ADH-stimulated conditions. The combination of enzyme assays and morphological examination shows that apical membrane uptake of HRP is stimulated during the interval immediately after removal of ADH.

Discussion

The ADH-elicited increase in collecting duct and toad urinary bladder transepithelial water permeability occurs within minutes of hormone application to the basolateral membrane. This dramatic change in apical membrane water permeability likely requires the activation or translocation of existing wa-

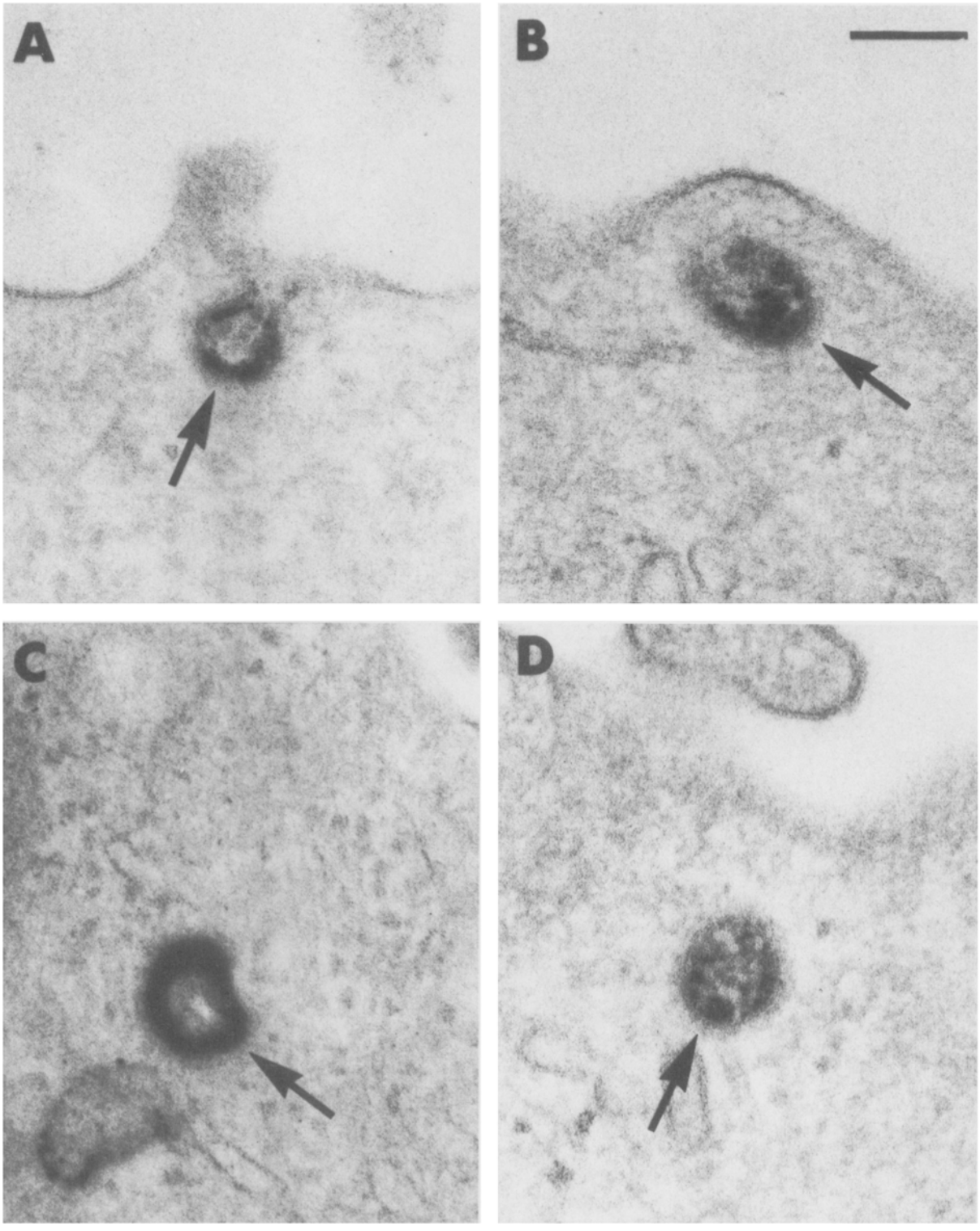

Fig. 4. HRP labels luminal membrane coated pits. Luminal membrane coated pits in both principal (*A* and *D*) and intercalated (*B* and *D*) cells were labeled with HRP by perfusing tubules with HRP for 1–5 min followed by perfusion with HRP-free saline for less than 1 min. Magnification 115,000× with the bar in (*B*) measuring 0.1 μm

