Renal Vacuolar H⁺-ATPase

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I. Introduction 1264
II. Structure and General Properties of Vacuolar H⁺-ATPase 1264
   A. General structure of the vacuolar H⁺-ATPase 1264
   B. Role of subunits of the vacuolar H⁺-ATPase 1265
   C. “Kidney-specific” subunits of the vacuolar H⁺-ATPase 1268
   D. General functional properties of vacuolar H⁺-ATPase 1268
III. Distribution of the Vacuolar H⁺-ATPase in the Kidney 1269
   A. Proximal tubule 1269
   B. Loop of Henle 1269
   C. Distal tubule 1270
   D. Connecting segment 1270
   E. Collecting duct 1271
   F. Distribution of the vacuolar H⁺-ATPase in other proton-transporting cells 1271
   G. Electron microscopy of the vacuolar H⁺-ATPase 1273
IV. Role of Vacuolar H⁺-ATPases in Kidney Acid-Base Transport 1275
   A. Proximal tubule 1275
   B. Loop of Henle and TAL 1275
   C. Late distal tubule, connecting segment, and cortical collecting duct 1276
   D. Medullary collecting duct 1277
V. Chloride Dependence of Vacuolar H⁺-ATPase Function 1278
   A. ClC-5 (and ClC-4) 1279
   B. AQP-6 1280
   C. CFTR 1280
VI. Endocytosis and Acidification of Intracellular Vesicles 1281
   A. Clathrin-Coated Pits/Vesicles 1281
   B. Endosomes, the Golgi/TGN, and lysosomes 1281
   C. Vesicle acidification and recruitment of endosomal coat components 1281
   D. Defective vesicle acidification and proximal tubule pathophysiology 1282
   E. Dent’s disease 1282
   F. Acquired Fanconi syndrome: cadmium nephrotoxicity 1283
VII. Interaction of the Vacuolar H⁺-ATPase With Proteins Other Than Its Own Subunits 1283
   A. Inhibitors and activators of the vacuolar H⁺-ATPase 1283
   B. Interaction of the vacuolar H⁺-ATPase with SNARE proteins 1283
   C. Subunit B1 of the vacuolar H⁺-ATPase is a PDZ binding protein 1284
   D. The vacuolar H⁺-ATPase directly interacts with the actin cytoskeleton 1285
   E. Interaction of the vacuolar H⁺-ATPase with enzymes of the glycolytic pathway 1285
   F. Interaction with other proteins 1285
VIII. Regulation of H⁺-ATPase Function and Localization 1285
   A. General mechanisms of regulation of vacuolar H⁺-ATPase activity 1285
   B. Polarized expression of the vacuolar H⁺-ATPase in intercalated cells 1286
   C. Adaptive regulation of vacuolar H⁺-ATPase activity 1288
   D. Hormonal regulation of vacuolar H⁺-ATPase activity 1289
IX. Modulations in Vacuolar H⁺-ATPase Function in Disease 1292
   A. Renal tubular acidosis 1293
   B. Mutations in the ATP6V1B1 (B1) subunit of the vacuolar H⁺-ATPase 1294
   C. Vacuolar H⁺-ATPase B1 subunit (Atp6v1b1)-deficient mice 1295
Wagner, Carsten A., Karin E. Finberg, Sylvie Breton, Vladimir Marshansky, Dennis Brown, and John P. Geibel. Renal Vacuolar H⁺-ATPase. Physiol Rev 84: 1263–1314; 2004; 10.1152/physrev.00045.2003.—Vacuolar H⁺-ATPases are ubiquitous multisubunit complexes mediating the ATP-dependent transport of protons. In addition to their role in acidifying the lumen of various intracellular organelles, vacuolar H⁺-ATPases fulfill special tasks in the kidney. Vacuolar H⁺-ATPases are expressed in the plasma membrane in the kidney almost along the entire length of the nephron with apical and/or basolateral localization patterns. In the proximal tubule, a high number of vacuolar H⁺-ATPases are also found in endosomes, which are acidified by the pump. In addition, vacuolar H⁺-ATPases contribute to proximal tubular bicarbonate reabsorption. The importance in final urinary acidification along the collecting system is highlighted by monogenic defects in two subunits (ATP6V0A4, ATP6V1B1) of the vacuolar H⁺-ATPase in patients with distal renal tubular acidosis. The activity of vacuolar H⁺-ATPases is tightly regulated by a variety of factors such as the acid-base or electrolyte status. This regulation is at least in part mediated by various hormones and protein-protein interactions between regulatory proteins and multiple subunits of the pump.

I. INTRODUCTION

Vacuolar H⁺-ATPases are ubiquitous multisubunit complexes mediating ATP-driven vectorial transport of protons across membranes. They are expressed in virtually all eukaryotic cells in intracellular membranes or in certain specialized cells also in the plasma membrane. Whereas intracellular pH is generally regulated by Na⁺/H⁺ and Na⁺-dependent and independent Cl⁻/HCO₃⁻ exchangers, the pH of many intracellular compartments such as the lysosome, the Golgi apparatus, secretory vesicles, and endosomes is regulated by vacuolar H⁺-ATPases (i.e., acidification of the compartment). The function of these organelles relies on an acidic intracellular pH to maintain optimal enzyme function. Disruption of this acidic intracellular pH leads to disturbance of organelle function and often to cell death. In neuronal cells, vacuolar H⁺-ATPases play an additional important role in synaptic transmission. Neurotransmitters taken up from the synaptic cleft are stored in vesicles acidified in an electrogenic manner by vacuolar H⁺-ATPases. The proton gradient (ΔpH) or the potential gradient (ΔV) generated by vacuolar H⁺-ATPases is then used by vesicular neurotransmitter transport proteins to accumulate neurotransmitters in storage vesicles (177). In contrast, vacuolar H⁺-ATPases localized in the plasma membrane mediate proton extrusion from the cell. Acidification of the cellular environment is intricately linked to specialized cell function as exemplified in osteoclasts where protons generated by the vacuolar H⁺-ATPases are used to dissolve bone matrix, or, macrophages where acidic pH is involved in killing and digesting neighboring cells or pathogens (513). In other cells vacuolar H⁺-ATPases mediate the regulation of extracellular pH of closed compartments such as in the inner ear and endolymph fluid (117, 167, 481), or the epididymis where seminal fluid is acidified (65, 68, 85). As reviewed here, in the kidney, in addition to the functions mentioned above, vacuolar H⁺-ATPases are involved in acid-base transport, thus contributing to overall body homeostasis.

Many of these functions have been reviewed elsewhere and are not the topic of this review (18, 174–177, 359, 384, 385, 392). This review focuses only on the function, regulation, and role of vacuolar H⁺-ATPases in renal physiology and pathophysiology. After a general introduction into the structure and function of the vacuolar H⁺-ATPase, we describe the localization of vacuolar H⁺-ATPases in the kidney and their role in acid-base transport as well as endo- and exocytosis. The regulation of these processes and the development of associated diseases caused by inherited or acquired states of vacuolar H⁺-ATPase dysfunction will also be addressed.

II. STRUCTURE AND GENERAL PROPERTIES OF VACUOLAR H⁺-ATPASE

A. General Structure of the Vacuolar H⁺-ATPase

Vacuolar H⁺-ATPases belong to the large superfamily of ATPases that is subdivided into three subclasses: 1) P-type ATPases such as Na⁺-K⁺-ATPases, Ca²⁺-ATPases, and H⁺-K⁺-ATPases; 2) mitochondrial F₁F₀-ATPases; and 3) V-type (vacuolar) H⁺-ATPases (http://www.gene.ucl.ac.uk/ nomenclature/) (370). Mitochondrial F₁F₀-ATPases and vacuolar H⁺-ATPases share many structural features such as subunit composition, high degrees of subunit similarities based on amino acid sequences, and subunit arrangement (212, 384). Functionally, however, they are distinguished by

D. ATP6V0A4 mutations in patients with autosomal recessive dRTA
E. Evidence for further genes
F. Vacuolar H⁺-ATPase loss or dysfunction in acquired diseases
X. Summary and Open Questions
the fact that $F_1F_0$-ATPases use a proton gradient for ATP synthesis, whereas vacuolar H$^+$-ATPases use ATP hydrolysis to generate a proton gradient. Some instances have been reported where vacuolar H$^+$-ATPases may be functionally reversed and act as proton-driven ATP synthetases (132, 241). $F_1F_0$-ATPase structure and function have been investigated in detail over the past years, and recently, the three-dimensional structures of the $F_1$ sector and stalk have been resolved at high resolution (357). Furthermore, the resultant structural changes that occur in response to different states of enzyme activity have also been observed and mapped (583). Consequently, vacuolar H$^+$-ATPase structure and function have been modelled based on results obtained from the $F_1F_0$-ATPases (384). In addition, much of the present knowledge on structure and function of vacuolar H$^+$-ATPases comes from experiments conducted with the yeast vacuolar H$^+$-ATPase, which also gave its name to this class of pump because of its role in acidifying the yeast food vacuole (384). Further extensive details on the structure of the vacuolar H$^+$-ATPase and the roles of the respective subunits are found in some recent reviews (175, 176, 212, 384, 392).

In general, vacuolar H$^+$-ATPases consist of two main domains, a peripheral catalytic V$_1$ domain (640 kDa) and a membrane-bound V$_0$ domain (240 kDa), together forming a protein complex of $\sim$900 kDa (Fig. 1). Both domains are connected through a stalklike structure that belongs to the V$_1$ domain (16–18, 177, 212, 384, 567). The stoichiometry of the vacuolar H$^+$-ATPase subunits in yeast is thought to be $A_3B_2C:D:E_2F:G_2:Ha:a:d:c''(c':c)_0$ (384, 420). The existence of the c$'$ subunit in mammals is not resolved yet (see Table 1, Ref. 471).

### B. Role of Subunits of the Vacuolar H$^+$-ATPase

#### 1. The V$_1$ domain

The cytosolic V$_1$ domain is composed of eight subunits denoted in capital letters (see Table 1). Homology to the $\alpha$- and $\beta$-subunits of the $F_1F_0$-ATPase, as well as mutational studies with yeast vacuolar H$^+$-ATPases, have established several functions for the A and B subunits. A and B subunits are arranged in an alternating manner in a pseudo-hexagonal head-piece (212). Whereas only one isoform of the A subunit has been identified, two highly homologous B subunits (ATP6V1B1 and ATP6V1B2) with a tissue- and cell-specific expression pattern exist in many species (53, 206, 389, 524, 556). Sequence comparisons demonstrate that the vacuolar H$^+$-ATPase B subunits share $\sim$20–25% amino acid identity not only with the vacuolar H$^+$-ATPase A subunit, but also with the $\beta$- and $\alpha$-subunits of the mitochondrial $F_1F_0$-ATPase, suggesting that all four of these proteins evolved from a common ancestral nucleotide binding protein. As predicted by homology to $\beta$- and $\alpha$-subunits of the $F_1F_0$-ATPase (1), both the A and B subunits of the vacuolar H$^+$-ATPase have been demonstrated to participate in nucleotide binding (591); however, genetic and biochemical evidence suggests that it is the nucleotide binding site on the A subunit that is catalytic (165, 546). Nevertheless, inhibition of vacuolar H$^+$-ATPase activity also occurred after modification of a single noncatalytic site of the B subunit by a photoactivated, nonhydrolyzable nucleotide analog, raising the possibility that the nucleotide binding site of the B subunit may play a role in the regulation of H$^+$-ATPase activity (527). In addition, the yeast vacuolar H$^+$-ATPase A subunit possesses a stretch of an additional 90 amino acids compared with $\alpha$-subunits. This additional segment may play a role in the coupling efficiency of ATP hydrolysis to proton transport and the dissociation of V$_1$ and V$_0$ complexes (461).

Studies in yeast have suggested that the B subunit plays an essential role in vacuolar H$^+$-ATPase function. In the yeast *Saccharomyces cerevisiae*, where the vacuolar H$^+$-ATPase mediates acidification of the vacuole, disruption of the gene encoding the B subunit results in a conditional lethal phenotype, in which mutants can grow only in media with a narrow acidity range around pH 5.5 (383, 579). Along the same line, knockout of the vha55, encoding a B subunit homolog of *Drosophila*, produced a larval lethal phenotype (126).

Comparison of the B1 and B2 subunit isoform amino acid sequences from both human and cow reveals that the central 409 amino acids are highly conserved, while the 20–25 amino acids at both the amino and carboxy termini have diverged greatly, thus raising the possibility that these terminal regions provide specialized isoform-specific functions of the B subunit (386, 421). It has been...
significantly homologous sequences. It is, however, unclear at present if a mammalian homolog exists (471). An alignment of the gene or protein with the human genome does not produce any significantly homologous sequences.

speculated that the two B isoforms may confer differences in vacuolar H\(^{+}\)-ATPases enzymatic activities or in vacuolar H\(^{+}\)-ATPases sorting capacities, or alternatively, that these two isoforms may differ in their ability to regulate the membrane density of the vacuolar H\(^{+}\)-ATPase (386). The carboxy terminus of the B1 subunit, but not the B2 subunit, terminates in a D-T-A-L sequence. This PDZ-binding motif is recognized by so-called PDZ proteins and mediates protein-protein interactions (see below). It has previously been suggested that interaction with PDZ domain-containing proteins may mediate tissue-specific interactions of the B1 subunit-containing vacuolar H\(^{+}\)-ATPase with other proteins, which could perhaps mediate targeting or trafficking of the complex (70). However, none of the ATP6V1B1 missense mutations identified to date in dRTA kindreds is located in the carboxy-terminal region of the protein (268, 492). Thus genetic studies of dRTA kindreds unfortunately have not shed light into the requirement of the region of the B1 protein essential for normal vacuolar H\(^{+}\)-ATPase-mediated proton secretion in intercalated cell (see below).

2. The V\(_{0}\) domain

The proton translocating channel is formed by the V\(_{0}\) domain involving the proteolipid c' and c'' subunits (420). Recent evidence from functional mutational analysis of the yeast Vph1 subunit (yeast analog of the mammalian a subunit) also suggests the involvement of the a subunit in the proton channel (304, 413). Targeted deletion of the

### Table 1. Human vacuolar H\(^{+}\)-ATPase subunits, chromosomal localization, and tissue distribution

<table>
<thead>
<tr>
<th>Name (Alternative Names)</th>
<th>Subunit/Function</th>
<th>Chromosomal Localization</th>
<th>Size, kDa</th>
<th>Tissue Localization</th>
<th>Accession Nos.</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP6V1A1 V(_{1}) subunit A1 ATP hydrolysis</td>
<td>3q13.31</td>
<td>70</td>
<td>Ubiquitous</td>
<td>L09235 NM_001690</td>
<td></td>
<td>523</td>
</tr>
<tr>
<td>ATP6V1B1 V(_{1}) subunit B1 ATP binding</td>
<td>2p13</td>
<td>56/58</td>
<td>Intercalated cells, inner ear, epididymis, ciliary body</td>
<td>AF107466 NM_001692</td>
<td></td>
<td>268, 386, 524</td>
</tr>
<tr>
<td>ATP6V1B2 V(_{1}) subunit B2 ATP binding</td>
<td>8p22-p21</td>
<td>56/58</td>
<td>Ubiquitous</td>
<td>L35249 NM_001693</td>
<td></td>
<td>49, 524</td>
</tr>
<tr>
<td>ATP6V1C1 V(_{1}) subunit C1</td>
<td>8p22-q21.3</td>
<td>42</td>
<td>Ubiquitous</td>
<td>X69151 NM_001695</td>
<td></td>
<td>471, 525</td>
</tr>
<tr>
<td>ATP6V1C2 V(_{1}) subunit C2</td>
<td>2p25.1</td>
<td>42</td>
<td>Placenta, kidney</td>
<td>AO039759 NM_144583</td>
<td></td>
<td>471</td>
</tr>
<tr>
<td>ATP6V1D V(_{1}) subunit D</td>
<td>14q23-q24.2</td>
<td>34</td>
<td>Ubiquitous</td>
<td>AF145316 NM_015904</td>
<td></td>
<td>525</td>
</tr>
<tr>
<td>ATP6V1E1 V(_{1}) subunit E1</td>
<td>22pter-q11.2</td>
<td>31</td>
<td>Ubiquitous</td>
<td>X76228 NM_001696</td>
<td></td>
<td>525</td>
</tr>
<tr>
<td>ATP6V1E2 V(_{1}) subunit E2</td>
<td>2p21</td>
<td>31</td>
<td>Testis</td>
<td>BC008891 NM_080653</td>
<td></td>
<td>251</td>
</tr>
<tr>
<td>ATP6V1EL1* V(_{1}) subunit E-like 1</td>
<td>2p23</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP6V1F V(_{1}) subunit F</td>
<td>7q33.1</td>
<td>13</td>
<td>Ubiquitous</td>
<td>AP038654 NM_004888</td>
<td></td>
<td>337, 471</td>
</tr>
<tr>
<td>ATP6V1G1 V(_{1}) subunit G1</td>
<td>9p21.3</td>
<td>13</td>
<td>Ubiquitous</td>
<td>Y14768 NM_138282</td>
<td></td>
<td>120, 387, 471</td>
</tr>
<tr>
<td>ATP6V1G2 V(_{1}) subunit G2</td>
<td>6p21.3</td>
<td>13</td>
<td>Brain</td>
<td>Y14768 NM_138282</td>
<td></td>
<td>120, 387, 471</td>
</tr>
<tr>
<td>ATP6V1G3 V(_{1}) subunit G3</td>
<td>1q22.2</td>
<td>13</td>
<td>Kidney</td>
<td>AF132945 NM_015941</td>
<td></td>
<td>293, 594</td>
</tr>
<tr>
<td>ATP6V1H V(_{1}) subunit H</td>
<td>8p22-q23.3</td>
<td>50/57</td>
<td>Ubiquitous</td>
<td>AF132945 NM_015941</td>
<td></td>
<td>293, 594</td>
</tr>
<tr>
<td>ATP6V0A1 V(_{0}) subunit a1</td>
<td>17q21</td>
<td>116</td>
<td>Ubiquitous</td>
<td>U73006 NM_065177</td>
<td></td>
<td>71, 412</td>
</tr>
<tr>
<td>ATP6V0A2 V(_{0}) subunit a2</td>
<td>12q24.31</td>
<td>116</td>
<td>Lung, kidney, spleen</td>
<td>AF112672 NM_012463</td>
<td></td>
<td>413</td>
</tr>
<tr>
<td>ATP6V0A3 V(_{0}) subunit a3</td>
<td>11q13.4-13.5</td>
<td>116</td>
<td>Osteoclasts</td>
<td>AF025374 NM_000619</td>
<td></td>
<td>181</td>
</tr>
<tr>
<td>ATP6V0A4 V(_{0}) subunit a4</td>
<td>7q33.34</td>
<td>116</td>
<td>Kidney, epididymis, inner ear</td>
<td>AF245517 NM_130841</td>
<td></td>
<td>474</td>
</tr>
<tr>
<td>ATP6V0B V(_{0}) subunit c'</td>
<td>1p32.3</td>
<td>21</td>
<td>Ubiquitous</td>
<td>NM_004047</td>
<td></td>
<td>393</td>
</tr>
<tr>
<td>ATP6V0C V(_{0}) subunit c</td>
<td>16p13.3</td>
<td>16</td>
<td>Ubiquitous</td>
<td>M62762 NM_001690</td>
<td></td>
<td>263</td>
</tr>
<tr>
<td>Vma1</td>
<td>V(_{0}) subunit c'</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP6V0D1 V(_{0}) subunit d1</td>
<td>16</td>
<td>38</td>
<td>Ubiquitous</td>
<td>X71490 NM_004691</td>
<td></td>
<td>471</td>
</tr>
<tr>
<td>ATP6V0D2 V(_{0}) subunit d2</td>
<td>8q21.13</td>
<td>38</td>
<td>Kidney, osteoclast, lung</td>
<td>AO075972 NM_152565</td>
<td></td>
<td>471</td>
</tr>
<tr>
<td>ATP6V0E V(_{0}) subunit e</td>
<td>5q35.2</td>
<td>9</td>
<td>Neuroendocrine tissues</td>
<td>Y12686 NM_003945</td>
<td></td>
<td>325</td>
</tr>
<tr>
<td>ATP6GPI (ATP6S1, Ac 45)</td>
<td>Interacting protein</td>
<td>45</td>
<td>Neuroendocrine tissues</td>
<td>D16469 NM_001183</td>
<td></td>
<td>244, 500</td>
</tr>
<tr>
<td>ATP6GPI (ATP6S2)</td>
<td>Interacting protein</td>
<td>Xq28</td>
<td>45</td>
<td>Neuroendocrine tissues</td>
<td>D16469 NM_001183</td>
<td></td>
</tr>
</tbody>
</table>

