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POLARIZED TARGETING OF V-ATPase IN KIDNEY EPITHELIAL CELLS

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Summary

The membrane-associated V-ATPase that plays an important role in the regulation of acid–base balance by the kidney is a multisubunit enzyme that is densely packed into specialized membrane domains in intercalated cells. Intercalated cells can be separated into at least two subtypes, A-cells and B-cells, based on their morphological features, the distribution of V-ATPase, and the presence or absence of a basolateral chloride/bicarbonate anion exchanger (AE1) exclusively in B-cells. A-cells secrete protons into the tubule lumen, whereas B-cells secrete bicarbonate. The relative amounts of V-ATPase and AE1 in the plasma membranes of A- and B-cells are modulated under different acid–base conditions and provide a sensitive means by which urinary acidification can be controlled. The mechanisms governing the movement of acid–base transporting proteins between intracellular vesicles and the plasma membrane are under investigation. The microtubular apparatus of the cell is involved in maintaining both apical and basolateral polarity of the enzyme, and different isoforms of V-ATPase subunits may also be involved in the selective targeting of V-ATPase to different membrane domains.

Introduction

A membrane-associated vacuolar H^+-ATPase (V-ATPase) plays an important role in physiologically regulated proton transport in the urinary tubule. In the proximal tubule, all epithelial cells have an apically located V-ATPase that contributes to proton secretion and bicarbonate reabsorption, whereas in the collecting duct, transepithelial proton transport is restricted to a subpopulation of cells known as intercalated cells. These cells, as well as related cells in other transporting epithelia, shuttle V-ATPase between a specialized intracellular vesicular compartment and the plasma membrane in response to changes in the acid–base balance of animals *in vivo*, as well as in isolated perfused collecting ducts *in vitro* (Bastani *et al.* 1991; Brown *et al.* 1987; Cannon *et al.* 1985; Dorup, 1986; Gluck *et al.* 1982; Madsen and Tisher, 1983, 1984; Schwartz and Al-Awqati, 1985; Schwartz *et al.* 1985; Stetson and Steinmetz, 1983; Van Adelsburg and Al-

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Awqati, 1986). In this way, both the concentration of V-ATPase in the plasma membrane and net acid or base secretion can be varied, providing a sensitive mechanism by which the kidney can regulate systemic acid–base balance.

V-ATPase molecules can be inserted into either the apical or basolateral plasma membrane of subtypes of intercalated cells (Brown *et al.* 1988*a*; Schwartz *et al.* 1985; Stetson and Steinmetz, 1985; Verlander *et al.* 1988). Because most membrane proteins have a well-defined apical or basolateral distribution in polarized epithelia (Caplan and Matlin, 1989; Simons, 1987), this property of the V-ATPase makes it an unusual membrane protein. Therefore, in addition to the importance of the membrane shuttling process in physiological regulation, the recycling of V-ATPase molecules to and from opposite membrane domains in intercalated cells provides a unique model for the study of the cell biological mechanisms involved in the insertion and retrieval of membrane proteins.

V-ATPase localization in intercalated cells

Intercalated cells are specialized acid-secreting cells in the urinary tubule. The availability of antibodies against V-ATPase isolated from bovine kidney medulla (Gluck and Caldwell, 1987, 1988) has allowed the precise immunolocalization of this molecule in these cells, as well as in other locations along the nephron (Brown *et al.* 1988*a*,*b*). These results provided direct evidence for the presence of different subtypes of intercalated cells that have V-ATPase in opposite plasma membrane domains in the cortical collecting duct (Fig. 1). Affinity-purified, polyclonal antibodies against three subunits (31, 56 and $70\times10^3 M_r$) of the purified bovine medullary V-ATPase were used for immunocytochemical studies in rat kidney. Using light microscopy, all intercalated cells were shown to be heavily labeled by antibodies against three pump subunits. In the renal medulla, the labeling was concentrated at the apical pole of intercalated cells (Fig. 1A) and all three ATPase subunits were co-localized to the same membrane domains. Using electron microscopy, immunogold labeling was localized on the cytoplasmic side of all membrane domains that showed a stud-like coating material, which we have identified as the cytoplasmic domain of the V-ATPase (Brown *et al.* 1987). This finding is consistent with physiological data showing that the medullary collecting duct is involved in hydrogen ion secretion (Atkins and Burg, 1985; Stone *et al.* 1983*a*).