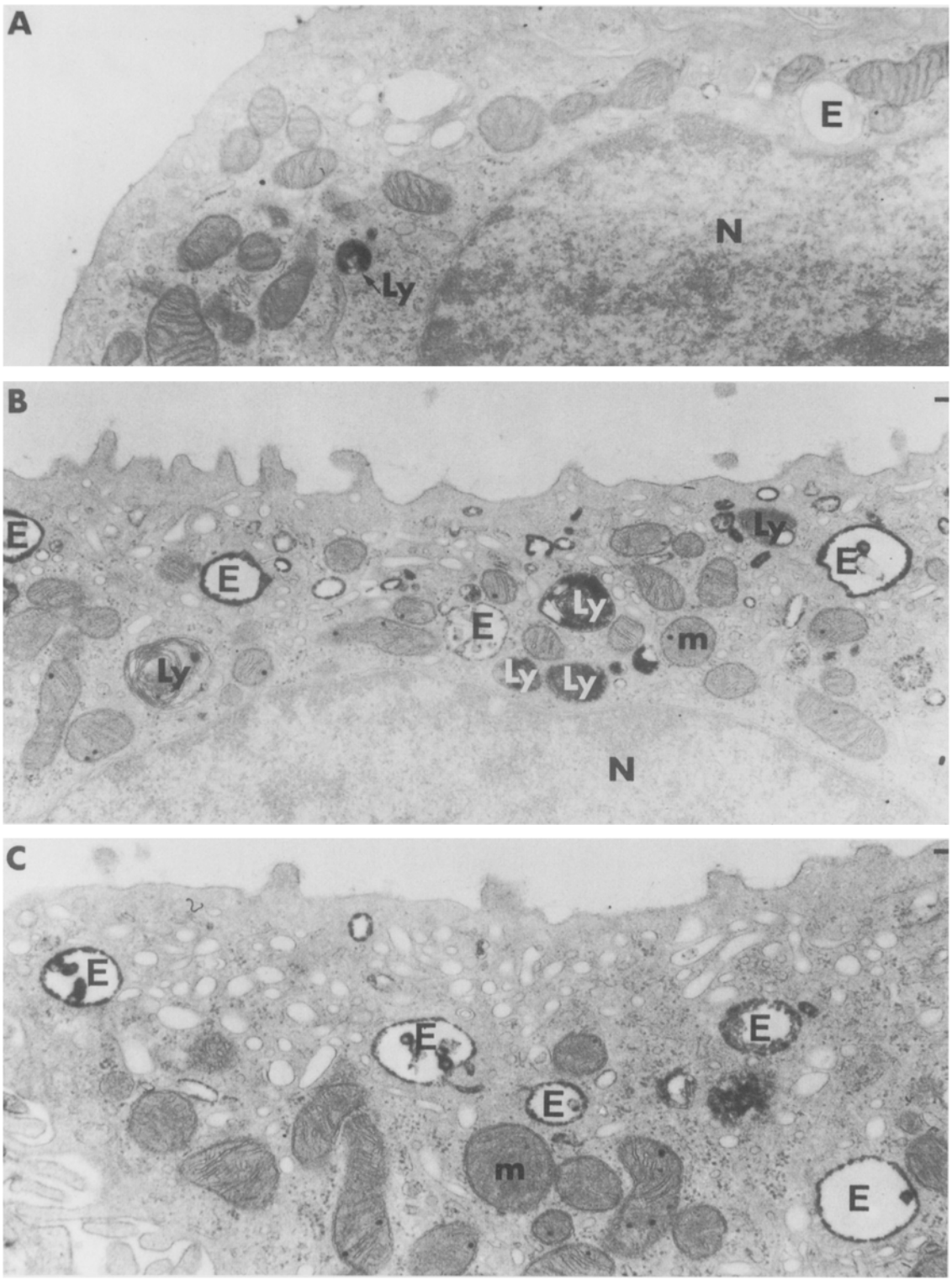

Fig. 5. HRP perfusion during ADH removal produces HRP uptake in intercalated cells. When tubules were subjected to protocol #3 (no ADH) shown in panel (*A*), there was HRP labeling of lysosomes (*Ly*) while many identifiable endosomes (*E*) were unlabeled. In contrast, when tubules were subjected to protocol #1 (ADH) stimulation and removal), many lysosomes (white *Ly*) and endosomes (*E*) were prominently labeled with HRP in intercalated cells (panels *B* and *C*). The symbols *N* and *M* designate a nucleus and mitochondrion, respectively, and the black *Ly* indicates a lysosome not labeled by HRP. Magnifications: (*A*) and (*B*) 25,000×, (*C*) 32,000×; all bars measure 0.1 μm