The names of the respective subunits are given according to the recently revised HUGO (Human Genome Organization) nomenclature (http://www.gene.ucl.ac.uk/nomenclature/) (473). ATP6 designates all subunits of the vacuolar H\(^{+}\)-ATPase as a subfamily of the superfamily of ATPases. V\(_{1}\) delineates subunits belonging to the peripheral, catalytic V\(_{1}\) domain, whereas V\(_{0}\) subunits belong to the membrane-bound V\(_{0}\) domain. * The ATP6V1EL1 subunit is found in the human genome database; however, the tissue distribution and function of this putative subunit have not been reported to date. The yeast (S. cerevisiae) protein Vma11 (V\(_{0}\) subunit C) is part of the proton-transferring proton channel. It is, however, unclear at present if a mammalian homolog exists (471). An alignment of the Vma11 gene or protein with the human genome does not produce any significantly homologous sequences.
proteolipid ATP6V0C (c) subunit in mouse leads to early embryonic lethality due to impaired nidation of the embryo (254). Proton translocation through the proton channel is potently inhibited in the nanomolar range by the macrolide antibiotics baflomycin A1 and concanamycin (59, 141). These selective vacuolar H⁺-ATPase inhibitors may act through interactions with the proteolipid c' and c'' subunits (58, 122, 248) as well as with the 100-kDa a subunit (590).

The a subunit (ATP6V0A1–4) of the V₀ domain has recently received special attention due to the fact that four isoforms of this subunit exist and that at least two human diseases are caused by mutations in two of these subunit isoforms (ATP6V0A3 and ATP6V0A4) (see below). In renal physiology, ATP6V0A4 (a4) is most interesting as this subunit isoform is only found in kidney and epididymis (472, 474). The ATP6V0A4 protein shares 61% amino acid identity with ATP6V0A1, which appears to be a widely transcribed a subunit isoform in mammals (412). The ATP6V0A4 protein also shares 47% identity with ATP6V0A3 (a3, also previously identified as OC116 or TCIRG1 on chromosome 11). Mutations in the ATP6V0A3 gene underlie one type of infantile malignant autosomal recessive osteopetrosis (181). ATP6V0A4 shares 40% amino acid identity with a subunit homologs as evolutionary distant as Neurospora and yeast, indicating the biological importance of this gene family (472).

Topological studies of yeast a subunit (Vph1p) of V-ATPase employing cysteine mutagenesis, labeling with membrane-permeant and -impermeant maleimides as well as using protease cleavage, led to an original topological model containing nine transmembrane domains in this subunit (305). Similarly, the alignment of mouse a1, a2, and a3 subunits with yeast Vph1p also predicted nine transmembrane domains (391). According to this model (272, 392) the amino-terminal domain is located on the cytoplasmic side of the membrane, while the carboxy terminus is located on the luminal side of the membrane. Hydropathy analysis of mouse a1, a2, a3, and a4 subunit isoforms performed in a different laboratory also predicted nine transmembrane domains in these subunits (397, 512). In contrast, another hydropathy analysis of the same mouse a1, a2, a3, and a4 isoform subunits as well as a human member (ATP6V0A4) of the a subunit family predicted six transmembrane domains (472, 494). According to this latter model, both amino-terminal and carboxy-terminal tails should be located on the cytoplasmic side of the membrane. Thus the transmembrane topology of the a subunits of the vacuolar H⁺-ATPase is still controversial, and additional structural/crystallography studies are needed to clarify this issue.

ATP6V0A4 contains several potential glycosylation sites, which were suggested to account for the difference between the 96-kDa predicted molecular mass of this subunit and the 116-kDa mass observed for previously reported a subunit homologs. However, analysis failed to detect either a signal sequence or a consensus sequence for polarized targeting to the lysosomal membrane or other organelles (472). In yeast and osteoclasts, pumps containing different a subunit isoforms localize to different intracellular organelles or reside in the cell membrane (271, 511). In addition, the coupling efficiency between ATP hydrolysis and proton pumping differed in pumps containing different a isoforms (271).

3. The stalk

The A and B subunits are connected to the V₀ domain through several subunits termed the "stalk," which also belongs to the V₁ domain. Homology modeling, using again the mitochondrial F₁F₀-ATPase, and cysteine cross-linking experiments in yeast, suggest that subunits G and E form part of the peripheral stalk connecting V₁ and V₀, whereas subunit D seems to localize to the central stalk (16). Further evidence from the crystal structure of the subunit C of Thermus thermophilus V-ATPase, homologous to eukaryotic subunit D of vacuolar H⁺-ATPases, suggests that this subunit belongs to the stalk and may help to attach the central stalk to the V₀ domain (257).

Direct protein-protein interactions have been demonstrated for the E and H subunits in which deletion of the carboxy terminus of the E subunit decreases ATP hydrolysis and proton transport activity (323). In addition, at least the amino terminus of the a subunit (Vph1 in yeast) which otherwise belongs to the V₀ domain also interacts with the A and H subunits forming a "stator"-like structure. Mutations introduced into the amino terminus of the yeast a subunit abolished proton translocation but not ATP hydrolysis, suggesting that the statorlike structure may be important for coupling ATP hydrolysis to proton translocation (294, 295).

On the basis of experiments using immobilized F₁ or V₁ sectors tagged with a bead on the stalk, rotations in both the mitochondrial F₁F₀-ATPase and a bacterial V₁-ATPase were observed (212, 252, 583). Similarly, tagging of the a subunit as part of the proton translocating pathway of the yeast vacuolar H⁺-ATPase showed counterclockwise rotations of the a subunit relative to the stalk G subunit (240, 586). Thus it is thought that ATP binding to the B subunit and subsequent hydrolysis leads to a rotation of the central stalk structure relative to the AₜBₜ domain (212, 583). This conformational change may then induce the motion of the ring structure formed by the c and a subunits, thus inducing the transfer of protons across the membrane (212, 384).

In addition to true vacuolar H⁺-ATPase subunits, associated proteins have been identified. Some of these proteins are expressed in a highly tissue-specific manner, suggesting that these proteins may be involved in tissue-specific functions of the vacuolar H⁺-ATPase or may be
involved in the targeting of the pump to specific intracellular structures (244, 500). Targeted disruption of the accessory Ac45 protein in mouse led to early developmental death of the blastocysts (450). In yeast, four homologous Vtc proteins (vacuolar transporter chaperones) have been identified associating with SNARE proteins and the V₀ domain of the vacuolar H⁺-ATPase. The function of these proteins is not fully understood yet; however, deletion of these proteins produces yeast strains with defects in vacuolar membrane fusion and H⁺-ATPase mediated acidification (369).

C. “Kidney-Specific” Subunits of the Vacular H⁺-ATPase

Of the identified subunits of the vacular H⁺-ATPase there are some isoforms that have been described in a limited number of tissues: these include the B₁ isoform (ATP6V1B1), the a₄ isoform (ATP6V0A4), the G₃ isoform (ATP6V1G3), the C₂ (ATP6V1C2), and the d₂ isoform (ATP6V0D2) (80, 169, 386, 471, 472, 499). All of these subunit isoforms appear to be expressed in the kidney, (ATP6V0D2) (80, 169, 386, 471, 472, 499). All of these subunit isoforms appear to be expressed in the kidney, particularly in intercalated cells (498). The B₁ isoform is part of the peripheral V₁ domain, and its yeast homolog is important for ATP binding before ATP hydrolysis by the A subunit (384). As mentioned above, two isoforms of the B subunit exist: the B₂ isoform (ATP6V1B2) is ubiquitously expressed (49, 524), whereas in the kidney the B₁ subunit is amplified in intercalated cells of the late distal tubule, connecting segment and cortical and medullary collecting duct (386), in narrow and clear cells of the epididymis (68, 80, 169), in the ciliary body of the eye (245, 558), and in the inner ear (interdental cell layer of the spiral limbus and some epithelial cells of the endolymphatic sac) (268). The a₄ subunit (ATP6V0A4) is one of four isoforms of the a subunit which forms part of the membrane-bound V₀ sector. All four a subunits are expressed in the kidney as detected by Northern blot and RT-PCR analysis (397, 472, 512). The a₄ isoform, however, is only expressed in narrow and clear cells of the epididymis (C. Pietremont, M. Futai, and S. Breton, unpublished observations; Ref. 472) the inner ear (492), and the kidney (proximal tubule, loop of Henle, and all subtypes of intercalated cells along the late distal tubule, connecting segment, and entire collecting duct) (472, 482). The distribution of the other a isoforms varies widely between tissues (347, 391, 512), but all isoforms are also expressed in the kidney with distribution patterns associated with various regions of the nephron in a differential manner (A. Hurtado-Lorenzo, D. Brown, M. Futai, and V. Marshansky, unpublished observations). Mutations in the a₃ isoform (ATP6V0A3 or TCIRG1) have been identified in a severe form of infantile osteopetrosis, a disease of the osteoclasts (181).

Mutations in two “kidney-specific” subunit isoforms, B₁ (ATP6V1B1) and a₄ (ATP6V0A4), have been identified in patients with inherited forms of distal renal tubular acidosis with and without sensorineural deafness (see below).

D. General Functional Properties of Vacular H⁺-ATPase

The general functional properties, such as pH dependence and ratio of ATP-hydrolysis to H⁺ pumping, have been examined mainly in yeast, turtle urinary bladder preparations, or membrane vesicles obtained from brain or kidney. Some information was also derived from other organisms such as plant vacuolar H⁺-ATPases showing a high degree of functional similarities.

As discussed in more detail in section v, the movement of H⁺ across membranes results in a net charge translocation unless the movement of other ions electrically compensates, and thus facilitates further H⁺ pumping. In addition to this electrical gradient and similar to any other membrane transport system, the chemical gradient for H⁺ and the pH difference across the membrane also affect the pumping rate of vacuolar H⁺-ATPases. In turtle bladder, a sigmoidal relationship between H⁺-transporting rate and H⁺ gradient was observed between a normal intracellular pH (~7.4) and extracellular pH from 4.5 to 7.0 (the rate being 0 below pH 4.5 and reaching saturation between pH 7.0 and 8.0) (14). Similarly, changes in the electrical gradient (i.e., an imposed voltage) resulted also in a sigmoidal curve for H⁺ transport in the range of a lumen-positive potential (relative to the serosa) of +120 mV (being zero) to −80 mV (being saturated between 0 and −30 mV) (14).

In addition to this intrinsic dependency on the transmembrane electrochemical gradient, the transport rate can also be modulated by alterations in the rate of ATP hydrolysis and its coupling to H⁺ translocation. A recent detailed analysis of current-voltage relationships in the absence and presence of several ions, ATP or ADP and imposing different pH gradients, described different coupling ratios for vacuolar H⁺-ATPases from yeast. In the presence of large pH gradients (4 pH units), the approximate ratio was 2 H⁺/ATP and increased to more than 4 H⁺/ATP for small or no pH gradients (273). Similar findings have also been reported from plant vacuolar H⁺-ATPases (125). Experiments on brain vesicular H⁺-ATPase suggested that the coupling efficiency can be modulated by the presence of Ca²⁺ or Mg²⁺ but with differential mechanisms. The effect of Ca²⁺ was dependent on the membrane potential, whereas Mg²⁺ supported vacuolar H⁺-ATPase activity independent from the voltage (121). Another factor influencing the coupling ratio of yeast vacuolar H⁺-ATPase is the availability of glucose (530), a fact that may be related to recent findings of interaction between vacuolar H⁺-ATPase subunits and
glomerular enzymes (see sect. VII). Even though the underlying molecular mechanism for these variable stoichiometries is unknown at present, some observations point to the involvement of at least two subunits. Changes in the coupling of ATP hydrolysis and H⁺ transport have been observed in some mutants of the V1 subunit of the V1/V1001 sector as well as in different isoforms of the a subunit in the V1/V1001 sector. As described above, ATP hydrolysis is mainly mediated by sites on the a subunit, and mutants have been identified in yeast vacuolar H⁺-ATPase that alter the efficiency of coupling between ATP hydrolysis and H⁺ translocation (461). Similarly, different coupling stoichiometries have been observed in yeast vacuolar H⁺-ATPase expressing different isoforms of the a subunit yeast homolog (271).

A complete uncoupling of ATP hydrolysis and H⁺ transport has been observed after application of azide, which inhibits proton transport without affecting ATP hydrolysis (528).

III. DISTRIBUTION OF THE VACUOLAR H⁺-ATPase IN THE KIDNEY

As discussed below, one of the major functions of the kidney is to maintain a constant plasma bicarbonate concentration by reabsorbing filtered bicarbonate and by regenerating bicarbonate that is consumed in buffering acids generated by metabolism. The vacuolar H⁺-ATPase is one of several acid-base regulating proteins that are involved in this process (222). While the vacuolar H⁺-ATPase is generally considered to be an “intracellular” protein, studies over the past several years have clearly shown that it is inserted into the plasma membrane of many cell types in the kidney and other tissues, where it plays a key role in different physiological processes including bicarbonate reabsorption, sperm maturation and storage, bone reabsorption, and hearing.

A. Proximal Tubule

Immunocytochemical observations using specific antibodies raised against various subunits of the vacuolar H⁺-ATPase, as well as numerous functional studies on intact tubules and isolated brush-border membrane vesicles, have shown that a bafilomycin- and N-ethylmaleimide (NEM)-sensitive vacuolar H⁺-ATPase is located in the apical membrane and in intracellular organelles of proximal tubule epithelial cells (146, 173, 191, 238, 260, 439, 440, 442, 543, 544). The 31-kDa E2 subunit of the vacuolar H⁺-ATPase is present in rat proximal tubule segments along with the “brain” or B2 (ATP6V1B2) isoform of the 56-kDa subunit. The “kidney” or B1 (ATP6V1B1) isoform is amplified in collecting duct intercalated cells (386) (see below). The B2 subunit isoform of the vacuolar H⁺-ATPase has a truncated carboxy-terminal domain that lacks the PDZ-binding domain found in the B1 (intercalated cell) isoform (386, 421). This subunit does not interact with the PDZ protein, NHERF1 in proximal tubules, and the localization of these proteins in the apical region of proximal tubule epithelial cells is distinct (70). While NHERF1 is expressed mainly in the brush-border microvilli (where it colocalizes with NHE-3), the vacuolar H⁺-ATPase is found in the submicrovillar region (Fig. 2, A and B). The functional relevance of expressing the B2 56-kDa isoform in proximal tubules is unknown, but it may allow the vacuolar H⁺-ATPase to function and recycle without interference from the complex PDZ and PDZ-binding protein interactions that would otherwise occur in this region of the cell.