When the kidney cortex was examined, a more complex picture emerged, and the findings directly confirmed the existence of at least two subpopulations of intercalated cells in cortical collecting ducts (Fig. 2), as proposed in previous studies (Madsen and Tisher, 1986; Schwartz *et al.* 1985). The cells, known as A-cells (acid-secreting) and B-cells (bicarbonate-secreting) have opposite polarities of plasma-membrane-associated proton pumps (Brown *et al.* 1988*a*,*b*). Similar subpopulations of carbonic-anhydrase-rich cells are found in the turtle bladder (Stetson *et al.* 1985; Stetson and Steinmetz, 1985). The A-cells showed predominantly apical labeling, whereas the B-cells had a clearly defined basolateral membrane localization of the proton pump (Fig. 1B). This finding was the first example of adjacent cells in the same epithelium maintaining opposite polarities of a major plasma membrane protein (Brown *et al.* 1988*a*). However, in

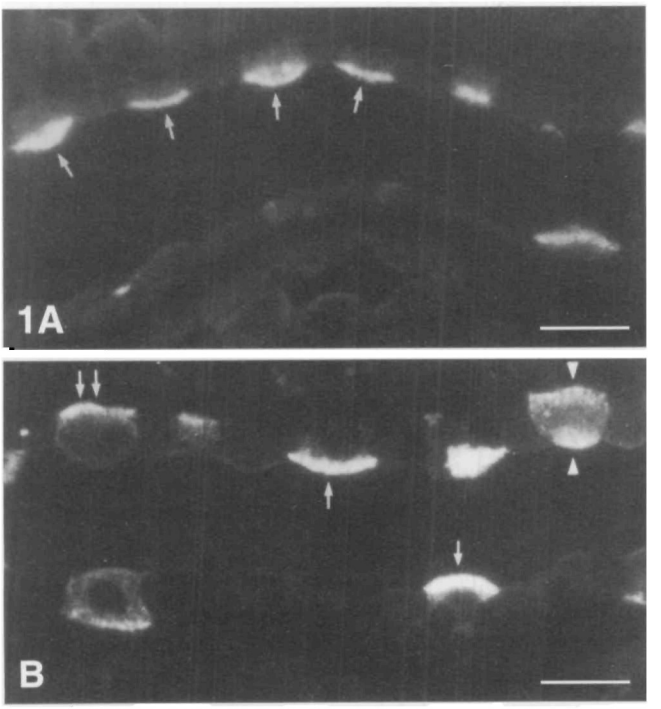


Fig. 1. Semithin resin-embedded sections of rat kidney, labeled with antibodies against vacuolar V-ATPase. (A) The inner stripe of the outer medulla; all intercalated cells show a pronounced apical staining (arrows) typical of the A-cells that are found in this region. Adjacent principal cells are only weakly stained. In contrast, intercalated cells in the cortical collecting duct (B) show apical (arrow), basolateral (double arrow) or diffuse/bipolar (arrowheads) staining. Scale bar, 20 μm.

addition, other immunopositive cells had a less well-polarized distribution of proton pumps, with the immunostaining distributed diffusely in the cytoplasm, or even appearing to be concentrated at both poles of the cell, close to the respective plasma membranes.

Subtypes of intercalated cells revealed by V-ATPase and band 3 staining

Immunocytochemical characterization of intercalated cells revealed subtypes that either exhibit or lack immunoreactive band 3 protein, a chloride/bicarbonate anion exchanger (AE1) that is abundant in the erythrocyte plasma membrane, in their basolateral membrane (Drenckhahn *et al.* 1985; Holthofer *et al.* 1987; Schuster *et al.* 1986; Wagner *et al.* 1987). In the case of the A-type cell, this basolateral anion exchanger works in concert with apical V-ATPase to ensure the extrusion of protons at the apical pole and HCO_3^- at the basolateral pole of the cell. Double staining revealed that all of the intercalated cells with basolateral or diffuse/bipolar staining for proton pumps were AE1-negative (Fig. 3). However, the double staining procedure also revealed a small number of AE1-negative cells that had a predominantly apical proton pump localization. These