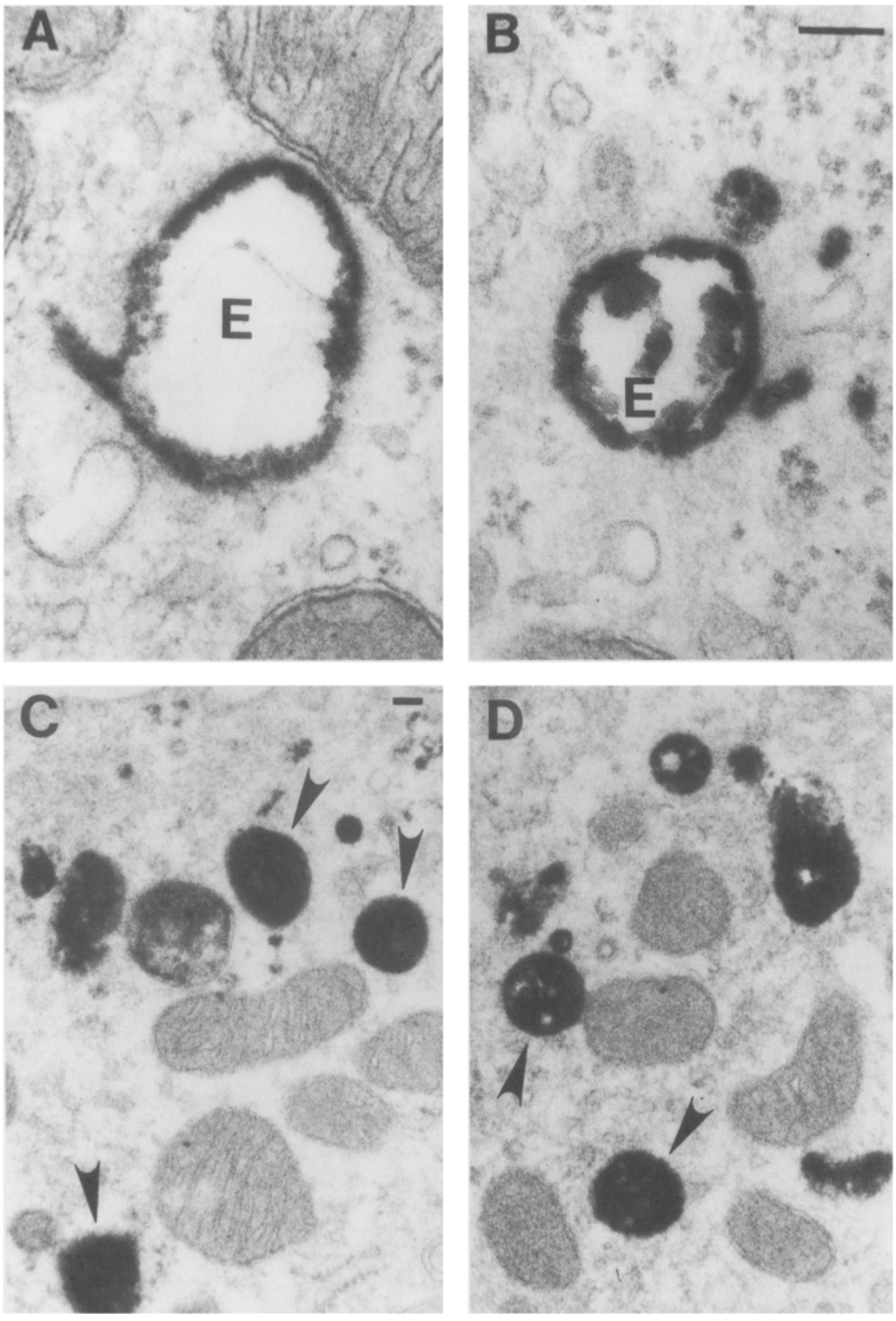

Fig. 6. HRP perfusion during ADH removal results in HRP labeling of lysosomes and endosomes in principal cells. Panels (*A*) and (*B*) show heavy HRP labeling of endosomes in principal cells subjected to protocol #1. In (*C*) and (*D*), lysosomes in principal cells are filled with dark HRP reaction product (arrows). Magnifications: (*A*) and (*B*) 90,000×, (*C*) 40,000×, and (*D*) 30,000×. All bars measure 0.1 μm

ter channels via a recruitment process rather than *de novo* synthesis. In toad bladder [3, 21], and cortical [26] as well as medullary collecting duct [6, 12, 13], particle aggregates identified by freeze-fracture electron microscopy appear in the apical membrane in a dose-response relationship with exposure to ADH and are believed to constitute or be closely related to water channels.