At the light microscope level, the proximal tubule vacuolar H⁺-ATPase localizes at the base of the brush border, which is the membrane domain showing a high level of clathrin-mediated endocytotic activity (430). The brush-border microvilli are also labeled to a variable extent, depending on the precise tubule segment examined and on the antibodies used for immunostaining (79, 232).

High-resolution immunogold labeling revealed that the proton pumps are not concentrated in the clathrin-coated domains of the apical membrane but are located at the neck of the apical invaginations, between clathrin-coated domains and the microvilli (79) (Fig. 2C). However, endosomes isolated from renal cortex, and which are derived mainly from proximal tubules, show a vigorous ATP-dependent acidification in vitro (Fig. 3). This acidification is inhibited by bafilomycin and confirms the role of the vacuolar H⁺-ATPase in acidifying proximal tubule endosomes. This acidification process is of critical importance in proximal tubule function, as will be discussed below.

B. Loop of Henle

As for the proximal tubule, proton and bicarbonate transport in the thick ascending limb of Henle’s loop are mediated by the vacuolar H⁺-ATPase, along with other transporters including the Na⁺/H⁺ exchanger NHE-3 (96, 554). Both the electroneutral NBC(N)1 (538) and electrogenic NBC4 (577) are expressed in the thick ascending limb (TAL). The Cl⁻/HCO₃⁻ exchanger AE2 has also been localized in the basolateral membrane of the TAL in rat and mouse kidney (12, 493) and might provide a potential route for bicarbonate reabsorption in this membrane domain.

The vacuolar H⁺-ATPase in this segment is located in numerous cytoplasmic vesicles, which are concentrated at the apical pole of the cell (Fig. 4A). Immunogold electron microscopy clearly shows that some vacuolar H⁺-ATPase is in fact in the apical plasma membrane, and some is associated with subapical vesicles (79). Further-
more, freeze-fracture studies have shown that the apical membrane of thick ascending limb cells contains clusters of rod-shaped intramembrane particles (Fig. 4, B and C), which are associated with the presence of the vacuolar H⁺-ATPase in some other cell types, such as intercalated cells (73). Subunit-specific antibodies show that the B2 isoform of the vacuolar H⁺-ATPase is expressed in the TAL (unpublished data). So far, the physiological regulation of vacuolar H⁺-ATPase recycling in the TAL has not been demonstrated.

In the thin limbs of Henle, plasma membrane vacuolar H⁺-ATPase was detected in the initial portion of the thin descending limb, immediately after the S3 segment of the proximal tubule (79). Both apical and basolateral membranes were labeled. A baflomycin-sensitive vacuolar H⁺-ATPase has also been detected by functional measurements in this initial segment (407). No other segments of long or short thin limbs have been reported to contain the vacuolar H⁺-ATPase.

C. Distal Tubule

In normal animals, bicarbonate reabsorption in this segment is negligible, but it can be increased by as much as fivefold in acidotic animals (307). By immunostaining, the vacuolar H⁺-ATPase (containing the B1 56-kDa subunit) is present at relatively high levels on the apical plasma membrane of distal convoluted tubule (DCT) cells, where it forms a very sharp line at the level of the membrane (79). Relatively little staining of intracellular vesicles is seen in the DCT, and the capacity of the vacuolar H⁺-ATPase to recycle in this segment is unknown. The late DCT also expresses apical H⁺-ATPase in “DCT” cells, but in addition, intercalated cells with very high H⁺-ATPase expression make their first appearance in this tubule segment.

D. Connecting Segment

The connecting segment joins the DCT with the cortical collecting duct. In rabbits, this segment forms long arcades that are easily distinguished, whereas in other species such as rodents it is shorter and less distinct. Specific connecting tubule cells can be distinguished by their high content of calbindin 28 in the rat (316, 435), which is expressed at lower levels in the DCT and the collecting duct. Intercalated cells are present in the connecting segment, where they show the range of phenotypes described in more detail below for the cortical collecting duct. However, connecting segments tend to have a greater percentage of B-cells and more of the subclass of intercalated cells that have apical vacuolar H⁺-ATPase but no basolateral AE1 (278). In addition, connecting tubule cells have a distinct apical band of vacuolar H⁺-ATPase staining similar to the staining of DCT cells (79).

FIG. 2. A: proximal tubule stained for the H⁺-ATPase (green) and NHERF-1 (red). NHERF-1 is located in the brush-border microvilli, and the H⁺-ATPase is present in sub-microvillar vesicles and invaginations. The two proteins are not colocalized. Bar = 10 \( \mu \)m. B: a differential interference contrast image is shown for orientation purposes. C: localization of the E subunit of the H⁺-ATPase in a proximal tubule cell by immunogold electron microscopy. H⁺-ATPase-associated gold particles are concentrated at the base of the brush-border microvilli (small arrows) and are excluded from the clathrin-coated pit domain. The clathrin coat appears as an electron-lucent band on the cytoplasmic side of the membrane invaginations (arrowheads). Bar = 0.5 \( \mu \)m. [Modified from Breton et al. (70) and Brown et al. (79).]
lomycin (ATP, inhibitor bafilomycin V-ATPase cation occurs when ATP is added in the presence the specific inhibitor bafilomycin (ATP, +Baf). After the generation of an acidic lumen, the endosomal pH can be alkalinized by the subsequent addition of bafilomycin (Baf), which inhibits further proton translocation into the endosomes. Protons exit the endosomes into the bulk bath solution by passive proton leakage across the endosomal membrane (this experiment was performed in a sodium-free solution, thus excluding the contribution of a Na⁺/H⁺ exchanger to the observed alkalinization). This alkalinization can be further enhanced by the V-ATPase inhibitor CCCP and by the proton ionophore nigericin (Nig), which allows complete pH equilibration with the bath solution to occur. (From V. Marschansky, unpublished results.)

FIG. 3. Acridine orange fluorescence quenching can be used to measure the functional acidification capacity of endosomes isolated from rat kidney cortex. In the presence of ATP alone (ATP, −Baf), the rapid fluorescence quenching reflects V-ATPase-dependent proton translocation into the endosomal lumen. No detectable intraluminal acidification occurs when ATP is added in the presence of the specific V-ATPase inhibitor bafilomycin (ATP, +Baf). After the generation of an acidic lumen, the endosomal pH can be alkalinized by the subsequent addition of bafilomycin (Baf), which inhibits further proton translocation into the endosomes. Protons exit the endosomes into the bulk bath solution by passive proton leakage across the endosomal membrane (this experiment was performed in a sodium-free solution, thus excluding the contribution of a Na⁺/H⁺ exchanger to the observed alkalinization). This alkalinization can be further enhanced by the V-ATPase inhibitor CCCP and by the proton ionophore nigericin (Nig), which allows complete pH equilibration with the bath solution to occur. (From V. Marschansky, unpublished results.)

E. Collecting Duct

Intercalated cells of the collecting duct express the highest levels of vacuolar H⁺-ATPases among all acid-base transporting cells in the kidney (Fig. 5). The 56-kDa B1 subunit of the vacuolar H⁺-ATPase, which contains a carboxy-terminal PDZ-binding motif (DTAL) that interacts with NHERF1, is expressed in intercalated cells (70). Other subunits including subunits A, B2, E2, G1, G3, a4, and d1 are also expressed in kidney intercalated cells (78, 79, 411, 498, 499). The classification of intercalated cells grows more complex as more is learned about their functional properties. In the initial study showing the cellular distribution of the vacuolar H⁺-ATPase in different intercalated cells, it was recognized that (in addition to the staining of numerous cytoplasmic vesicles in most intercalated cells), A-cells have apical vacuolar H⁺-ATPase, B-cells have basolateral vacuolar H⁺-ATPase, but many cells have a diffuse or even a bipolar vacuolar H⁺-ATPase distribution (34, 78). In the cortical collecting duct, all subtypes of intercalated cells (IC) are detectable (Fig. 5A). In the outer stripe of the outer medulla, A-cells predominate, but a few residual B-cells can be found. In the inner stripe of the outer medulla, only A-IC are present, and they represent ~40% of the epithelial cell population of the collecting duct. In the inner medulla, the epithelium initially contains between 5 and 10% A-IC, and these cells disappear from the epithelium in the middle and terminal portions of the inner medullary collecting duct (79, 114).

A-IC can be distinguished from B-IC by the presence of the anion exchanger AE-1 (band 3) on their basolateral plasma membrane (Fig. 5A). This protein is undetectable in typical B-IC (11). Subsequent studies showed that not only were cells with discrete basolateral vacuolar H⁺-ATPase staining AE-1-negative, but that those with a more diffuse or bipolar staining pattern were also AE-1 negative (11, 34). In addition, a few cells with discrete apical vacuolar H⁺-ATPase localization (which would otherwise have been classified as A-cells) were also AE-1 negative (11). Thus intercalated cells have been identified with unique apical staining, and unique basolateral staining, as well as combinations of both apical and basolateral staining (34). While tight (plasma membrane) or diffuse (cytoplasmic vesicles) apical vacuolar H⁺-ATPase staining is always found in cells that have basolateral AE-1, some cortical collecting duct cells with apical vacuolar H⁺-ATPase have no detectable AE-1. Al-Awqati and colleagues (455) were the first to suggest that these cell types might be interconvertible due to plasticity of epithelial cell polarity. The presence of the vacuolar H⁺-ATPase on numerous intracellular vesicles that can shuttle the protein to and from the plasma membrane supports this idea. The complex relationship among these different intercalated cell phenotypes has subsequently been explored in many studies that will be discussed in more detail below. Finally, it should be noted that the relative proportions of the different phenotypic intercalated cell variants found on the cortical region differ among species (278) and may be related to factors including diet.

The “kidney” 56-kDa B1 (ATP6V1B1) subunit is also present at lower levels in principal cells, where it is associated with endosomes that contain aquaporin (AQP)-2 water channels (447). Since these endosomes do not acidify their lumen, it was proposed that this vacuolar H⁺-ATPase subunit might be involved in the recycling of AQP-2 water channels in a way that is independent of its proton pumping activity (218).

F. Distribution of the Vacuolar H⁺-ATPase in Other Proton-Transporting Cells

The vacuolar H⁺-ATPase plays a pivotal role in various transporting epithelia that are not directly in-
involved in systemic acid-base balance. This pump can also act as an energizer of plasma membranes, particularly the apical membranes of epithelial cells, by imposing proton electrochemical gradients across the membrane, which provides the driving force for a variety of ion transport processes (566). Thus the vacuolar H⁺-ATPase is found on the plasma membranes of a variety of cell types in addition to those described above in the kidney.

1. Epididymis/vas deferens

The epididymis and vas deferens of the male reproductive tract contain a subpopulation of epithelial cells that express very high levels of the vacuolar H⁺-ATPase on their apical membrane and intracellular vesicles (68, 80). Numerous subunits of the H⁺-ATPase, including the B₁, E₁, A (70 kD), and a₄ subunits, are expressed in these epithelial cells (69, 80, 169; Breton et al., unpublished data.)

FIG. 4. A: immunofluorescence localization of the E₁ subunit of the H⁺-ATPase in thick ascending limbs of Henle. A bright staining is seen in the apical membrane and subapical vesicles. Bar = 10 μm. B: freeze-fracture electron micrograph of the basolateral (BL) and apical (Ap) membrane of cells from the thick ascending limb of Henle. The localization of mitochondria within the basolateral membrane invaginations is characteristic of this kidney segment. The lipid bilayer of the apical membrane contains typical rod-shaped intramembranous particles (IMPs). Bar = 0.5 μm. C: higher magnification image showing the apical membrane subdomains containing clusters of rod-shaped IMPs. These rod-shaped IMPs are thought to represent part of the V₀ sector of the H⁺-ATPase. Bar = 80 nm. (From D. Brown and S. Breton, unpublished data.)
Mutations of the B1 subunit have been related to sensorineural deafness (268) probably due to alkalinization of the endolymph and subsequent impairment of the contractile response of hair cells to mechanostimuli. These results support the notion that active acidification of the endolymph by the vacuolar H⁺-ATPase is essential for adequate auditory function.

3. Osteoclasts

Osteoclasts are specialized macrophages that are involved in bone remodeling. Osteoclasts attach themselves to bone matrix and reabsorb mineralized bone by creating an acidic environment between their bone-facing apical plasma membrane and the bone surface. Their apical membrane “ruffled border” contains high levels of the vacuolar H⁺-ATPase, which works in parallel with a “basolateral” Cl⁻/HCO₃⁻ exchanger AE1 (35). The vacuolar H⁺-ATPase 56-kDa subunit expressed in osteoclasts is the “brain B2” isoform (298), whereas the 116-kDa (a) subunit isoform is the a3 (ATP6V0A3). Mutations in the gene encoding for the a3 subunit have been identified in patients suffering from infantile malignant osteopetrosis, a disease where the bone marrow cavity is not formed due to impaired osteoclast activity (181). The vacuolar H⁺-ATPase is, therefore, a major player in modulating bone resorption and might be a suitable target for therapeutic intervention for osteoporosis and other skeletal diseases.

G. Electron Microscopy of the Vacuolar H⁺-ATPase

As described above, the vacuolar H⁺-ATPase is a complex, multisubunit protein that comprises a transmembrane Vₒ sector and a much larger cytoplasmic V₁ sector. Because of its large size, the vacuolar H⁺-ATPase can be visualized by electron microscopy using several techniques.

1. Conventional electron microscopy

Electron microscopic studies on ion-transporting epithelia in insects revealed a dense array of 10-nm studlike projections associated with some plasma membrane domains (217). These structures, named “portasomes” (227), closely resembled the mitochondrial F₁Fₒ-ATPase (102). Similar structures were identified on the plasma membranes of proton transporting epithelial cells of the turtle urinary bladder (484, 485) and of kidney intercalated cells (83, 331, 333, 378, 486), where they coated not only the plasma membrane (Fig. 6, A and C) but also the cytoplasmic surface of many intracellular vesicles. These 10-nm projections were identified as the Vₒ sector of the vacuolar H⁺-ATPase by direct immunogold labeling of intercalated cells and help maintain a low luminal pH and low bicarbonate concentration that are critical for sperm cell maturation and storage in the epididymis. Sperm motility is triggered, during ejaculation, by neutralization of the epididymal fluid by the prostatic and seminal vesicle fluid.

2. Inner ear

In the inner ear, immunocytochemical data showed that epithelial cells lining the endolymphatic sac and interdental cells of the cochlea express subunits B1 and E1 of the proton pump on their apical membrane (138, 481).
lated cell plasma membranes with specific antivacuolar H^+-ATPase subunit antibodies (Fig. 6B) (77).

2. Rapid-freeze, deep-etch electron microscopy

This membrane-coating material was identified as the V1 sector of the vacuolar H^+-ATPase complex by rapid-freeze, deep-etch electron microscopy. The underside of fragments of apical plasma membrane from toad urinary bladder epithelium was examined following rapid-freezing and rotary shadowing with platinum and carbon (77). Figure 6D shows the underside of the plasma membrane from a proton-secreting mitochondria-rich (MR) cell. The paracrystalline arrays of densely packed, 10-nm-diameter projections were also found on the cytoplasmic surface of vesicles inside these cells. Identical structures were seen when the purified enzyme was incorporated into liposomes and examined by the rapid-freeze, deep-etch procedure, confirming their identity as the vacuolar H^+-ATPase (77). The vacuolar H^+-ATPase complex on the contractile vacuole of Paramecium was also examined by the rapid-freeze, deep-etch technique, and similar studs (referred to as “pegs”) were detected (115, 237). Coupled with immunocytochemical labeling at the light and electron microscopic level with specific antivacuolar H^+-ATPase subunit antibodies, this work provided direct evidence that the portasomes originally observed in insect cells were composed of the cytoplasmic V1 sectors of the vacuolar H^+-ATPase.

FIG. 6. A: electron micrograph of the apical region of a kidney collecting duct intercalated cell. The underside of the plasma membrane is decorated with an array of electron-dense projections, or studs, which measure ~20 nm in length (arrows). These studs represent the V1 sectors of the H^+-ATPase. Bar = 60 nm. B: localization of the H^+-ATPase by immunogold electron microscopy in a single intercalated cell apical microvillus. The submembrane electron-dense studs are less discernable under these conditions, but the gold particles are closely associated with this dense band on the cytoplasmic surface of the membrane (arrows). Bar = 40 nm. C: high-magnification image showing the H^+-ATPase-associated studs on the cytoplasmic side of the plasma membrane (arrows). Bar = 60 nm. D: apical plasma membrane of a proton-secreting mitochondria-rich cell from a toad urinary bladder visualized after rapid-freeze, deep-etch treatment. Each individual stud represents the V1 sector of a single H^+-ATPase molecule. The 10-nm-diameter structures form a tightly packed, paracrystalline array. This illustrates the dense clustering of H^+-ATPase molecules in these specialized membrane domains. Bar = 30 nm. [Modified from Brown et al. (77).]
3. Freeze-fracture electron microscopy and rod-shaped intramembranous particles

By freeze-fracture electron microscopy, the lipid bilayer of many cells that express high levels of the vacuolar H\(^+\)-ATPase contains characteristic, elongated, or rod-shaped intramembranous particles (IMPs) (73). These particles are usually assumed to represent the transmembrane V\(_0\) sector of the vacuolar H\(^+\)-ATPase (Fig. 4C). In some instances, an increase in the proton secretory activity of membranes has been associated with an increase in the number and density of rod-shaped IMPs in the plasma membrane (286, 486) or with the apparent activation of intercalated cells (486). However, while all membrane domains that contain rod-shaped IMPs also contain the vacuolar H\(^+\)-ATPase as detected by immunocytochemistry, some cells that have abundant membrane-associated vacuolar H\(^+\)-ATPase do not show rod-shaped IMPs when examined by the freeze-fracture technique. Renal proximal tubules, for example, have apical plasma membrane vacuolar H\(^+\)-ATPase but no rod-shaped IMPs (401). Furthermore, H\(^+\)-ATPase-rich narrow cells in the proximal regions of the epididymis have many rod-shaped IMPs (81), whereas those in the distal epididymis, which are known as clear cells, have no detectable rod-shaped IMPs (82). The precise relationship between the characteristic rod-shaped IMPs and the vacuolar H\(^+\)-ATPase remains to be established but by analogy with the F\(_0\)F\(_1\) ATP synthase (92), it is possible that they represent dimeric structures comprised of two V\(_0\) domains (which form the rod-shaped IMP) and two V\(_1\) domains, which are visible on the cytoplasmic surface of the membrane as studs or portasomes.