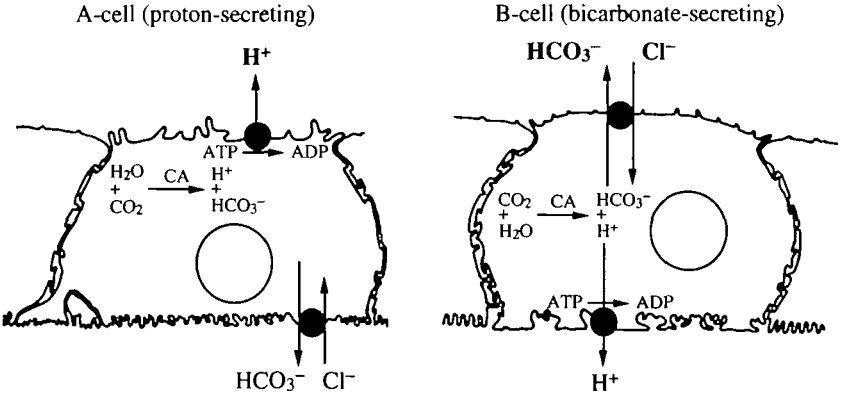


Fig. 2. Schematic representation of the two major subtypes of intercalated cells that are present in the cortical collecting duct. Both cell types contain cytosolic carbonic anhydrase II (CA), which is responsible for the intracellular production of proteins and bicarbonate. The type A, proton-secreting cell has an apical V-ATPase and a basolaterally located chloride/bicarbonate anion exchanger (AE1) that is a band-3-like protein. This exchanger can be immunolocalized with anti-band-3 antibodies. The bicarbonate-secreting B-cell has a V-ATPase of opposite polarity, which can be localized in the basolateral membrane (shown here), but which can also assume a diffuse or bipolar distribution within the cell (see Fig. 1B). An apical chloride/bicarbonate exchanger works in conjunction with apical proton pumps to extrude bicarbonate, but this exchanger has eluded all attempts at immunocytochemical detection with available antibodies against erythroid band 3 or antibodies against kidney band-3-related protein. Adapted from (Madsen *et al.* 1991).

studies demonstrated that cells with opposite polarities of a proton-pumping ATPase co-exist in the cortical collecting duct, as proposed on the basis of earlier studies. Similar results were obtained in the rabbit cortical collecting duct, except that well-polarized basolateral staining for V-ATPase was not observed in the B-cells (Schuster *et al.* 1991). The AE1-negative B-cells showed diffuse cytoplasmic staining for V-ATPase. Taken together, the immunocytochemical data are consistent with physiological data showing that the cortical collecting duct can secrete either net bicarbonate or net acid under different physiological conditions, but that the medullary collecting duct does not secrete bicarbonate (Koeppen and Helman, 1982; Star *et al.* 1985; Stone *et al.* 1983*b*).

Adaptive responses of intercalated cells to acid–base conditions

It has been proposed that individual intercalated cells are interconvertible between the A and B configurations and can reverse the polarity of important transporting proteins in response to acid–base loads (Schwartz *et al.* 1985). This attractive hypothesis has great significance for cell biology, because it implies that the same protein(s) could modify its polarized insertion in an epithelial cell in response to some external stimulus. However, some quantitative studies based on cell morphology alone (Bastani *et al.* 1991; Dorup, 1986; Madsen and Tisher, 1983, 1984) and immunocytochemical staining with H^+-ATPase antibodies (Bastani *et al.* 1991) have shown that although there is, indeed, a