In the toad bladder, HRP has been extensively used as a marker of ADH-induced fluid-phase endocytosis [5, 10, 14, 15, 27, 28, 41] in an effort to identify specific membrane components which are inserted or removed following hormone stimulation. HRP uptake from the solution bathing the apical plasma membrane of granular cells occurs at an extremely low rate in the absence of stimulation by ADH (5, 10, 15, 27]. In contrast, under conditions where net osmotic water flow occurs in an apical to basolateral direction during ADH stimulation [5, 15, 27] or when ADH is removed from the serosal solution [14, 28, 41], apical membrane endocytosis of HRP by granular cells is increased dramatically, and, according to recent studies [5], HRP is endocytosed into vesicles containing intramembrane particle aggregates. Thus, it is reasonably well-established that in the ADH-stimulated toad bladder, water-permeable membrane units are inserted into the apical plasma membrane from intracellular particle aggregate-containing vesicles and are retrieved from the plasma membrane into the same vesicles. There is no evidence that coated pits are involved in the endocytosis of water-permeable apical membrane.

The apical membrane events initiated by ADH removal in the collecting duct are not known. As discussed in the Introduction, there is no evidence of apical membrane retrieval during ADH withdrawal and the accompanying decrease in transepithelial water permeability. In addition, there is little evidence regarding the nature of the structure possibly involved in the insertion and retrieval of apical particle aggregates. To evaluate apical membrane retrieval, we measured the uptake of HRP from the lumen of isolated perfused rabbit cortical collecting tubules. Electron-microscopic examination of the tubules showed minimal evidence of HRP adherent to the luminal membrane and no evidence of adherence or uptake into cells by way of the basolateral membrane (Fig. 1). Therefore, our measurements of HRP uptake represent true endocytosis of HRP at the apical membrane. We observed a marked increase in HRP uptake in ADH-stimulated tubules perfused with HRP during the period immediately after hormone removal as compared to unstimulated tubules or tubules exposed continuously to ADH during the HRP luminal perfusion period.

We frequently observed coated pits on the apical membranes of both principal and intercalated cells of the rabbit cortical collecting tubule (Fig. 3). These coated pit structures closely resemble clathrin-coated pits found on the surface of many cells, but the participation of clathrin in principal and intercalated cell endocytosis will require definitive immunolocalization. Visualization of coated pits filled with HRP required that we rapidly fix the tubule after minimal perfusion with HRP-free saline (Fig. 4). Apparently, the rate of endocytosis via apical membrane coated pits is such that HRP is rapidly transferred from coated pits to endosomes. Prolongation of the interval between the end of HRP perfusion and fixation results in a diminution in the number of HRP-labeled coated pits and endosomes and an increase in HRP-labeling of lysosomes (Figs. 5 and 6). Our results indicate that internalization of apical membrane via this endocytic pathway is stimulated by removal of ADH from the peritubular bathing medium. In the course of these studies, we did not observe HRP reaction product in tubular-shaped vesicles resembling aggrephores of the toad bladder in either principal or intercalated cells.

There is controversy regarding the participation of intercalated cells in the ADH-elicited increase in water permeability of the collecting duct. Although there is evidence that intercalated cells do not bind ADH [23], recent direct measurements suggest strongly that intercalated cells respond to ADH with an increase in apical membrane water permeability comparable to the increase observed in principal cells [38]. Unfortunately, our studies were not designed for quantitative evaluation of endocytosis in specific cell types. Nevertheless, the presence of HRP uptake in intercalated cells (Fig. 5) and principal cells (Fig. 6), combined with the quantitative increase in HRP uptake measured in the entire tubule (Table) following ADH withdrawal indicates that quantitative electron-microscope evaluation of endocytosis in the two cell types would be valuable in resolving the issue. In addition, direct examination of the membrane particle aggregate content of these HRP-labeled vesicles through use of a newly described label-fracture approach [5] should be undertaken using the conditions defined by our experiments.

To conclude, this investigation extends the earlier observation [1, 2] that ADH stimulation of rat collecting ducts causes the appearance of particle aggregates in apical membrane coated pits. Our studies are the first to demonstrate directly that increased apical membrane endocytosis occurs via coated pits in rabbit cortical collecting duct following removal of ADH from the peritubular bathing medium. This endocytosis occurs when water per-

meability is declining rapidly [9]. We suggest that in rabbit cortical collecting ducts, the water permeability response to ADH is reversed by retrieval of water-permeable membrane from the apical plasma membrane via coated pits.

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