IV. ROLE OF VACUOLAR H\(^+\)-ATPases IN KIDNEY ACID-BASE TRANSPORT

Overall body pH (acid-base) homeostasis is controlled mainly by the exhalation of CO\(_2\) and by the reabsorption, generation, or secretion of bicarbonate as well as the secretion of acid and acid equivalents by the kidneys (for review, see Ref. 222). About 180–200 liters of blood are filtered daily in the glomeruli of the kidneys; 99% of the filtered load has to be reabsorbed along the nephron to avoid excessive loss of solutes and fluid. This is achieved by the reabsorption of solutes (i.e., amino acids, phosphate, glucose), NaCl, bicarbonate, and water mediated by specialized transport systems.

A. Proximal Tubule

Bicarbonate reabsorption in the proximal tubule accounts for \(\sim 70–80\)\% of the filtered load and occurs mainly in the initial segments (222). It is mediated by the concerted action of several transport proteins and enzymes located on the apical and basolateral membranes (Fig. 7A). On the apical side two main transport systems mediate H\(^+\) secretion, the first step in bicarbonate reabsorption. The bulk of proton secretion involves Na\(^+\)/H\(^+\) exchange, and several isoforms of Na\(^+\)/H\(^+\) exchangers have been localized in the brush-border membrane of the proximal tubule, including NHE-2, NHE-3, and NHE-8 (209). About 50% of overall apical NHE activity may be mediated by NHE-3, the remainder by another isoform(s) (111). Genetic knock-out of NHE-2 has no effect on renal function, whereas complete or kidney-specific knock-out of NHE-3 results in a reduction of proximal tubular bicarbonate reabsorption (555) as well as Na\(^+\) and water loss (451, 573). Up to 40% of proximal tubule bicarbonate reabsorption is Na\(^+\) independent and is sensitive to the vacuolar H\(^+\)-ATPase inhibitor bafilomycin (103, 555), and it has thus been postulated to be mediated by vacuolar H\(^+\)-ATPases expressed in the brush-border membrane. The extent of Na\(^+\)-independent, bafilomycin-sensitive bicarbonate reabsorption varies among different species, being more extensive in carnivores than in herbivores (146). Indeed, vacuolar H\(^+\)-ATPases are strongly expressed in the proximal tubule (see below), and the activity has been measured as NEM-, bafilomycin-, or concanamycin-sensitive H\(^+\) extrusion in several proximal tubular preparations such as in brush-border membrane vesicles (101, 234, 260, 329, 446, 475), isolated proximal tubule fragments (146, 173, 276, 543, 544, 596), and isolated perfused proximal tubules (191, 334) and using in vivo microperfusion experiments (103, 555).

After secretion, H\(^+\) combines with the filtered HCO\(_3^-\) to produce H\(_2\)O and CO\(_2\), a process catalyzed by the membrane-bound carbonic anhydrase IV. CO\(_2\) then diffuses into the proximal tubule cells where it reacts with H\(_2\)O (catalyzed by the cytosolic carbonic anhydrase II isoform) to produce H\(^+\) and HCO\(_3^-\). The generated HCO\(_3^-\) is exported into blood via the basolateral electronegative Na\(^+\)/HCO\(_3^-\) exchanger (kNBC-1, SLC4A4), whereas the proton is recycled by the Na\(^+\)/H\(^+\) exchangers and vacuolar H\(^+\)-ATPase. As bicarbonate reabsorption in the proximal tubule is intricately linked to Na\(^+\) and water reabsorption and thus to the regulation of extracellular volume, the transport processes involved in bicarbonate reabsorption are tightly regulated by hormones and metabolic status as described in detail below (see sect. viii).

B. Loop of Henle and TAL

About 15–20% of the filtered bicarbonate is reabsorbed in the loop of Henle, mainly in the TAL involving Na\(^+\)/H\(^+\) exchange and NEM-sensitive vacuolar H\(^+\)-ATPase (56, 97, 98, 557). The main Na\(^+\)/H\(^+\) exchanger isoform is NHE-3, but NHE-2 may also play a role (98, 554). The reabsorbed HCO\(_3^-\) is excreted into blood possi-
The remaining 5% of the filtered bicarbonate is reabsorbed in the late distal tubule (also called DCT2) and connecting segment. The first intercalated cells are found in the DCT2 and express vacuolar H⁺-ATPases (51). In addition to the reabsorption of filtered bicarbonate, the kidney has to generate bicarbonate to buffer ~70 meq of acid produced by daily metabolism. The main role of type A intercalated cell is the net excretion of protons through apically localized vacuolar H⁺-ATPases where the H⁺ is produced by the cytosolic carbonic anhydrase II (Fig. 7B). The generated HCO₃⁻ is released into the blood by the basolateral kidney-specific isoform of the Cl⁻/HCO₃⁻ exchanger band 3/AE-1 (SLC4A1) (10, 72). In contrast, under conditions where bicarbonate has to be secreted such as in metabolic alkalosis, type B intercalated cells are activated (183, 333, 350, 351, 436, 542). This subtype of intercalated cells is mainly found in the connecting segment and cortical collecting duct (278, 289, 505). A few B-IC are also present in the initial portion of the outer medullary collecting duct. Type B intercalated cells secrete bicarbonate into the urine via an apically located Cl⁻/HCO₃⁻ exchanger, whereas basolaterally expressed vacuolar H⁺-ATPases extrude protons into the interstitium (Fig. 7C) (154, 155, 358). This apical Cl⁻/HCO₃⁻ exchanger is insensitive to DIDS in the presence of Cl⁻ but becomes sensitive in its absence (154). The molecular identity of this apical Cl⁻/HCO₃⁻ exchanger is presently under discussion. Two Cl⁻/HCO₃⁻ exchangers have been identified in type B intercalated cells: pendrin (282, 436, 550) and AE-4 (514). Pendrin resides in the apical membrane of all non-type A intercalated cells (282, 436, 550) and is regulated by acid-base status (542), and genetic loss of pendrin function (PDS knock-out mice) leads to reduction of bicarbonate secretion in the isolated perfused cortical col-

**FIG. 7.** Schematic model of transport processes involved in renal bicarbonate absorption and proton secretion. A: in the proximal tubule, protons are secreted via apical Na⁺/H⁺ exchangers and vacuolar H⁺-ATPases. The secreted H⁺ combine with filtered HCO₃⁻ under the influence of a membrane-bound carbonic anhydrase (CA IV) to form H₂O and CO₂. After diffusion into the cell, CO₂ is rehydrated by the cytosolic carbonic anhydrase II (CA II), the H⁺ secreted again, and the HCO₃⁻ released into the interstitium via the basolateral Na⁺/HCO₃⁻ cotransporter NBC1. B: type A intercalated cells are characterized by the expression of the basolateral Cl⁻/HCO₃⁻ exchanger AE-1 and the presence of an apical vacuolar H⁺-ATPase. H⁺ and HCO₃⁻ are formed by a cytosolic CA II and secreted into the lumen and the interstitium, respectively. Type A intercalated cells may also express an apical H⁺/K⁺-ATPase. C: type B intercalated cells are characterized by the absence of the AE-1 Cl⁻/HCO₃⁻ exchanger on the basolateral side and the presence of the vacuolar H⁺-ATPase which can be found on both sides of the cell. In addition, type B intercalated cells express an apical Cl⁻/HCO₃⁻ exchanger that may be represented by pendrin. As suggested by some authors, a third subtype of intercalated cells, non-A/non-B, is characterized by the apical expression of vacuolar H⁺-ATPases and the absence of AE-1.

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lecting ducts (436). Pendrin seems to have some sensitivity to DIDS (428, 476). AE-4 is DIDS insensitive and is also expressed in non-type A intercalated cells; however, its subcellular localization seems to be species specific (i.e., apical in rabbit, basolateral in mouse and rat) and its physiological role is, therefore, uncertain (285, 514).

As already mentioned, at least two subtypes of intercalated cells can be distinguished based on morphological and functional criteria. Acid-secreting type A intercalated cells express vacuolar H\(^+\)-ATPases on the apical side, extrude protons into the lumen, and are characterized by the basolateral expression of the kidney-specific truncated version of the band 3/AE1 (SLC4A1) Cl\(^-\)/HCO\(_3\)\(^-\) exchanger isoform (11). In contrast, type B intercalated cells secrete bicarbonate and express vacuolar H\(^+\)-ATPases on the basolateral side and a Cl\(^-\)/HCO\(_3\)\(^-\) exchanger (not identical to AE-1) on the apical pole (155). Some studies have attempted to distinguish a third subtype of intercalated cells, non-type A/B intercalated cells (also called \(\gamma\)-intercalated cells) which appear to express vacuolar H\(^+\)-ATPases and DIDS-insensitive Cl\(^-\)/HCO\(_3\)\(^-\) exchangers on both membranes (155, 278, 505). These cells would be capable of both bicarbonate or acid secretion. This subtype of cell has been found only in the connecting tubule and cortical collecting duct. Type A and B intercalated cells can be activated in response to several stimuli, and the potential conversion between both phenotypes is discussed below.

### D. Medullary Collecting Duct

The medullary collecting duct consists of several functionally and morphologically distinct segments. The outer medullary collecting duct is composed of two main cell types: intercalated cells mainly of the acid-secretory A type (11) (a few B-IC can be detected in the outer stripe, but not in the inner stripe) and principal cells involved in water, Na\(^+\), and K\(^+\) transport. Proton secretion is mediated by vacuolar H\(^+\)-ATPases as evident from in vitro perfusion studies of isolated outer medullary collecting ducts. Bicarbonate absorption in isolated perfused outer medullary collecting ducts showed Cl\(^-\) dependence from the bath side, which is explained by the presence of the basolateral Cl\(^-\)/HCO\(_3\)\(^-\) exchanger AE-1/band 3 (490). The apical step of bicarbonate reabsorption is not Cl\(^-\) dependent (see also sect. v). Proton secretion and bicarbonate absorption in perfused tubules is stimulated in animals with metabolic acidosis and reduced in animals with metabolic alkalosis (see also sect. viii). Similarly, the activity of the vacuolar H\(^+\)-ATPase in this segment is under the control of several hormones as will be discussed later.

An important feature of the medullary collecting duct is the use of so-called titratable acids, phosphate, citrate, and ammonia/ammonium to buffer excreted protons (223, 560). The buffering of protons is necessary to maintain a favorable proton gradient across the apical membrane to facilitate vacuolar H\(^+\)-ATPase activity. Without titratable acids, the proton gradient would exceed more than 3–4 pH units and reduce the efficiency of urinary acidification. Phosphate and citrate are freely filtered and mostly reabsorbed in the proximal tubule, and thus their availability is limited under normal conditions. In contrast, ammonia (NH\(_3\)) and ammonium (NH\(_4\)\(^+\)) are produced in the proximal tubule by the metabolism of glutamine and glutamate, which in turn generates newly formed bicarbonate ions (373). NH\(_4\)\(^+\) is secreted into urine via the Na\(^+\)/H\(^+\) exchanger (with NH\(_4\)\(^+\) substituting for H\(^+\)), and NH\(_4\)\(^+\) diffuses into the tubular lumen, where it is protonated to form NH\(_3\). Secreted NH\(_4\)\(^+\) is reabsorbed in the TAL limb via the Na\(^+\)/K\(^+\)/2Cl\(^-\) symporter NKCC2/BSC1 (NH\(_4\)\(^+\) substituting for K\(^+\)) and accumulates in the medullary interstitium. NH\(_3\) in is in chemical equilibrium with NH\(_3\) which can freely diffuse across the medullary collecting duct epithelium to reach the luminal side of the tubule. Luminal NH\(_3\) is protonated to NH\(_4\)\(^+\) following proton secretion by intercalated cells. NH\(_4\)\(^+\) is then trapped in the collecting duct lumen, because this segment is not permeant to NH\(_3\). Several transport pathways for NH\(_3\)/NH\(_4\)\(^+\) in the collecting duct have been suggested including the Na\(^+\)-K\(^+\)-ATPase (547, 548), the Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter NKCC1 (SLC12A2) (249), and recently members of the Rhesus surface antigen determining Rh protein family, RhGB and RhCB (151, 231, 313, 314, 341, 425, 532).

In addition to vacuolar H\(^+\)-ATPases, a H\(^+\)-K\(^+\)-ATPase has been found in the outer medullary collecting duct based on immunohistochemistry (163, 533, 570), and the inhibition of proton secretion by the SCH28080 compound, a selective inhibitor of H\(^+\)-K\(^+\)-ATPase activity (200, 569). It has, however, remained controversial to what extent the colonic, gastric, or both isoforms of H\(^+\)-K\(^+\)-ATPase underlie this activity as the use of pharmacological tools (ouabain, SCH28080, or omeprazole) and attempts to identify the molecular nature (immunohistochemistry, Northern, in situ hybridization) have produced partially conflicting data (95, 163, 164, 200, 291, 296, 374, 375, 551). It has, however, emerged that the contribution of H\(^+\)-K\(^+\)-ATPases to overall proton secretion in this segment and thus to bicarbonate reabsorption is minor and that H\(^+\)-K\(^+\)-ATPases may rather play an important role as a potassium scavenging pathway in systemic potassium depletion (20, 144, 199, 216). The role and regulation of renal H\(^+\)-K\(^+\)-ATPase activity have been reviewed recently (144, 258, 467, 571).

The inner medullary collecting duct can be divided into at least two separate segments: the initial portion and the terminal region which comprises approximately the last two-thirds of the IMCD (114, 330). Intercalated cells gradually disappear from the initial third of the IMCD, and only so-called “IMCD” cells (“principal cells” in the IMCD...
are sufficiently distinct from principal cells in the outer medulla to warrant a different name) are found in the terminal portion of this tubule segment. These cells express AQP-2 and are involved in the final concentration of urine. Under nonphysiological conditions, expression of intercalated cells and vacuolar H\(^+\)-ATPases may also occur in the later part of the IMCD as demonstrated in a knock-out mouse model for the AQP-1 water channel. Knock-out mouse models for the AQP-1 water channel were used to investigate the role of AQP-1 in water transport in various tubule segments, and their expression was increased, and IMCD cells were detected with strong apical expression of vacuolar H\(^+\)-ATPases (280).

**V. CHLORIDE DEPENDENCE OF VACUOLAR H\(^+\)-ATPASE FUNCTION**

Translocation of H\(^+\) across membranes by the vacuolar H\(^+\)-ATPase is electrogenic, rendering the cell interior negative with respect to the exterior. This process generates both a transmembrane potential in addition to a chemical gradient, resulting in a self-limiting activity of the vacuolar H\(^+\)-ATPase. Modelling of acidification rates reported from several intracellular organelles has confirmed the presence of base dependence of a chloridion conductance. In many intracellular organelles and at cell plasma membranes, a parallel Cl\(^-\) conductance provides an electrical shunt compensating for the positive charge transferred by the pump and thus dissipating the electrical gradient (345, 346, 355, 384, 385, 392).

The presence of this parallel Cl\(^-\) conductance has been found in most intracellular organelles that are acidified by vacuolar H\(^+\)-ATPases such as lysosomes (396), endosomes (24, 345, 346, 427, 439, 479, 580), the trans-Golgi network (TGN) (129, 574), and neurotransmitter storage vesicles (348, 487). In most cases, the presence of an anion conductance was inferred from the higher rate of acidification of the organelles in the presence of chloride, or the generation of a higher transmembrane potential in its absence. More recently, direct measurements of intracellular chloride during acidification alone or in parallel with monitoring intracellular acidification confirmed the direct relationship between both processes. An increase in endosomal chloride concentration was observed in parallel with endosomal pH acidification by ~1.5 pH units. Both processes were blocked by inhibition of vacuolar H\(^+\)-ATPase activity with baflomycin (479).

The chloride dependence of plasma membrane H\(^+\)-ATPase is, however, a much more complex issue because of the expression of numerous other conductances or electrogenic transporters in apical and basolateral membrane domains. In the kidney, it is beyond doubt that vacuolar H\(^+\)-ATPase-dependent acidification is Cl\(^-\) dependent in endosomal fractions (24, 238, 345, 346, 427, 439) and in brush-border membrane vesicles (270, 288, 343, 441, 442). However, the role of chloride in apical H\(^+\)-ATPase-dependent proton secretion has been examined in only a few studies.

In isolated rat proximal tubules, vacuolar H\(^+\)-ATPase-dependent H\(^+\) extrusion was reduced after pre-incubation in Cl\(^-\)-free media (596). In another study, angiotensin II was shown to stimulate H\(^+\)-ATPase-dependent proton extrusion, via a process involving a Cl\(^-\)-dependent insertion of vesicles into the brush-border membrane (543). In addition, in mouse proximal tubules, the insertion of vacuolar H\(^+\)-ATPase containing vesicles was delayed in the absence of chloride (334).

No studies on the Cl\(^-\) dependence of vacuolar H\(^+\)-ATPase activity have been reported for the thin loop of Henle and the TAL. In the late distal tubule, in vivo microperfusion experiments demonstrated a reduction of electrogenic H\(^+\) secretion by the Cl\(^-\) channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), indicating coupling between H\(^+\)-ATPase and Cl\(^-\) transport (166).