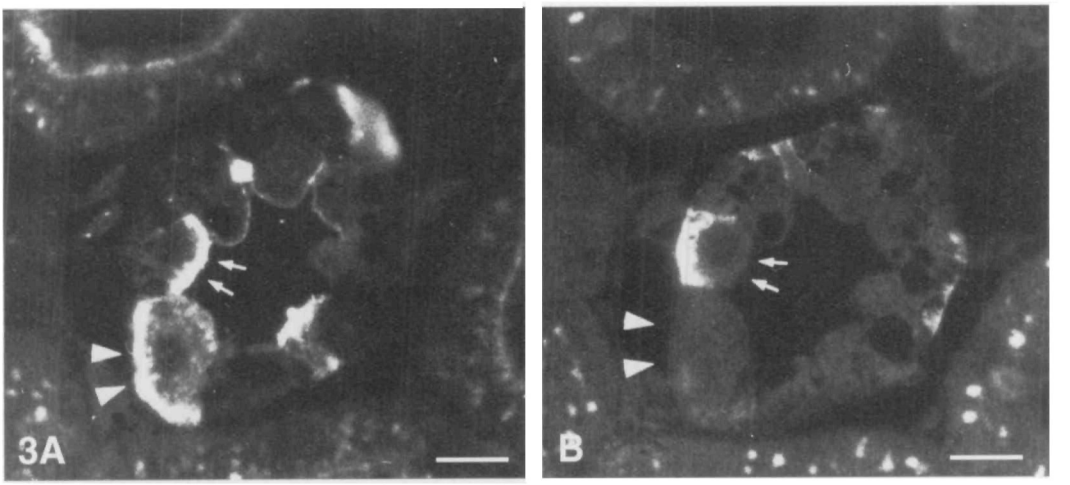


Fig. 3. Adjacent semithin sections of LX-112-embedded rat kidney showing a connecting tubule from the cortex. (A) Indirect immunofluorescence labeling of the vacuolar V-ATPase, demonstrating the heavy apical labeling of some intercalated cells (arrows), while another cell has a predominant basolateral staining, with some diffuse cytoplasmic fluorescence (arrowheads). When the same cells were examined with an anti-band-3 antibody (B), the cell with apical V-ATPase had basolateral band 3 (arrows), whereas no staining was detectable in the cell with basolateral V-ATPase (arrowheads). These two cell types represent A and B intercalated cells, respectively. Note that a delicate apical staining with anti-V-ATPase antibody was found in the connecting tubule cells adjacent to the intercalated cells. A typical subapical staining for V-ATPase was also seen in the proximal tubule in the top left-hand corner of the figure. Scale bar, 7 μm. From Alper *et al.* (1989).

marked change in the cellular distribution of cytoplasmic vesicles and membrane domains containing proton pumps during adaptation to acidosis or alkalosis, this result does not necessarily involve an interconversion of A- and B-cells. It is more likely that A-cells can increase the number of proton pumps in their apical plasma membrane during acidosis by exocytosis, and that these pumps are removed from the membrane during alkalosis (Bastani *et al.* 1991). Moreover, we have recently shown that basolateral staining for AE1 is considerably reduced in cortical A-cells during alkalosis, which would further reduce the activity of A-cells under these conditions (Alper *et al.* 1991). While these changes are occurring in A-cells, B-cells are also responding to acidosis by removing proton pumps from their basolateral membrane (Bastani *et al.* 1991); in parallel, acidosis stimulates endocytosis of apical plasma membrane in these B-cells (Satlin and Schwartz, 1989). Indeed a vigorous apical endocytosis in B-cells (identified by double staining to reveal basolateral proton pumps) can be detected even in normal control rats, using fluid-phase markers of endocytosis (D. Brown and I. Sabolic, unpublished observations). Thus, although A and B intercalated cells can both modulate their plasma membrane content of V-ATPase and anion exchanger, there is still no direct evidence for interconversion of these two cell types *in vivo*.

If the simple reversal of polarity model of A- and B-cells is correct, then apically located AE1 should be detectable in B-cells. However, efforts by several groups to reveal

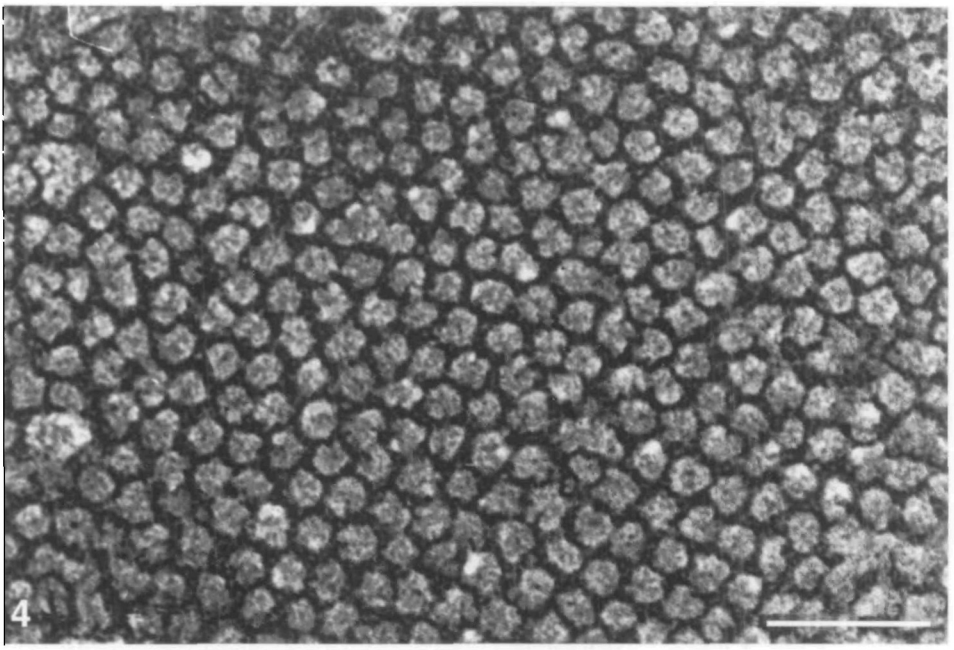


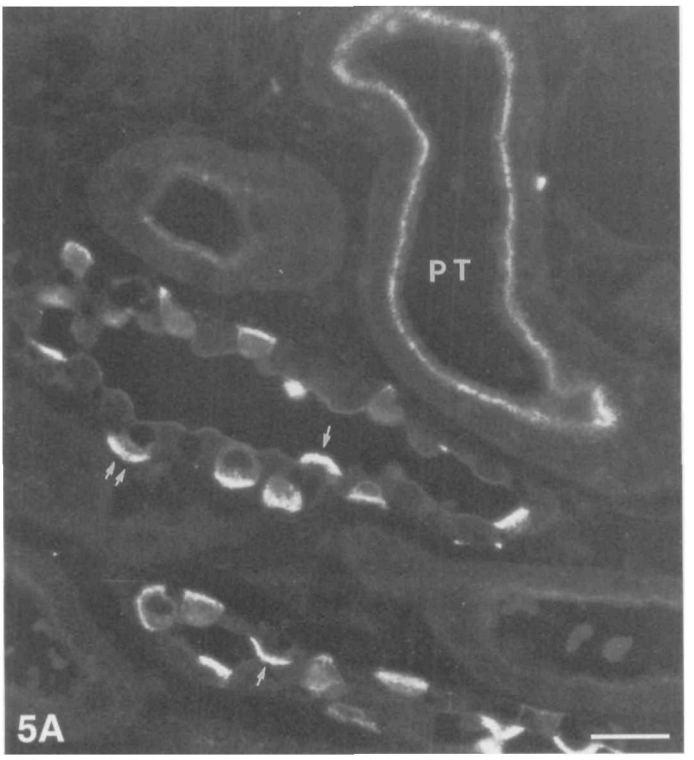
Fig. 4. Replica of a rapid-freeze, deep-etch preparation of a membrane fragment from a proton-secreting cell in toad urinary bladder. The underside of the plasma membrane shows regions in which stud-like projections, representing the cytoplasmic domains of V-ATPase molecules are arranged into hexagonally packed, paracrystalline arrays. Scale bar, 50 nm.

this exchanger by immunocytochemistry have failed. Nevertheless, physiological data do support the existence of an apical anion exchanger in B-cells (Satlin and Schwartz, 1989), but show that it has certain characteristics that are distinct from the AE1 exchanger in the basolateral membrane of A-cells. Thus, either the apical anion exchanger in B-cells is antigenically distinct from basolateral AE1 of A-cells (Alper *et al.* 1988), or important epitopes are in some way masked in the apical membrane of intact tissue. In the turtle bladder, the functional characteristics of the apical anion exchanger are also somewhat different from those of the basolateral exchanger (Kohn *et al.* 1990), implying that it may be a different (but perhaps related) protein.

Structure of V-ATPase revealed by rapid-freeze, deep-etching

The intercalated cell membrane domains implicated in proton secretion, as well as intracellular vesicles that shuttle V-ATPase to and from the plasma membrane, have a