In isolated rabbit medullary collecting duct, bicarbonate reabsorption is independent of the presence of luminal chloride but is completely abolished in the absence of basolateral chloride, presumably due to impairment of peritubular bicarbonate exit in exchange for Cl\(^-\) via the Cl\(^-\)/HCO\(_3\) exchange AE-1 (490). This result indicated the involvement of basolateral Cl\(^-\) in net proton secretion measured in the presence of bicarbonate. However, further studies on mouse cortical collecting duct fragments incubated in the absence of bicarbonate clearly showed that vacuolar H\(^+\)-ATPase proton extrusion is independent of chloride (541). In contrast, proton extrusion in proximal tubules was Cl\(^-\) dependent and was inhibited by the Cl\(^-\) channel blocker NPPB (541).

These results demonstrate the complexity of the complete pathway for vacuolar H\(^+\)-ATPase-dependent proton secretion in a whole cell setting. Mathematical analysis of rat collecting duct acid/base transport has led to a proposed model involving either 1) basolateral Cl\(^-\) / HCO\(_3\) exchange via AE-1, coupled to chloride recycling across the membrane via a chloride conductance; or 2) electrogenic basolateral bicarbonate exit via a conductive pathway, possibly Cl\(^-\) channels (560). In support of this model, increasing evidence showing the absence of a chloride requirement for vacuolar H\(^+\)-ATPase-dependent proton secretion in nonrenal systems. In isolated vas deferens, baflomycin-sensitive proton secretion is independent of Cl\(^-\) but requires the enzymatic activity of carbonic anhydrase II (65). In the absence of chloride, the chloride channel inhibitor diphenylamine-2-carboxylate (DPC) markedly inhibited baflomycin-sensitive apical proton efflux, indicating that Cl\(^-\) channels...
might contribute to basolateral extrusion of HCO$_3^-$ under these conditions. In addition, vacuolar H$^+$-ATPase-mediated acidification of the secretory pathway in a pituitary cell line was independent of the presence of chloride (574). There is also evidence that, in the TGN, K$^+$ may serve, in addition to Cl$^-$, to maintain electroneutrality during proton transport (129).

The underlying mechanisms for the apparent differences in the requirement for chloride in the different nephron segments have remained elusive to date, and there is still some controversy as to the molecular identity of the associated Cl$^-$-conducting proteins for each segment even though several promising candidates have been identified, namely, ClC-5, AQP-6, and cystic fibrosis transmembrane conductance regulator (CFTR). An order of anion selectivity with I$^-$, NO$_3^-$, and SCN$^-$ over Cl$^-$ and Br$^-$ has been described (283, 288), suggestive of the involvement of members of the CIC Cl$^-$ channel family. Furthermore, a similar Cl$^-$ conductance was found in brush-border membranes prepared from CFTR knock-out mice, excluding CFTR as an important contributor to the observed Cl$^-$ conductance (283), despite earlier reports indicating a similar pharmacology and the presence of CFTR in brush-border membrane preparations (47). Furthermore, the endosomal Cl$^-$ conductance is strongly stimulated by activation of the cAMP/protein kinase A (PKA) pathway (24). These candidates will be discussed in more detail in the following sections.

A. ClC-5 (and ClC-4)

ClC-5 belongs to the superfamily of voltage-gated Cl$^-$ channels and is highly abundant in kidney (for review, see Refs. 261, 545). Immunolocalization studies found a predominantly intracellular expression in the proximal tubule colocalizing with the vacuolar H$^+$-ATPase in the apical compartment (131, 214, 326, 448). Furthermore, expression has been reported in intercalated cells in the collecting duct (131, 214, 326, 448). However, the identification of the channel in type A or B intercalated cells remains controversial; mRNA was identified only in type A cells (395), whereas immunohistochemistry suggested expression of ClC-5 also in type B cells (214). The finding that ClC-5 and the vacuolar H$^+$-ATPase are present in similar intracellular structures and the fact that mutations in ClC-5 in humans cause Dent’s disease, which displays a defective proximal tubular endocytosis of low-molecular-weight proteins, prompted the idea that ClC-5 may be the Cl$^-$ conductive pathway necessary for endosomal acidification (see also below). Indeed, two mouse models for Dent’s disease with a complete genetic knock-out of ClC-5 suffer from low-molecular-weight proteinuria and have a reduced rate of receptor-mediated endocytosis (418, 552). Coexpression of ClC-5 with vacuolar H$^+$-ATPases and localization to endosomes have also been reported from other tissues such as small and large intestine or epididymis (256, 522). However, in epididymal clear cells, ClC-5 does not completely colocalize with the vacuolar H$^+$-ATPase, and some H$^+$-ATPase-containing intracellular vesicles are clearly negative for ClC-5 (256). Thus ClC-5 might be involved in the acidification of a subpopulation of H$^+$-ATPase-containing vesicles, while another unidentified conductance is involved in the acidification of the remaining vesicles.

In addition, the Cl$^-$ conductance in proximal tubular endosomes is highly stimulated by activation of PKA, whereas ClC-5 appears not to be regulated by PKA (24, 427, 561). ClC-5 chloride conductance is highly pH sensitive and is strongly inhibited at a pH below 6.5, suggesting that ClC-5 could only contribute to the initial phase of acidification (140, 182, 362). Moreover, recent data from one ClC-5 mouse model show that the Cl$^-$-dependent acidification rate of endosomes was only slightly reduced, suggesting that ClC-5 is not the major Cl$^-$ channel involved in endosomal acidification by the vacuolar H$^+$-ATPase (215). Some ClC-5 protein seems to be expressed also in the brush-border membrane of the proximal tubule and in the apical membrane of intercalated cells in the cortical collecting duct together with vacuolar H$^+$-ATPases (131, 214, 448). Experiments with isolated proximal tubules and cortical collecting ducts from a mouse model with highly reduced ClC-5 channel expression (327) found no differences in plasma membrane vacuolar H$^+$-ATPase activity in both proximal tubules and cortical collecting ducts (541). This recent evidence suggests that ClC-5 is not the only Cl$^-$ channel playing an important role in proximal tubule acidification and transferrin receptor trafficking was demonstrated (363). In cultured intestinal (Caco-2) and renal (LLC-PK$_1$) cells, both ClC-5 and ClC-4 channels are partially colocalized with transferrin-positive endosomes and are colocalized with the early endosomal marker Rab5a. Moreover, ClC-4 is expressed in kidney proximal tubule cells in situ, where it is localized in apical vesicles and colocalizes with internalized FITC-dextran. Finally, functional studies demonstrated that both ClC-4 and ClC-5 contribute to the efficient endosomal acidification and participate in the regulation of transferrin receptor trafficking. Coimmunoprecipitation of ClC-4 and ClC-5 from rat kidney homogenates and Chinese hamster ovary (CHO) cells was also demonstrated. While the molecular
basis for the interaction between CIC-4 and CIC-5 remains unclear, since biochemical studies do not distinguish between a direct and indirect interaction, the authors hypothesized a direct heterodimerization of CIC-4 and CIC-5. The possible involvement of CIC-4 in renal function both in health and disease was also proposed (363).

Interestingly, a recent study investigated the abundance and localization of the 31-kDa E1 subunit in renal biopsies from patients with Dent’s disease and with other unrelated diseases, suggesting that the abundance of the E1 subunit was strongly decreased and that vacuolar H^+-ATPase staining was found in the basolateral membrane of proximal tubules and absent from intercalated cells (367). However, it would be expected that patients having such a dramatic reduction in vacuolar H^+-ATPase expression in intercalated cells would suffer from a marked distal renal tubular acidosis, similar to that seen in inherited disorders of vacuolar H^+-ATPase function (10) and in some patients with loss of intercalated cell H^+-ATPase due to Sjogren’s syndrome (128). The patients included in this study did not show any abnormality in their acid-base status. It will be interesting to see if the mouse models with CIC-5 knock-down or knock-out show similar changes in vacuolar H^+-ATPase abundance or distribution.

B. AQP-6

Aquaporin water channels facilitate the movement of water across membranes, usually plasma membranes; some AQP family members also allow the transport of other uncharged ions or solutes such as urea or glycerol (3). AQP-6, however, displays different characteristics, namely, a gated anion conductance activated at a low pH (584). The anion conductance of AQP-6 is activated by mercury, unlike most other AQP family members whose water permeability is inhibited by mercurial compounds. Surprisingly, the anion conductance also accepts nitrate as a substrate with an even higher selectivity than chloride (250). In contrast, the water permeability of AQP-6 is comparably low. AQP-6 protein expression is restricted to intracellular subapical vesicles in the proximal tubule and to vesicles in type A intercalated cells colocalizing with vacuolar H^+-ATPase in both segments (584, 585). There is no direct evidence at present that AQP-6 is providing a Cl^- shunt for vacuolar H^+-ATPase activity in vesicles expressing both proteins.

C. CFTR

The CFTR, defective in the inherited disease cystic fibrosis, serves at least two functions, i.e., as an anion channel as well as a regulator of other membrane transport proteins (457). CFTR protein is expressed in the kidney in renal endosomes, microsome, or brush-border membrane preparations (48, 364, 457) and has been localized by immunohistochemistry to the apical side of proximal and distal tubules (118). Initially no expression of CFTR was detected in the collecting duct, but subsequent studies showed mRNA, protein, and CFTR-like currents in collecting ducts or collecting duct-derived cells (130, 247, 508). A particularly high expression of CFTR mRNA has been reported for intercalated cells with the highest level in immunosorted type B intercalated cells (508).

A role for CFTR in the regulation of vacuolar H^+-ATPase-dependent acidification has been proposed, but this is controversial. The ability of CFTR to mediate HCO_3^- transport and more importantly to regulate HCO_3^- transporters has been demonstrated. Whereas CFTR itself is permeable for HCO_3^- (110, 419), the physiological significance of direct HCO_3^- transport by CFTR is only minor as other anions have a higher permeability. In addition, CFTR is involved in the regulation of several transporters mediating HCO_3^- transport (300, 408), most of which belong to the SLC26 family of anion exchangers, namely, DRA (SLC26A4), pendrin (SLC26A4), and CFEX (PAT-1)(SLC26A6) (284). The fact that cells from cystic fibrosis patients show also defects in apparently unrelated membrane proteins and properties prompted the idea that CFTR may affect more general cell functions. These include protein processing in the Golgi or export of proteins into the membrane (60), Al-Awqati and colleagues (31) reported less accumulation of the weak base DAMP, a marker of acidic compartments, in the Golgi network, and prelysosome as well as a reduced Cl^- dependent acidification rate of acidic vesicles in cells from cystic fibrosis patients. This suggested a role of CFTR in vacuolar H^+-ATPase-dependent acidification of intracellular organelles (31). In contrast, several groups used direct intracellular pH measurements in living cells to show a similar steady-state pH of the TGN in nonepithelial and epithelial cell lines with endogenous or transfected CFTR, as well as with nonfunctional ΔF508CFTR expression (459). One study in genetically matching lung cells expressing wild-type CFTR or ΔF508CFTR reported an even more acidic pH of the TGN in the ΔF508CFTR cell line (104). Also, after stimulation of cAMP-dependent Cl^- conductances, no difference in the acidification rates of several intracellular organelles could be seen (52, 459). The trafficking of the M2 influenza virus protein, which is dependent on the proper acidification and pH of the TGN, was not slowed down or altered in cystic fibrosis cells, suggesting that also on a functional level no evidence could be obtained for a disturbed acidification of intracellular organelles in cystic fibrosis (198). Thus the bulk of the evidence obtained indicates that CFTR has no detectable functional role in determining the pH of endosomes.
VI. ENDOCYTOSIS AND ACIDIFICATION OF INTRACELLULAR VESICLES

The vacuolar H\(^+\)-ATPase was originally isolated from intracellular organelles and has a key function in many facets of organelle function. Many of the membrane-bound compartments that comprise the secretory and endocytotic pathways in epithelial and nonepithelial cells are acidified via the action of a vacuolar H\(^+\)-ATPase (188, 345, 346, 354, 355). As discussed above in this review, development of maximal acidification of these structures depends on the presence of a parallel anion (usually chloride) conductance in the same membrane to dissipate the positive potential that would otherwise result from accumulation of protons within the vesicles (24, 343, 479). Thus defects in either the vacuolar H\(^+\)-ATPase or the chloride conductance pathway can result in a failure of organelles and vesicles to acidify appropriately. As discussed in the following sections, this has important consequences for renal tubular function.

A. Clathrin-Coated Pits/Vesicles

Many cell surface proteins and receptors are internalized via a clathrin-mediated mechanism in renal tubule epithelial cells. For example, the apical membrane of the proximal tubule is highly specialized for clathrin-mediated endocytosis of cell surface and filtered proteins (50, 158, 246, 320, 343, 344, 430). In the collecting duct, recycling of AQP-2 water channels also involves a clathrin-mediated internalization step (497). Over two decades ago, isolated clathrin-coated vesicles were shown to contain a vacuolar H\(^+\)-ATPase (178, 576), but whether proton pumps are present on all clathrin-coated vesicles remains controversial. Isolated clathrin-coated vesicles represent a heterogeneous preparation that is derived from cell surface-coated pits and from intracellular membranes, predominantly the TGN (211). Some lines of evidence suggest that while clathrin-coated vesicles derived from the TGN may indeed be acidic compartments, clathrin-coated vesicles involved in the internalization of cell surface proteins may not acidify. Isolated endosomes that are involved in clathrin-mediated AQP-2 internalization, do not acidify their lumen and lack functionally important subunits of the vacuolar H\(^+\)-ATPase (303, 447). Similar data were obtained on toad bladder endosomes that in-
tion of COP proteins, in particular β-COP and small GTPases of the ADP-ribosylation factor (ARF) family, with some vesicles depends on the generation of an acidic luminal pH (15, 213, 338, 589). Thus neutralization of vesicle luminal pH may inhibit some steps of the intracellular trafficking pathway by preventing the recruitment of coat proteins that are required for vesicle formation and budding.

In renal proximal tubules, the apical plasma membrane and subapical vesicles contain several proteins that are important for regulation of the endocytotic and recycling pathways. These include not only the endocytotic receptor proteins such as megalin and cubulin, but also the vacuolar H⁺-ATPase, Arf1 and Arf6, rab5 and rab 11, and an Arf6 GDP/GTP exchange factor (GEF) known as ARNO (ADP ribosylation factor nucleotide binding site opener) (338). A recent study showed that Arf6 and ARNO, but not Arf1, are recruited from the cytosol to proximal tubule endosomal membranes in response to a decrease in pH of the endosomal lumen (338). In contrast, work on cell culture models demonstrated that Arf1 but not Arf6 was recruited to endosomes in a pH-dependent manner (213). Both Arf1 and Arf6 can, however, be recruited to the same endosomal membranes in a GTP-dependent manner (338). In view of the complexity of the endocytotic process, and the likelihood that these studies were examining heterogeneous endosomal populations (e.g., early endosomes, recycling endosomes) from different cell types, the extent and specificity of pH-dependent vesicle coat recruitment may vary under different cellular conditions. For example, in the proximal tubule, the initial clathrin-coated pits at the cell surface, as well as the clathrin-coated vesicles that they produce, may not be acidic compartments (see above). Therefore, in this cell type the earliest stage of endocytosis may involve the GTP-dependent recruitment of Arf1 to the membrane. Subsequently, Arf6 may be recruited to early endosomes in a pH-dependent step, since these vesicles contain a functional vacuolar H⁺-ATPase that generates an acidic luminal pH.

Regardless of the nature of the coat proteins and effectors that are recruited in response to an acidic luminal pH, it has been proposed that an endosomal pH-sensing protein (PSP) is responsible for initiating this process (15, 338, 344). The sensor is likely to be a transmembrane protein, with a luminal domain that detects a drop in endosomal pH and in some way transmits this information to its cytosolic domain. A pH-induced change in the conformation of the cytosolic domain would be the signal for the recruitment of key components of the endosomal coat, including Arf proteins and regulators of Arf function such as ARNO. While this sensor remains to be identified, it is clear that transmembrane signaling is a common phenomenon that regulates a variety of cellular processes, most notably receptor ligand-induced cascades. A pH sensor would, therefore, add to the growing list of signals that can be transmitted from one side of a biological membrane to the other.

D. Defective Vesicle Acidification and Proximal Tubule Pathophysiology

Most epithelial cell types along the urinary tubule show a remarkable ability to modulate the composition and function of their plasma membranes by membrane protein recycling (75, 86). Because intracellular vesicle trafficking involves the sequential passage of transported molecules through a series of acidified compartments, it is to be expected that perturbation of the acidification process will lead to tubule dysfunction and potentially to pathophysiological states. This section will focus on the importance of vesicle acidification in proximal tubule function. Proximal tubules have an extensive and specialized apical membrane endocytotic and recycling apparatus that serves 1) to reabsorb low-molecular-weight proteins that traverse the glomerular filtration barrier and 2) to recycle apical membrane proteins such as megalin, Na⁺/Pi cotransporters, glucose transporters, and many other functionally critical apical membrane proteins.

E. Dent’s Disease

This disease, characterized by a Fanconi-like syndrome and nephrolithiasis, is caused by a loss of function mutation in a chloride channel, CIC-5 (see above) (171, 172, 315, 483). Similar, but variable, pathophysologies have been duplicated in various strains of CIC-5 knockout mice (215, 418, 465, 552). The most likely cause of the low-molecular-weight proteinuria seen in Dent’s disease patients and in CIC-5 knockout mice is defective endocytosis of filtered proteins and defective recycling of apical membrane proteins. Decreased endosomal acidification results from the loss of a functional vesicular chloride channel such that the intravesicular positive electrical potential cannot be dissipated. This severely limits the extent to which the endosomal lumen can be acidified. Evidence in favor of this hypothesis is that both the CIC-5 channel and the vacuolar H⁺-ATPase are located in apical endosomes in the renal proximal tubule (214, 326, 448) and that proximal tubule endosomes from CIC-5 knockout mice have a reduced capacity to acidify in response to ATP addition compared with endosomes from normal animals (215).