Fig. 5. Semithin sections of rat kidney cortex showing immunostaining for V-ATPase in normal (A) and colchicine-treated (B) kidney. In the normal kidney, intercalated cells show typical apical (arrow) and basolateral (double arrow) staining, and a dense band of staining is located at the base of the proximal tubule brush border (PT). After colchicine treatment (B), all intercalated cells show a diffuse, cytoplasmic staining of V-ATPase (arrows) and the apical band in the proximal tubule (PT) is disrupted. Scale bars, 20 μm (A) and 30 μm (B).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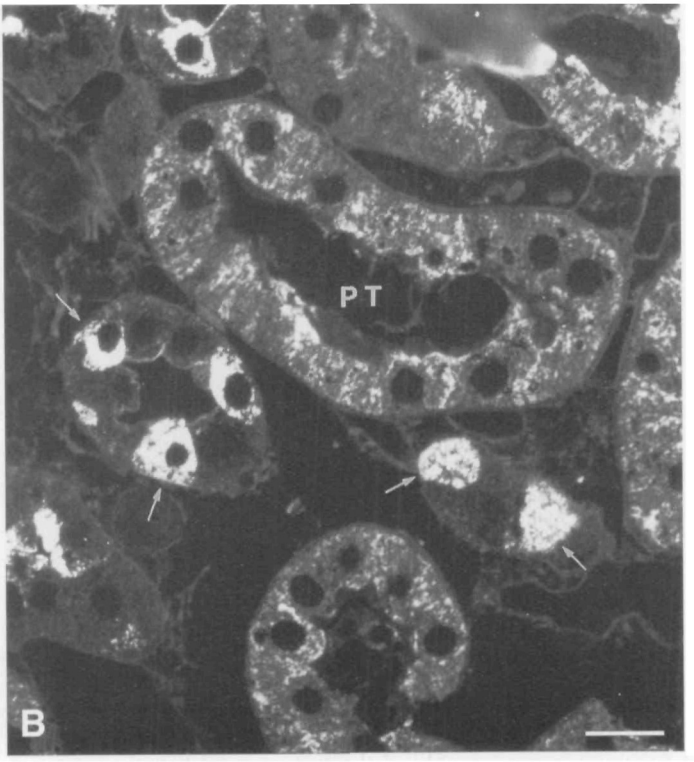
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distinctive stud-like coating material on their cytoplasmic leaflet (Brown *et al.* 1987; Griffith *et al.* 1968; Madsen and Tisher, 1986; Stetson *et al.* 1980). Turning to the toad urinary bladder as a model epithelium, we used the rapid-freeze, deep-etch procedure to examine the structure of this coating material in pieces of membrane removed from intact proton-secreting cells by adherence to poly-L-lysine-coated coverslips (Brown *et al.* 1987). After rotary-shadowing these membrane fragments with platinum and carbon, a remarkable array of hexogonally packed 10 nm globular subunits was visualized on the underside of the plasma membrane from these cells (Fig. 4). In addition, a similar paracrystalline array of particles was found on the cytoplasmic surface of some intracellular vesicles. Affinity-purified V-ATPase reconstituted into phospholipid vesicles and examined by the same technique exhibited an identical hexagonally packed array of particles, confirming that the structures visualized in proton-secreting cells *in vivo* were indeed the cytoplasmic domains of the V-ATPase molecule. How the different subunits of the V-ATPase are arranged in these membrane-associated particles is unknown and is the subject of continuing studies.

Role of microtubules in determining V-ATPase polarity

It has been known for some time that acidification in the turtle bladder is reduced, but not completely eliminated, by microtubule disruption with colchicine (Stetson and Steinmetz, 1983). Studies on other polarized epithelial cells have suggested that microtubules are involved in the transport of membrane proteins to the apical pole of epithelial cells (Achler *et al.* 1989; Gutmann *et al.* 1989; Ojakian and Schwimmer, 1988), whereas basolateral targetting of membrane proteins is less, if at all, dependent on intact microtubules. Therefore, we postulated that colchicine treatment of intercalated cells might lead to the inhibition of apical proton pump insertion in type A cells, whereas basolateral insertion of V-ATPase in type B cells would be relatively unaffected. In fact, we found that membrane insertion of V-ATPase was equally affected in both A and B intercalated cells, so that, in the cortex of colchicine-treated rats, A- and B-cells could no longer be distinguished by their patterns of V-ATPase staining (Brown *et al.* 1990). All intercalated cells showed a diffuse cytoplasmic staining of the pump, because the V-ATPase-containing vesicles were scattered throughout the cytoplasm (Fig. 5). The acidosis-induced insertion of V-ATPase into the apical membrane of A-type cells is inhibited by colchicine (D. Brown and I. Sabolic, unpublished data), explaining previous results that this drug inhibits the stimulation of acidification in turtle bladder.

However, two other basolateral membrane proteins in the intercalated cells, AE1 and a facilitated glucose transporter Glut-1 (Thorens *et al.* 1990), retained a strong basolateral staining pattern (not shown), implying that colchicine was selective for certain proteins. In the medullary collecting duct, the A-cells also showed a diffuse staining pattern for V-ATPase – no cells showed basolateral staining after colchicine treatment, despite the proximity of many positive vesicles close to the basolateral plasma membrane of these cells.