However, the precise role of the CIC-5 channel in vesicle acidification remains a matter of debate for two reasons. First, PKA-dependent chloride channels have been found in proximal tubule endosomes (24), but so far the chloride conductance of CIC-5 has not been shown to be phosphorylation dependent. Second, when expressed...
in oocytes, ClC-5 produces an outward current (i.e., chloride moves into the cytosol) at positive membrane potentials and does not carry a significant current at normal physiological membrane potentials. This characteristic is, of course, the opposite of what would be required to move chloride into the endosomal lumen and has been discussed in a recent review (568). Finally, ClC-5 is also expressed in collecting tube intercalated cells and in epididymal clear cells, in parallel with the vacuolar H^+/H^+ATPase (214, 256, 395, 448), but no perturbation of intercalated cell function has yet been described as a result of loss of ClC-5 function. Thus, while ClC-5 perturbation does have at least some of the predicted effects on renal tubule function, further work will be required to understand more fully the precise mechanisms underlying these functional defects. Thus it is interesting that a recent report demonstrates reduced expression of the endocytic receptor proteins megalin and cubulin in a ClC-5-deficient mouse model which could partly explain the endocytotic defect (112).

F. Acquired Fanconi Syndrome: Cadmium Nephrotoxicity

Both in humans and in experimental animals, chronic exposure to the heavy metal cadmium results in a Fanconi syndrome caused by proximal tubule dysfunction (2, 208, 234, 236, 279, 299, 394, 506). While cadmium has a variety of effects on various components of epithelial cells, including microtubule depolymerization (443, 445) and Na^+-K^+-ATPase inhibition (506), it also has a direct inhibitory effect on the vacuolar H^+-ATPase, leading to defective endosomal acidification (234). Interestingly, cadmium intoxication also affects vacuolar H^+-ATPase function and luminal acidification in the male reproductive tract (93, 94, 235). Thus, by inhibiting intracellular vacuolar H^+-ATPase activity, cadmium intoxication may affect proximal tubule function in a way similar to that described above in Dent’s disease. In support of this theory, cadmium toxicity data from rats show impaired recycling of apical membrane proteins (445) and defective endocytosis of filtered markers (234). However, depolymerization of microtubules in the proximal tubule by drugs such as colchicine have similar effects on membrane protein recycling (44, 84, 153) and therefore the extent to which the cadmium effect on membrane trafficking is due to impaired vesicle acidification, or to its effect on microtubules, remains uncertain.

VII. INTERACTION OF THE VACUOLAR H^+-ATPASE WITH PROTEINS OTHER THAN ITS OWN SUBUNITS

Increasing evidence over the last decade has shown that the vacuolar H^+-ATPase has the capacity to bind to numerous regulatory proteins. It, therefore, appears that the cascade of events that ultimately result in modulating vacuolar H^+-ATPase assembly, targeting, and activity might be governed at different levels via protein-protein interaction.

A. Inhibitors and Activators of the Vacuolar H^+-ATPase

The first indication that the vacuolar H^+-ATPase might interact with proteins other than its own subunits was the finding that small-molecular-weight proteins, present in the cytosol, can directly modulate the activity of the vacuolar H^+-ATPase. A protein of 6 kDa was purified from bovine kidney cytosol and was shown to inhibit both ATPase activity and proton translocation (593). The same group also demonstrated the presence of a larger molecular weight cytosolic protein of 35 kDa, which activates the vacuolar H^+-ATPase (592). In addition, a distinct 6-kDa protein was isolated from bovine brain, where it activates the clathrin-coated vesicle vacuolar H^+-ATPase (575). However, since the publication of these reports in the early 1990s, no information regarding any physiological role has emerged. However, numerous reports have indicated direct interaction between various subunits of the vacuolar H^+-ATPase and other regulatory proteins.

B. Interaction of the Vacuolar H^+-ATPase With SNARE Proteins

The molecular mechanisms responsible for the regulation of endo- and exocytic processes in transporting epithelia are still not completely understood. Several studies have indicated that these processes require components similar to those involved in the shuttleing of synaptic vesicles, which occurs in the central nervous system (8, 66, 179, 180, 335, 336, 390). Syntaxin (STX1), SNAP-25 (25-kDa synaptosome associated protein), and VAMP (vesicle-associated membrane protein, also called synaptobrevin) were the first identified SNARE (soluble NEM-sensitive factor attachment protein receptor) proteins (reviewed in Refs. 108, 228). These proteins are involved in the trafficking and fusion of synaptic vesicles. SNAREs were initially classified into v-SNAREs and t-SNAREs, whether they were localized in the vesicles or their target membranes, respectively (477). More recently, a new nomenclature was proposed to avoid confusion in the case of homotypic membrane fusion, and the SNAREs were renamed R-SNAREs (arginine-containing SNAREs) or Q-SNAREs (glutamine-containing SNAREs) (159). So far, more than 30 mammalian SNARE proteins have been identified and classified into three distinct groups, the
syntaxin, VAMP, or SNAP-25 families, based on their sequence homology and domain structure.

In the original model, the specificity of docking and fusion of cytoplasmic vesicles with the plasma membrane was proposed to be mediated by SNARE proteins and to require the participation of soluble factors such as NSF and α-SNAP (416, 478). Further studies have proposed that SNAREs cannot act alone in vesicle docking and that additional factors, including small GTPases of the Rab family and associated tethering proteins, participate in the targeting of exocytic vesicles to their appropriate membrane domain (416, 417). VAMP2 (or synaptobrevin 2), cellubrevin, the ubiquitously expressed analog of synaptobrevin (352), α-SNAP, and NSF as well as syntaxin-4 were reported to be present in inner medullary collecting duct principal cells and were proposed to be involved in the recycling of the vasopressin-regulated water channel AQP-2 (179, 180, 262, 335, 336, 390). SNARE proteins are present in kidney intercalated cells (66, 255, 336, 390), and in proton secreting cells of the male reproductive tract, which resemble intercalated cells (67), indicating their involvement in the regulation of key membrane proteins expressed by these cells.

Some subunits of the vacuolar H\(^+\)-ATPase have the capacity to bind to SNARE proteins, indicating that the SNARE machinery might participate in vacuolar H\(^+\)-ATPase trafficking. In rat synaptosomes, subunits c, d (Ac39), and a (Ac116) of the vacuolar H\(^+\)-ATPase are associated with the synaptobrevin-synaptophysin complex, suggesting a potential role in recruiting the proton pump into synaptic vesicles (187). Subunit d of the vacuolar H\(^+\)-ATPase is homologous to physophillin, a cytosolic synaptophysin-binding protein (463). In nonneuronal cells, a similar interaction between the vacuolar H\(^+\)-ATPase and some SNARE proteins has been shown. In proton-secreting cells of the male reproductive tract, vacuolar H\(^+\)-ATPase is actively recycled in a manner similar to kidney intercalated cells, and net proton secretion requires the participation of the v-SNARE cellubrevin (67). In cultured inner medullary collecting duct (IMCD) cells, acid-induced vacuolar H\(^+\)-ATPase exocytosis is inhibited by clostridial toxins, which are specific SNARE proteases (30), and subunit E1 of the vacuolar H\(^+\)-ATPase coimmunoprecipitates with α-SNAP, SNAP-23, syntaxin, and VAMP2 (29, 30). A recent report from the same group showed that interaction between syntaxin-1A and the vacuolar H\(^+\)-ATPase is involved in the exocytosis of the pump to the apical membrane of IMCD cells (310).

While SNARE proteins have clearly been implicated in the attachment of vesicles to their target membranes, the subsequent process of membrane fusion still remains largely uncharacterized. The V\(_0\) domain of the vacuolar H\(^+\)-ATPase, particularly subunit c, was proposed to be a major player in the lipid bilayer mixing that occurs during membrane fusion (45, 415). It was proposed that following trans-SNARE pairing, V\(_0\) sectors from opposing membranes form complexes that make a continuous, proteolipid-lined channel at the fusion site. Radial expansion of this protein pore provides a mechanism for membrane fusion. While extremely interesting, this model was disputed because disruption of the c subunit gene does not lead to the fragmentation of the yeast vacuole that would be predicted from that model (517; and reviewed in Ref. 392).

In summary, it appears that the vacuolar H\(^+\)-ATPase participates in different events that take place during the exocytic process, including an early step that involves the targeting of the vesicles to their target membrane as well as the final step of membrane fusion. Therefore, the vacuolar H\(^+\)-ATPase might be involved in its own targeting as well as in a more generalized targeting of other membrane proteins that share the same transporting vesicles.

### C. Subunit B1 of the Vacuolar H\(^+\)-ATPase is a PDZ Binding Protein

In addition to SNARE proteins, the vacuolar H\(^+\)-ATPase has the capacity to interact with various proteins, indicating its role in a multitude of regulatory functions. As mentioned above, the B1 (ATP6V1B1) subunit of the vacuolar H\(^+\)-ATPase possesses a COOH-terminal “DTAL” motif typical of a PDZ-interacting domain (386, 421). Coimmunoprecipitation and pull-down assays demonstrated the association of the B1 subunit with the PDZ protein NHERF-1 in rat kidney (70). Subunit E1 was also present in these samples via association with the B1 subunit. In type B intercalated cells, NHERF-1 colocalizes with the vacuolar H\(^+\)-ATPase in either the apical or basolateral pole, but NHERF-1 is not detectable in type A intercalated cells. This indicates that interaction of the vacuolar H\(^+\)-ATPase with NHERF-1 might play a role in generating, maintaining, or modulating the variable vacuolar H\(^+\)-ATPase polarity that characterizes the B-cell phenotype. In the proximal tubule, while NHERF-1 is located in the apical brush border, the vacuolar H\(^+\)-ATPase is located in a distinct membrane domain at the base of the brush border (Fig. 2, A and B), consistent with the expression of the truncated B2 subunit isoform, lacking the DTAL motif, in this tubule segment.

Association between the B1 subunit and another PDZ binding protein, the Na\(^+\)-bicarbonate transporter NBC3, was demonstrated in kidney lysates (423). The PDZ interacting domains of both the B1 subunit and NBC3 are essential for the interaction to occur, and NHERF-1 is a component of the complex. NBC3 and the B1 subunit colocalize in A- and B-intercalated cells, and it was speculated that NBC3 may modulate the activity of the vacuolar H\(^+\)-ATPase by altering the local concentration of bicarbonate and/or by protein-protein interaction through NHERF-1 or other PDZ proteins.
D. The Vacuolar H\textsuperscript{+}-ATPase Directly Interacts With the Actin Cytoskeleton

In addition to the indirect interaction between the vacuolar H\textsuperscript{+}-ATPase and the actin cytoskeleton that is provided via NHERF binding, the V\textsubscript{i} complex of the pump can directly interact with actin (242, 537). The amino termini of both the B1 and B2 subunits of the vacuolar H\textsuperscript{+}-ATPase contain high-affinity F-actin binding sites (242). With the use of short peptide sequences, an 11-amino acid binding motif was isolated in both B subunits at amino acids 49–59 (B1) and 55–65 (B2) (107). Because the amino terminus of the B subunit is predicted to be located in the region of the V\textsubscript{i} sector that extends away from the plasma membrane insertion site, it was suggested that the F-actin binding site was ideally positioned to mediate interaction between membrane-bound vacuolar H\textsuperscript{+}-ATPase and the actin cytoskeleton. This hypothesis is consistent with the association of F-actin and vacuolar H\textsuperscript{+}-ATPase-containing vesicles that was observed by freeze-fracture, deep-etch electron microscopy in the toad urinary bladder (77). More recently, a direct interaction between F-actin and subunit C was reported (537). The C subunit is a constituent part of the V\textsubscript{i}V\textsubscript{0} holoenzyme but not of the V\textsubscript{0} complex of the vacuolar H\textsuperscript{+}-ATPase. It was proposed that subunit C plays a crucial role in controlling binding of the vacuolar H\textsuperscript{+}-ATPase to the actin cytoskeleton when the pump is inserted into the plasma membrane (537).

E. Interaction of the Vacuolar H\textsuperscript{+}-ATPase With Enzymes of the Glycolytic Pathway

In yeast, glucose deprivation induces disassembly of the V\textsubscript{i} domain from the plasma membrane V\textsubscript{0} domain (409), suggesting a potential coupling between vacuolar H\textsuperscript{+}-ATPase activity and glycolysis. In support of this notion, direct binding of the glycolytic enzyme aldolase with yeast subunits a, B, and E was recently shown (321, 322). This direct association suggests the possibility that aldolase may be involved in regulating the activity, distribution, or assembly of the vacuolar H\textsuperscript{+}-ATPase. Indeed, assembly of the V\textsubscript{i} and V\textsubscript{0} vectors of the vacuolar H\textsuperscript{+}-ATPase was disturbed in an aldolase-deficient yeast strain (322). In addition, evidence for a direct interaction of the cytoplasmic carboxy-terminal domain of the human V\textsubscript{0} subunit a4 with phosphofructokinase I, another enzyme of the glycolytic pathway (494), further indicated potential regulatory mechanism(s) between vacuolar H\textsuperscript{+}-ATPase function and energy supply. However, as discussed above, this is a controversial issue since according to one of the models with nine transmembrane domains in the a subunit (392, 397), the carboxy-terminal tail should be located inside the vesicle lumen and would not be accessible for binding to cytosolic proteins.

F. Interaction With Other Proteins

The H subunit of the vacuolar H\textsuperscript{+}-ATPase interacts with the human immunodeficiency virus protein Nef, and this interaction was proposed to facilitate the internalization of CD4 in T cells (324). The carboxy-terminal portion of subunit H shares significant homology with adaptor protein complexes and is similar to the β-subunit of COPI coatamer complexes (196). This subunit binds to the carboxy-terminal loop of Nef and to the adaptor protein complex 2 (AP-2) (197). Therefore, subunit H can act as an adaptor protein for interactions between Nef and AP-2. In addition, the primary component of the proton-forming pore of the vacuolar H\textsuperscript{+}-ATPase subunit c was shown to interact with the bovine papillomavirus E5 protein and platelet-derived growth factor receptor (207), as well as with β\textsubscript{1}-integrin (469).

In summary, as more effort is devoted to characterizing the targeting, assembly, and regulation of the vacuolar H\textsuperscript{+}-ATPase, the number of proteins that interact with the pump is constantly growing. These proteins are involved in a variety of cell functions and are present in different cell types, indicating that a tight coupling with vacuolar H\textsuperscript{+}-ATPase activity is a key mechanism in the physiology and pathophysiology of many biological systems.

VIII. REGULATION OF H\textsuperscript{+}-ATPASE FUNCTION AND LOCALIZATION

Regulation of vacuolar H\textsuperscript{+}-ATPase function may occur at several levels, such as increased transcription/translation of vacuolar H\textsuperscript{+}-ATPases, assembly or disassembly of V\textsubscript{i} and V\textsubscript{0} domains, trafficking and sorting into and from the membrane, interactions with other (activator or inhibitory) proteins, or by altering the abundance of cells expressing vacuolar H\textsuperscript{+}-ATPases (polarity of intercalated cells).

A. General Mechanisms of Regulation of Vacuolar H\textsuperscript{+}-ATPase Activity

Much of the evidence for different mechanisms of regulation of vacuolar H\textsuperscript{+}-ATPase activity comes again from studies in yeast. Some of these mechanisms such as activator or inhibitory proteins have already been mentioned in other paragraphs or will be described below (e.g., regulation of polar expression, sorting).

1. Assembly and disassembly of vacuolar H\textsuperscript{+}-ATPases

As described above, vacuolar H\textsuperscript{+}-ATPases comprise two sectors, the V\textsubscript{i} and V\textsubscript{0} domains, which can be assembled and disassembled as a means to regulate holoenzyme
activity (266). The regulation of vacuolar H\(^+\)-ATPase activity and consequently acidification capacity of lysosomes by assembly of \(V_0\) and \(V_1\) sectors has been recently demonstrated during maturation of the dendritic cell, both in vitro and in intact cells (513). Regulation of H\(^+\)-ATPase activity by the recruitment of \(V_0\) and \(V_1\) sectors has also been demonstrated in insects during the molting cycle (496, 588). Similarly, in yeast, glucose availability regulates the activity of the vacuolar H\(^+\)-ATPase in the food vacuole by inducing assembly of its two domains (409). Likewise, glucose withdrawal induces a rapid disassembly of vacuolar H\(^+\)-ATPases (265). In yeast, the glucose-induced assembly is at least in part regulated by an associated protein complex named RAVE [regulator of the (H\(^+\))-ATPase of the vacuolar and endosomal membranes] and which consists of proteins (Skp1, Rav1, and Rav2) belonging to the SCF ubiquitin ligase family and interacting proteins (460). This complex seems to be essential for normal vacuolar H\(^+\)-ATPase assembly as yeast strains lacking some of its subunits are unable to properly assemble functional vacuolar H\(^+\)-ATPase complexes (470). Interestingly, disassembly of vacuolar H\(^+\)-ATPases requires microtubules, whereas assembly is not sensitive to disruptors of the microtubular network, suggesting that different mechanisms underlie assembly and disassembly (578). In addition, stimuli leading to disassembly of vacuolar H\(^+\)-ATPases in yeast affect only pumps containing the Stv1 subunit but not the Vph1 subunit (yeast homologs of mammalian \(a\) subunits), suggesting that the yeast homologs of the \(a\) subunit influence or control assembly/disassembly (271). In the bovine renal cell line MDBK, assembly of vacuolar H\(^+\)-ATPases during biogenesis of the holoenzyme has been studied, and it was suggested that most of the assembly takes place in the Golgi but that there is a large cytosolic pool of \(V_1\) domains (372). Unfortunately, there is no evidence at present demonstrating a role for assembly or disassembly of the vacuolar H\(^+\)-ATPase holoenzymes in the regulation of plasma membrane vacuolar H\(^+\)-ATPase activity in the kidney.