A similar effect of colchicine treatment on the distribution of V-ATPase was seen in the proximal tubule, where the normally tight apical band of staining was replaced by a

scattered distribution of positive vesicles throughout the cell cytoplasm (Brown *et al.* 1990). Because apical and basolateral membrane vesicles can be readily prepared from kidney cortex (Sabolic and Burckhardt, 1983, 1990), we examined the distribution of *N*-ethylmaleimide (NEM)-sensitive ATPase activity (a biochemical assay for V-ATPase) in these membrane fractions before and after colchicine treatment of rats. As expected, colchicine treatment greatly reduced the apical membrane NEM-sensitive ATPase activity (Fig. 6), confirming the reduction in apical V-ATPase content detected by immunocytochemistry as well as by Western blotting of isolated plasma membrane vesicles (data not shown). However, no increase in basolateral NEM-sensitive ATPase was found after colchicine treatment, showing that basolateral insertion of 'misdirected' vesicles did not occur.

Taken together with other studies on apical membrane proteins in the kidney (Gutmann *et al.* 1989), these results suggest (i) that microtubules can be involved in the targetting of intracellular vesicles to both the apical and basolateral pole of epithelial cells, (ii) that microtubules are not involved in the final fusion event, since 'misdirected' apical vesicles do not fuse with the basolateral plasma membrane, and (iii) that the effect of microtubule disruption of membrane protein distribution is protein-specific, rather than membrane-specific. Thus, membrane proteins that turn over or are recycled rapidly may be more susceptible to the effects of microtubule disruption than membrane proteins that have a longer time of residency in the membrane.

A-cell-specific isoform of the V-ATPase $56\times10^3 M_r$ subunit in the kidney

The relative distribution of V-ATPase among various intracellular compartments and the plasma membrane varies greatly in different cell types along the urinary tubule. cDNA cloning was used to search for isoforms of pump subunits in the kidney and other

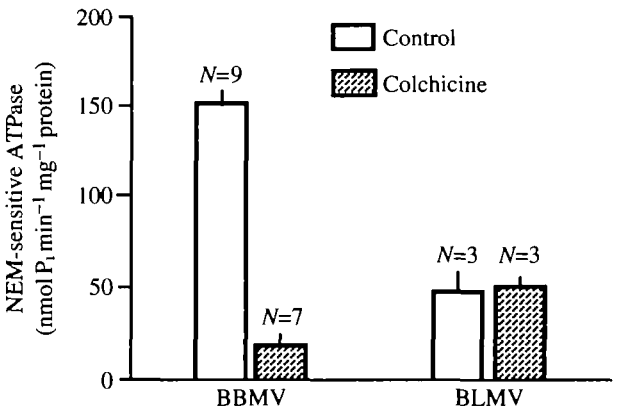

Fig. 6. Measurement of NEM-sensitive ATPase activity in brush-border membrane vesicles (BBMV) and basolateral plasma membrane vesicles (BLMV) from normal and colchicine-treated rat kidneys. Colchicine-treated rats showed a large reduction in brush-border NEM-sensitive ATPase activity compared to control rats, but the level of activity in basolateral membrane preparations was the same in both groups of animals. Values are mean + S.D.

tissues that might provide clues concerning the mechanisms responsible for this differential intracellular targetting (Hirsch *et al.* 1988; Nelson *et al.* 1992; Wang and Gluck, 1990; Wang *et al.* 1989). Isoforms of the 56×10^3 M_r subunit were detected that shared sequence similarity in the internal region of the molecule, but which showed divergence in the amino- and carboxy-terminal regions. mRNA hybridization for one

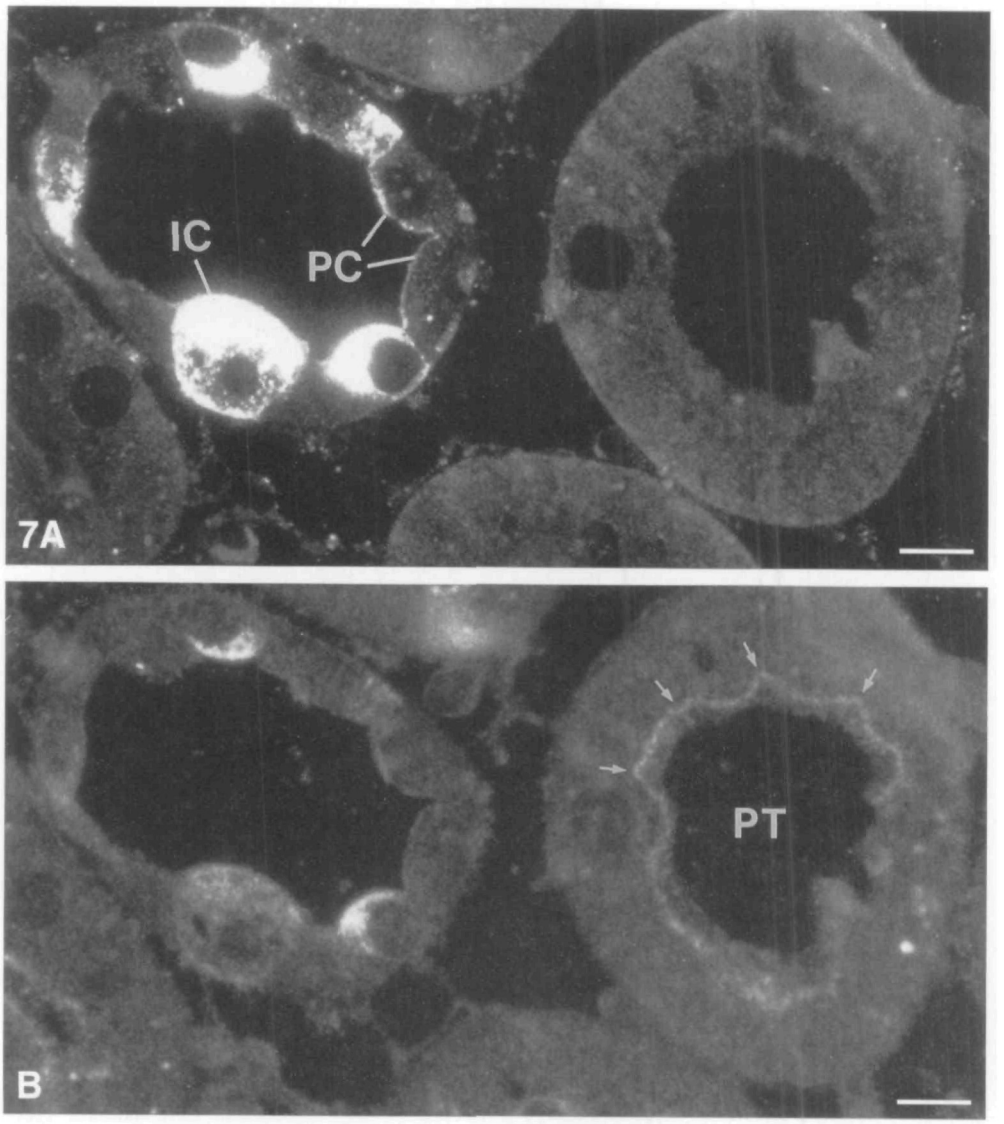

Fig. 7. Double immunostaining of rat cortex with a monoclonal antibody against the 31×10^3 M_r subunit of V-ATPase (A) and a polyclonal antibody against a peptide unique to the 'kidney' form of the V-ATPase 56×10^3 M_r subunit (B). Both antibodies label intercalated cells (IC), but only the monoclonal anti-31×10^3 M_r antibody labels the proximal tubule (PT) apical membrane (arrows). In contrast, antibody to the 56×10^3 M_r subunit also labels the apical membrane of principal cells (PC) in the cortical collecting duct; these cells are not labeled with the anti-31×10^3 M_r antibody. Scale bar, 10 μm.

form (the 'kidney' isoform) revealed that it was present only in kidney cortex and medulla, whereas the other isoform was also present in other tissues. Specific antibodies against a C-terminal peptide found only in the 'kidney' isoform showed intense localization in intercalated cells of the cortex, together with a weaker staining of principal cells and distal tubule cells. Immunostaining with this antibody was completely absent in the proximal tubule. In contrast, antibodies against shared epitopes from the internal sequence of the $56\times10^3 M_r$ subunits stained proximal tubule cells in addition to the other cell types (Nelson *et al.* 1992).

Double staining with the 'kidney' isoform-specific antibody and a monoclonal antibody against the $31\times10^3 M_r$ subunit of the V-ATPase shows this remarkable cell specificity of labeling with the two antibodies. The $31\times10^3 M_r$ antibody labels both collecting duct epithelial cells and proximal tubule cells, whereas proximal tubules are unstained with the C-terminal anti-$56\times10^3 M_r$ antibody (Fig. 7). Further examination of distinct isoforms of V-ATPase subunits may provide important information related to the intracellular targetting and functional distribution of this enzyme in kidney epithelial cells. Indeed, isoforms of the $31\times10^3 M_r$ subunit appear to be present on brush-border microvilli and intracellular organelles in the proximal tubule (Wang *et al.* 1989).

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