2. Altered abundance of vacuolar H\(^+\)-ATPases

Changes in the abundance of vacuolar H\(^+\)-ATPases or of some of its subunits are potentially involved in the regulation of vacuolar H\(^+\)-ATPase activity in the kidney. Fejes-Toth and Naray-Fejes-Toth (162) reported increased mRNA levels for the E1 (31 kDa) subunit in immuno-sorted type A intercalated cells from rabbits during metabolic acidosis and increased mRNA in type B intercalated cells during metabolic alkalinosis. These results are in contrast to other studies examining protein abundance of several vacuolar H\(^+\)-ATPase subunits in different models of acid-base and electrolyte disturbances. In these studies, no changes in protein abundance could be found under similar conditions (34, 482). It remains to be resolved if the reported changes in mRNA levels reflect a technical problem of the semi-quantitative PCR used or if changes in mRNA occur but do not translate into protein expression. However, an increase in the amount of E1 subunit protein was detected by Western blotting in the medulla of kidneys from acetazolamide-treated rats (25).

3. Disulfide bridges and changes in ATP-coupling efficiency

Formation of disulfide bridges between several cysteine residues through oxidative stimuli inhibits vacuolar H\(^+\)-ATPase activity, a process that is not easily reversed by normal concentrations of intracellular glutathione (176). Furthermore, a yeast mutant has been identified lacking an enzyme involved in cysteine synthesis and thus having lower intracellular glutathione levels. Vacuolar acidification is strongly reduced due to an inhibition of the vacuolar H\(^+\)-ATPase, and this inhibition is prevented either by supplying glutathione or a mutation of the cysteine residue in the vacuolar H\(^+\)-ATPase sensitive to oxidation (400). It is, however, not clear if this oxidative inhibition of vacuolar H\(^+\)-ATPase activity has physiological implications or represents a pathophysiological process.

Another means of regulation of vacuolar H\(^+\)-ATPase activity may be changes in the coupling efficiency between ATP hydrolysis and proton pumping. Indeed, it has been observed that pumps containing different isoforms of the \(a\) subunit display small changes in their coupling efficiency (271). In yeast, pumps containing either the Vph1 or the Stv1 a subunit homologs are localized to different intracellular compartments and show distinct coupling efficiencies (271). Similarly, a differential expression of a subunits has been observed in osteoclasts (511) and mouse kidney, suggesting that pumps containing distinct a subunit isoforms may serve different functions or may be regulated differently (Wagner and Marshansky, unpublished observations). In addition to changes in coupling, ADP generated during ATP hydrolysis has an inhibitory effect on vacuolar H\(^+\)-ATPase activity providing a possible feedback mechanism (274).

B. Polarized Expression of the Vacuolar H\(^+\)-ATPase in Intercalated Cells

Collecting duct intercalated cells express high levels of vacuolar H\(^+\)-ATPase in intracellular vesicles and/or on their plasma membrane, and as detailed above, the distribution and phenotype of intercalated cells vary in the different regions of the collecting duct. Although all intercalated cells can modulate their cell surface expression of vacuolar H\(^+\)-ATPase by vesicle trafficking, the situation is
especially complex in the cortical collecting duct and the connecting segment of the urinary tubule. Under different physiological conditions of acidosis or alkalosis, the cortical collecting duct can secrete either net acid or net base (317, 350). In the most simplified scenario, these functions are achieved in the cortical collecting duct by type A- and type B-intercalated cells, respectively (5, 333). Net proton secretion by type A-intercalated cells (A-IC) replenishes body bicarbonate levels, while bicarbonate secretion by type B-intercalated cells (B-IC) helps to restore bicarbonate concentration during metabolic alkalosis. The medullary collecting duct is capable only of net acid secretion, and accordingly, only A-IC are detectable in the medulla (64, 79, 278, 505).

1. Acid-base regulation of vacuolar H\(^+\)-ATPase polarity in intercalated cells

In the kidney, proton secretion by intercalated cells is regulated to a great extent by modulating the amount of vacuolar H\(^+\)-ATPase at the cell surface. Vacuolar H\(^+\)-ATPase proteins are shuttled to and from the apical membrane via specialized intracellular acidic vesicles (5, 34, 73, 74, 333, 438, 454, 515). Observations on mice, rats, and rabbits in which systemic acidosis has been induced show that the number of cortical intercalated cells with apical membrane vacuolar H\(^+\)-ATPase expression increases and the number of cells with basolateral vacuolar H\(^+\)-ATPase expression decreases (34, 332, 331, 482). Conversely, in systemic alkalosis, the number of cells with basolateral vacuolar H\(^+\)-ATPase expression increases, whereas apical vacuolar H\(^+\)-ATPase expression decreases. These are precisely the cellular changes that would be expected to increase net acid secretion (to compensate for acidosis) and increase net bicarbonate secretion (to compensate for metabolic alkalosis). These cellular changes could occur via different mechanisms, although the physiological result would be the same in all cases.

2. Plasticity of the intercalated cell phenotype

In their initial study on the regulation of proton secretion by intercalated cells, Schwartz et al. (455) proposed that A and B cells were functionally distinct manifestations of a single type of intercalated cell. According to this model, A cells with apical vacuolar H\(^+\)-ATPase and basolateral anion exchanger (AE-1) can repolarize, presumably by transcytosis, to produce B cells, which have a mirror-image polarity, with basolateral vacuolar H\(^+\)-ATPase and apical AE-1. This attractive hypothesis has received considerable support from studies showing that a novel matrix protein, hensin, could reverse the functional phenotype of cultured intercalated cells (6). Thus bicarbonate-secreting intercalated cells in culture could be converted to proton-secreting cells by growing them on a matrix containing hensin (502). Most recently, it has been shown using collecting ducts incubated in vitro that anti-hensin antibodies applied to the basolateral bathing medium can inhibit the induction of acid secretion and bicarbonate reabsorption that normally occurs after incubation of the tubules in acidic medium (456). Hensin was proposed to induce terminal differentiation in intercalated cells, which was reflected by the A-cell phenotype, a process that was inhibited when hensin function was blocked by antibodies. This change in functional activity of intercalated cells involved the concerted action of microtubules and microfilaments, and also required de novo protein synthesis (456). The various patterns of vacuolar H\(^+\)-ATPase distribution that are observed in intercalated cells, ranging from strict apical (A cells), strict basolateral (B cells), diffuse cytoplasmic with little or no apparent membrane staining, and bipolar localization, support the concept that all intercalated cells are variants of the same cell type and that the precise cellular location of the vacuolar H\(^+\)-ATPase is determined by prevailing physiological acid-base status. Furthermore, other work has shown that apical and basolateral anion exchangers (detected either functionally or by immunocytochemistry) can be regulated and/or internalized under appropriate acid-base conditions (183, 438, 449, 542).

Some observations, however, remain to be reconciled with the simple notion that A and B cells are phenotypic variants of the same cell type that can rapidly remodel. For example, medullary A cells with the appearance of B cells have never been described in adult kidney. Intercalated cells in the medulla retain their A-cell phenotype under all experimental conditions. This could indicate that their A-cell phenotype is irreversibly fixed, or that experimental conditions in vivo have not yet been found to convert them to B cells. However, both A- and B-intercalated cells are present in inner medulla of neonatal rats, and the number of B cells is greater in pups from alkalotic mothers (55, 377). In addition, in the cortex, the relative numbers of A versus B cells are constantly changing in response to variations in acid-base status. Thus the model of one plastic intercalated cell phenotype would require that the hensin-induced terminal differentiation of B cells into A intercalated cells be a reversible phenomenon so that the appropriate response to alkalosis could occur (i.e., the production of more B-type intercalated cells by A-cell “dedifferentiation”).

The nature of the apical anion exchanger in B cells was also a matter of considerable debate because it was not detectable using antibodies against AE-1 (11), and AE-1 mRNA was expressed only at very low levels in immunosorted B cells (160). In cultured intercalated cells, the apical anion exchanger was identified as AE-1 (521), whereas in the kidney collecting duct, pendrin has now been identified as at least one major player responsible for apical anion exchange in B-intercalated cells (282, 436,
550). Furthermore, apical pendrin is relocated to the cytosol of B-intercalated cells in acid-loaded mouse kidney, consistent with previous reports of acid-induced apical anion exchanger internalization in rabbit collecting ducts (449). Finally, A- and B-intercalated cells have been distinguished by a differential expression of other proteins, including NHERF-1 (70). Clearly, the change in phenotype from A- to B-intercalated cells involves more than a simple change in the polarity of membrane transporters. This aspect of phenotypic response was already implied from recent data, discussed above, showing that protein synthesis is required to elicit these changes in isolated perfused collecting tubules (70, 456).

3. Plasticity of the principal and intercalated cell phenotypes

In addition to changes within the intercalated cell population, there is some evidence that principal cells and intercalated cells may interconvert. This would allow even more flexibility and adaptation to specific physiological stresses in the kidney. Early studies showed that the number of cells identified as intercalated or principal cells could vary under a variety of physiological conditions, including acid-base changes, dietary potassium intake, and hydration state of the animal (539), although most of these studies were performed before the development of cell-specific antibodies and were based on light and electron microscopic appearance only. In neonatal rats, intercalated cells are found in the tip of the papilla, but gradually disappear from this region as the pups develop (243, 379). While selective shedding and apoptosis may explain some of this loss of intercalated cells (277), a conversion to principal cells has not been ruled out. Among the data that support a conversion process between principal and intercalated cells are 1) some cells that express principal and intercalated cell markers (AQP-2 and vacuolar H\(^+\)-ATPase) can be found in the cortical regions of neonatal rats (444); 2) inhibition of carbonic anhydrases with acetazolamide treatment of adult rats increases the number of cells identified as intercalated or principal cells (452); and 3) a conversion of type A intercalated cells to type B intercalated cells has also been reported in brush-border membrane vesicles, most likely reflecting stimulation of the pump in proximal tubules (101). The molecular mechanisms leading to an increased membrane insertion or activity of vacuolar H\(^+\)-ATPase subunits in the luminal membrane of type A intercalated cells in the late distal tubule, the connecting segment, and the collecting duct. This translocation of vacuolar H\(^+\)-ATPase into the membrane is paralleled by increased activity of NEM-, bafilomycin- or concanamycin-sensitive ATPase activity as well as proton extrusion (276). Increased vacuolar H\(^+\)-ATPase activity has also been reported in brush-border membrane vesicles, most likely reflecting stimulation of the pump in proximal tubules (101). The molecular mechanisms leading to an increased membrane insertion or activity of vacuolar H\(^+\)-ATPases in metabolic acidosis has not been elucidated. An increase in the ambient CO\(_2\), however, may play a role. In isolated perfused rabbit proximal tubules and collecting ducts, a rise in CO\(_2\) leads to the exocytic insertion of vacuolar H\(^+\)-ATPases, thus increasing vacuolar H\(^+\)-ATPase activity in the plasma membrane (453). In turtle bladder proton-secreting cells, this process was dependent on an increase in intracellular calcium (520).

C. Adaptive Regulation of Vacuolar H\(^+\)-ATPase Activity

Work in whole animals using in vivo microperfusion or renal clearance experiments, as well as in vitro perfusion studies with isolated tubule segments obtained from adapted or treated animals, have firmly established over the last three decades that the vacuolar H\(^+\)-ATPase plays an important role in the adaptive response of the kidney to altered acid-base or electrolyte status. In addition, studies using cell cultures and isolated perfused tubules in vitro have examined mechanisms underlying the adaptive regulation of vacuolar H\(^+\)-ATPases by acidosis and alkalosis, as well as by hormones.

1. Metabolic acidosis

In animal models, metabolic acidosis is associated with increased bicarbonate reabsorption in some segments (but not the TAL) as well as with increased proton secretion along the different segments of the collecting duct. This acid-base disturbance, which is often induced by the addition of NH\(_4\)Cl to the drinking water (34, 101, 162, 276), induces a more pronounced expression of vacuolar H\(^+\)-ATPase subunits in the luminal membrane of type A intercalated cells in the late distal tubule, the connecting segment, and the collecting duct. This translocation of vacuolar H\(^+\)-ATPase into the membrane is paralleled by increased activity of NEM-, bafilomycin- or concanamycin-sensitive ATPase activity as well as proton extrusion (276). Increased vacuolar H\(^+\)-ATPase activity has also been observed in brush-border membrane vesicles, with increased bicarbonate reabsorption in some segments (but not the TAL) as well as with increased proton secretion along the different segments of the collecting duct. This acid-base disturbance, which is often induced by the addition of NH\(_4\)Cl to the drinking water (34, 101, 162, 276), induces a more pronounced expression of vacuolar H\(^+\)-ATPase subunits in the luminal membrane of type A intercalated cells in the late distal tubule, the connecting segment, and the collecting duct. This translocation of vacuolar H\(^+\)-ATPase into the membrane is paralleled by increased activity of NEM-, bafilomycin- or concanamycin-sensitive ATPase activity as well as proton extrusion (276). Increased vacuolar H\(^+\)-ATPase activity has also been reported in brush-border membrane vesicles, most likely reflecting stimulation of the pump in proximal tubules (101). The molecular mechanisms leading to an increased membrane insertion or activity of vacuolar H\(^+\)-ATPases in metabolic acidosis has not been elucidated. An increase in the ambient CO\(_2\), however, may play a role. In isolated perfused rabbit proximal tubules and collecting ducts, a rise in CO\(_2\) leads to the exocytic insertion of vacuolar H\(^+\)-ATPases, thus increasing vacuolar H\(^+\)-ATPase activity in the plasma membrane (453). In turtle bladder proton-secreting cells, this process was dependent on an increase in intracellular calcium (520).

2. Metabolic alkalosis

Metabolic alkalosis is compensated by the kidney by reducing bicarbonate reabsorption and generation in the collecting duct with increased bicarbonate secretion by type B intercalated cells (405). Accordingly, an inactivation of type A intercalated cells can be observed in parallel with an activation of type B intercalated cells with concomitant shifts of vacuolar H\(^+\)-ATPase immunoreactivity from the apical pole to subapical compartments in type A intercalated cells and a more pronounced basolateral staining in type B intercalated cells (34). In addition,
more type B intercalated cells may be found in chronic metabolic alkalosis as discussed above. In dissected nephron segments, a decrease in NEM-sensitive ATPase activity was observed in the medullary part of the thick ascending limb and the outer medullary collecting duct after oral NaHCO₃ loading (276). Similarly, a reduction in luminal acidification in the inner medullary collecting duct was described (46). In contrast, in a model using furosemide-induced metabolic alkalosis, an increase in H⁺-ATPase activity in dissected nephron segments was measured in the cortical and medullary collecting duct (147). The induction of volume contraction by the chronic furosemide application and the resulting high aldosterone levels and hypokalemia in this model make the interpretation of these findings difficult. There is also evidence that increased vacuolar H⁺-ATPase activity or membrane expression in type A intercalated cells may be at least partly responsible for the development of metabolic alkalosis as seen in the setting of chronic potassium depletion (see below).

3. Respiratory acidosis and alkalosis

Increased or decreased ventilation results in hypo- or hypercapnia, respectively, and subsequent metabolic alkalosis or acidosis. In healthy subjects, renal excretion of acid or base equivalents adapts rapidly and clears metabolism from additional protons or bicarbonate. In chronic but not acute hypercapnia (respiratory acidosis), an increase in NEM-sensitive ATPase activity in the proximal tubule and cortical collecting duct was found that was independent from potassium and aldosterone levels (149). Similarly, in acute respiratory alkalosis the luminal acidification rate in the inner medullary collecting duct is decreased (46). Moreover, during respiratory acidosis, morphological changes occur in the intercalated cells of the outer medullary collecting duct consistent with insertion of subapical vesicles, and activation of acid secretion was observed (331). Acute and chronic hypocapnia (respiratory alkalosis) has no effect on NEM-sensitive ATPase activity along the nephron (149).

4. Electrolyte disturbances

Alterations of electrolyte homeostasis are often associated with changes of systemic acid-base homeostasis as a consequence of altered renal transport processes as well as due to the shift of electrolytes between intra- and extracellular compartments in exchange for protons. Hypokalemia leads to metabolic alkalosis, which is in part caused by the shift of intracellular potassium to the extracellular space, resulting in the uptake of protons into cells. Hypokalemia can be caused by low potassium in the diet or more acutely by the application of loop diuretics (147). In the proximal tubule, the Na⁺/bicarbonate co-transporter NBC-1 is stimulated (13), and in the distal tubule and cortical collecting duct hypertrophy of intercalated cells is found (152, 486) and a more pronounced apical location of the vacuolar H⁺-ATPase associated with increased activity has been observed (26, 28, 466). These transport mechanisms are likely to contribute to the development of metabolic alkalosis under these conditions. The effect of hyperkalemia on vacuolar H⁺-ATPase has not been reported.

Chronic Na⁺ depletion leads to activation of the renin-angiotensin-aldosterone axis (see below) and to metabolic alkalosis. Stimulation of vacuolar H⁺-ATPase activity by both angiotensin II and aldosterone has been reported for various nephron segments and cell types and is discussed below.

In a rat model for acute chloride depletion alkalosis, a redistribution of vacuolar H⁺-ATPases has been noted during the compensatory bicarbonate secretion consistent with activation of type B intercalated cells and inactivation of type A intercalated cells (201, 531). Vacuolar H⁺-ATPases appeared strongly basolateral in B-IC and were mainly found in subapical storage vesicles in A-IC (531).

D. Hormonal Regulation of Vacuolar H⁺-ATPase Activity

Direct hormonal regulation of vacuolar H⁺-ATPase activity in the kidney has not been extensively studied. Many conclusions on the effect of several hormones are rather indirect, and their purported actions are due to their effect on bicarbonate reabsorption or secretion in the respective nephron segments or cells.

1. Regulation of bicarbonate reabsorption and vacuolar H⁺-ATPase activity by angiotensin II and aldosterone

A) THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM. The main function of the angiotensin II-aldosterone system is the maintenance of extracellular Na⁺ and volume homeostasis by controlling renal Na⁺ reabsorption through the stimulation of Na⁺-reabsorptive transport such as the epithelial Na⁺ channel and Na⁺ -K⁺-ATPases (9, 253, 534, 535). In addition, angiotensin II is a potent vasoconstrictor and has also direct stimulatory effects on epithelial Na⁺ transport. Besides its effects on Na⁺ homeostasis and blood pressure regulation, the renin-angiotensin-aldosterone system has also a profound influence on the renal handling of acid-base transport through both direct hormonal action and indirect pathways involving alterations of overall electrolyte and water balance (i.e., increased K⁺ secretion, water retention, and decreased vasopressin release).

B) ANGIOTENSIN II. Angiotensin II is mainly formed by renin-mediated activation of circulating angiotensinogen...
originating from the liver and after activation of angiotensin I by angiotensin converting enzymes (ACE). In the kidney, angiotensin II is filtered in the glomerulus, but intraluminal angiotensin II concentrations greater than those in the blood have been found (381, 382). Recent data demonstrated that angiotensinogen, renin, and ACE are also present along the nephron and that angiotensinogen is secreted into the lumen and activated there forming a paracrine renin-angiotensin II system along the nephron (424, 432). Angiotensin II signals mainly through two main receptor subtypes, AT1 and AT2 (9, 19, 253). Both receptors are expressed in the kidney, AT1 being the predominant subtype found in vessels and almost ubiquitously along the nephron possibly including the intercalated cells of the collecting duct (9, 90, 139, 226, 361). In general, the AT1 receptor mediates effects leading to increased Na+/HCO₃⁻, and fluid reabsorption along the nephron, which together with the induced vasoconstriction result in a rise of blood pressure (9, 43, 192, 253, 306, 307, 553). For these short-term effects, concentrations of angiotensin II in the picomolar range are needed in contrast to (high) nanomolar concentrations that will activate the AT2 receptor (19, 221). The AT2 receptor seems to antagonize the effects of the AT1 receptor on transport and blood pressure (221). Several groups have shown either in whole animal or cell culture models that angiotensin II is a potent activator of bicarbonate reabsorbing and proton secretory mechanisms along the nephron, thus stimulating overall bicarbonate reabsorption. The target transport mechanisms include the Na⁺/HCO₃⁻ co-transporter and Na⁺/H⁺ exchanger (at least NHE-3) in the proximal tubule and the vacuolar H⁺-ATPase in the proximal tubule, the distal convoluted tubule, and intercalated cells of the cortical collecting duct (43, 192, 307, 312, 540, 543, 553). Studies in whole animals and in humans show a stimulatory effect of angiotensin II on acid secretion and bicarbonate reabsorption (233, 306, 307). In all cases where the pharmacological profile was determined, stimulation occurred through AT1 receptors (233). In contrast, one study reported a reduction in cortical collecting duct vacuolar H⁺-ATPase enzymatic activity in response to angiotensin II (510), another an increase of bicarbonate secretion in isolated rabbit cortical collecting duct (559). In addition, in perfused rat outer medullary collecting duct, a reduction in bicarbonate reabsorption after application of angiotensin II was found (549).

The regulation of acid-base transport in the kidney by angiotensin II depends on the concentration used: in the proximal tubule, stimulation of bicarbonate reabsorption, Na⁺/H⁺ exchange, and Na⁺/HCO₃⁻ cotransport was shown at low concentrations (<10⁻⁶ M), while inhibition of bicarbonate reabsorption was shown at concentrations higher than 10⁻⁸ M (105, 134, 192, 195, 312). The stimulation observed at low angiotensin II concentrations was attributable to inhibition of the production of cAMP via the G protein G₁, while the inhibition of bicarbonate transport was due to an elevation of intracellular calcium (105, 301). AT₁ receptors couple intracellularly mainly to the phospholipase C, Ca₂⁺, and protein kinase C pathway, but may also activate tyrosine kinases (19, 57, 133). Thus it could be speculated that most stimulatory effects of angiotensin II on the vacuolar H⁺-ATPase should be mediated via these signal pathways. However, this has not been tested in native tissue to date. In a subclone of Madin-Darby canine kidney (MDCK) cells, however, with some characteristics of intercalated cells, angiotensin II increases intracellular Ca²⁺ and stimulates vacuolar H⁺-ATPase activity. Chelating intracellular Ca²⁺ abolished the stimulatory effect of angiotensin II (398). In isolated rat proximal tubules and in intercalated cells of isolated mouse cortical collecting ducts, the stimulation of vacuolar H⁺-ATPase was prevented by colchicine disrupting the microtubular network (540, 543). This suggests that vacuolar H⁺-ATPases, some of its subunits, or other stimulatory proteins may be trafficked to the membrane.

In addition to acute effects of angiotensin II on vacuolar H⁺-ATPase activity, AT1 receptor blockade in an animal model with heminephrectomy has been shown to reduce the adaptive increase of expression of the 31-kDa (ATP6V1E1, E1) subunit (308, 309).

C) ALDOSTERONE. Aldosterone is synthesized and released from the zona glomerulosa of the adrenal glands in response to several stimuli such as angiotensin II, hypokalemia, or metabolic acidosis. It serves primarily to enhance Na⁺ (re)absorption in kidney and colon, thereby regulating extracellular volume and blood pressure (534, 535). The action of aldosterone is classically mediated after binding to the mineralocorticoid receptor by altering transcription of target genes. The specificity of aldosterone action is furthermore achieved by concomitant expression of the 11β-hydroxysteroid dehydrogenase 2 enzyme (11β-HSD2), which protects the mineralocorticoid receptor from activation by glucocorticoids. In the kidney, mineralocorticoid receptors are expressed along the entire length of the connecting segment and collecting duct with only a small portion of cells negative for staining (157, 318). Expression of 11β-HSD2 has been found along the complete aldosterone-sensitive distal nephron; the localization to intercalated cells has, however, remained controversial (63, 376, 462). It is therefore possible that intercalated cells represent a direct target for the genomic effects of aldosterone.

Aldosterone regulates cell and transport functions through both rapid, nongenomic effects and transcription-dependent mechanisms that occur either early (within 30 min) or up to several hours (early genes), or alternatively start only after several hours (late genes) (for review, see Refs. 319, 488, 534, 535). Furthermore, aldosterone influences cell and transport functions either through direct changes in transcription/translation of target proteins or it
may act through intermediate proteins such as signaling molecules like K-ras or SGK (488, 534).

On a functional level, several strong links between aldosterone and proton secretion in the collecting duct have been found. 1) Stimulation of electrogenic Na\(^+\) reabsorption through the luminal epithelial Na\(^+\) channel is stimulated by aldosterone (488, 535). The resulting lumen-negative potential is thought to increase the electrical driving force for H\(^+\) secretion in the cortical collecting duct (76, 222). 2) A direct action of aldosterone on H\(^+\) secretion via transcriptional/translational pathways has been shown in turtle bladder and in rat medullary collecting duct (7, 143, 368, 491). 3) Na\(^+\) depletion in whole animal studies causes increased urinary H\(^+\) secretion, which is independent of the concomitant hypokalemia (148). 4) Metabolic acidosis increases aldosterone secretion from the adrenal glands (23, 219). 5) Adrenalectomy or inhibition of mineralocorticoid receptors cause metabolic acidosis (type IV dRTA, see below) in rat and human (124, 233, 458). 6) dRTA due to adrenalectomy can only be corrected if aldosterone is administered. 7) A number of monogenic diseases interfering with aldosterone synthesis, signaling, or its target proteins affect H\(^+\) secretion in the collecting duct leading to metabolic acidosis or alkalosis (see also below) (22, 39, 145, 311).

Even though aldosterone stimulated H\(^+\)-ATPase-dependent bicarbonate reabsorption in all collecting duct segments, different sensitivities of vacuolar H\(^+\)-ATPase enzymatic activity in distinct collecting duct segments have been described (275). Whereas overall enzymatic vacuolar H\(^+\)-ATPase activity was not altered in the cortical collecting duct, it was increased in the outer medullary collecting duct. The effect of aldosterone was independent of Na\(^+\) in medullary collecting ducts perfused in vitro, pointing also to mechanisms in addition to changes in electrical driving force (491). Also, even though aldosterone stimulated glucose utilization in turtle bladder, vacuolar H\(^+\)-ATPase stimulation was not linked to increased ATP availability (4). In addition, in isolated collecting duct segments, the action of aldosterone (incubation over several hours) was blocked by inhibitors of RNA and protein synthesis (7, 275). Finally, the effect of aldosterone is not mediated through the induction of hypokalemia or increased K\(^+\) secretion (148), which are also known to stimulate vacuolar H\(^+\)-ATPases in the collecting duct (28, 466). Recently, we found a rapid nongenomic effect of aldosterone on vacuolar H\(^+\)-ATPase-mediated H\(^+\) secretion in intercalated cells in isolated mouse outer medullary collecting ducts (572). The stimulatory effect of aldosterone could not be prevented by the mineralocorticoid receptor blocker spironolactone or by inhibition of RNA or protein synthesis. However, colchicine disruption of microtubules inhibited the aldosterone-induced stimulation, suggesting the involvement of vesicle trafficking processes in the response. In addition, inhibition of protein kinase C-dependent processes with chelerythrine abolished the stimulatory effect of aldosterone, pointing to a role of protein kinase C in this signaling cascade (572).

Taken together, these results suggest that aldosterone affects vacuolar H\(^+\)-ATPase activity in the collecting duct through different mechanisms that may be segment and cell type specific. Some of the aldosterone actions may require protein synthesis and/or protein trafficking.

D) ENDOTHELIN. Endothelin-1, a potent vasoconstrictor produced in endothelial cells, has recently emerged as a major hormone that regulates acid-base handling by the kidney. In cell culture, secretion of endothelin-1 is increased from renal endothelial cells in response to acidosis (565), and higher levels of endothelin-1 are found in animals with induced metabolic acidosis (562). The effects of endothelin-1 on acid-base transport are mainly mediated through ET\(_B\) receptors along the nephron (562). In the proximal tubule, endothelin-1 stimulates bicarbonate reabsorption through Na\(^+\)/H\(^+\) exchangers (292). In the distal tubule and collecting duct, endothelin receptor blockade (ET\(_B\)) inhibits acid-diet induced luminal acidification, and ET\(_B\) endothelin receptor activation decreases reduced HCO\(_3\) secretion and increases vacuolar H\(^+\)-ATPase-mediated proton secretion (562, 564). In addition, the adaptational increase in bicarbonate reabsorption and H\(^+\) secretion in the remnant kidney in nephrectomy animal models is blunted by blockade of ET\(_B\) receptors (563).

E) KALLIKREIN. The serine-protease kallikrein activates kininogen producing kinin which may be involved in regulating blood pressure by reducing renal Na\(^+\) absorption and dilating vessels (536). Particularly high concentrations of kallikrein are produced and secreted into the urine in the collecting duct (340). In metabolic acidosis kallikrein levels are increased, whereas in metabolic alkalosis kallikrein concentrations are reduced, suggesting a link with renal acid-base handling (340). Inhibition of kallikrein activity increases renal bicarbonate loss and induces mild metabolic acidosis (340). In the collecting duct, kallikrein reduces bicarbonate secretion and increases bicarbonate reabsorption (340). In MDCK cells with some features of intercalated cells, kallikrein inhibits a stilbene-sensitive Cl\(^-\)/HCO\(_3\)\(^-\) exchanger (518). It is, however, questionable if MDCK cells represent a reliable model for type B intercalated cells, and it is also unclear if the investigated Cl\(^-\)/HCO\(_3\)\(^-\) exchanger represents the type B intercalated cell apical stilbene-insensitive bicarbonate secreting transporter (e.g., pendrin).

F) THYROID HORMONES. There is limited information that thyroid hormones may influence vacuolar H\(^+\)-ATPase activity. Thyroid hormone stimulates HCO\(_3\) reabsorption in proximal tubules through Na\(^+\)/H\(^+\) exchange (339) and in distal tubules and collecting ducts possibly through vacuolar H\(^+\)-ATPases (339). Collecting duct HCO\(_3\) transport...
from patients with hypothyroidism is reduced, and these patients often present with distal renal tubular acidosis (403). Also, autoimmune thyroiditis is often associated with dRTA, even though it is not known whether dRTA develops secondary to hypothyroidism or is part of the autoimmune reaction (587).

G) CALCITONIN. Calcitonin, produced by thyroid parafollicular C cells, stimulates bone calcium accumulation and inhibits osteoclast activity, reduces renal Ca\(^{2+}\) excretion, and thus preserves whole body Ca\(^{2+}\) (123, 464). In metabolic acidosis, calcium carbonate is released from bone to buffer H\(^{+}\) resulting in rickets or osteomalacia if the underlying metabolic acidosis is left untreated (91, 135, 136, 302). In addition to its direct effects on body calcium, calcitonin also increases bicarbonate reabsorption in the cortical collecting duct by stimulating H\(^+\) -ATPase activity (371). In contrast, in MDCK cells, ADH stimulates vacuolar H\(^+\)-ATPase activity both through V\(_1\) and V\(_2\) receptors (399). Activation of V\(_1\) receptors raises intracellular Ca\(^{2+}\), whereas V\(_2\) receptors were coupled to the cAMP/PKA pathway. The stimulatory action of ADH required activation of both receptors and the synergy of the Ca\(^{2+}\)/protein kinase C and cAMP/PKA pathways (399).

2. Other hormones

Several other hormones or mediators have been shown to exert regulatory effects on bicarbonate transport and/or vacuolar H\(^+\)-ATPase activity (see Table 2). The physiological significance has not been clearly established for all of these effects. Isoproterenol reduces cortical collecting duct bicarbonate reabsorption and induces bicarbonate secretion via stimulation of the B-cell apical Cl\(^-\)/HCO\(_3^-\) exchanger (229, 516). Similarly, the vasoactive intestinal polypeptide (VIP) reverts bicarbonate reabsorption to secretion (307). In the medullary collecting duct, stimulation of H\(^+\) secretion has been shown by maneuvers increasing cAMP (230). In contrast, nitric oxide and cGMP inhibit H\(^+\)-ATPase activity in cortical collecting ducts (509). However, the physiological agonists for the activation of these pathways have not been identified yet.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Effect on Bicarbonate Reabsorption/Vacuolar H(^+)-ATPase Activity</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II</td>
<td>PT and CCD: stimulates bicarb. reabs. via Na(^+)/H(^+) (PT) and H(^+)-ATPase (PT, CCD)</td>
<td>192, 307, 540, 543</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>Stimulates bicarb. reabs. and H(^+)-ATPase activity directly (OMCD) and indirectly (CCD)</td>
<td>143, 148, 189, 287, 368, 491, 572</td>
</tr>
<tr>
<td>Endothelin</td>
<td>PT: stimulates bicarb. reabs. via Na(^+)/H(^+); CCD: stimulates A-IC, inhibits B-IC activity</td>
<td>292, 562–565</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>CCD: stimulates bicarb. reabs. (A-IC)</td>
<td>123, 464</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>CCD: stimulates bicarbonate secretion (B-IC)</td>
<td>230, 307</td>
</tr>
<tr>
<td>Vasopressin (ADH)</td>
<td>CCD: increases bicarb. reabs. (V1) or reduces (V2)</td>
<td>32, 307, 371, 399</td>
</tr>
<tr>
<td>Vasoactive intestinal polypeptide (VIP)</td>
<td>CCD: reduces bicarb. reabs.</td>
<td>307</td>
</tr>
<tr>
<td>NO</td>
<td>CCD: reduces bicarb. reab.</td>
<td>509</td>
</tr>
<tr>
<td>Kallikrein</td>
<td>CCD: increases bicarb. reab.</td>
<td>340, 518</td>
</tr>
<tr>
<td>Thyroid hormone</td>
<td>Stimulates final urinary acidification</td>
<td>339</td>
</tr>
</tbody>
</table>

A-IC, type A intercalated cells; B-IO, type B intercalated cells; PT, proximal tubule; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; bicarb. reab, bicarbonate reabsorption.
(diabetic nephropathy) or hypertension. These diseases also affect tubular function in several ways, not only reducing the transport capacity but also decreasing ammonium/ammonia production in the proximal tubule, which is necessary for final urinary acidification. On the basis of the site of impaired nephron function, acquired or inherited disorders leading to reduced bicarbonate reabsorption or proton secretion have been classified into four types of renal tubular acidosis.

A. Renal Tubular Acidosis

The various forms of renal tubular acidosis (RTA) are defined as a clinically and etiologically heterogeneous group of pathophysiological states that result from failure of the normal renal mechanisms that regulate systemic pH homeostasis. The clinical presentations of these states vary due to differences in the nephron region contributing to the tubular dysfunction. However, all of these states are characterized by metabolic acidosis accompanied by high serum chloride levels (145).

Type I (or distal) RTA (dRTA) results from a direct failure of the distal nephron to secrete acid into the tubular lumen. The generally accepted diagnostic criterion is the inability to acidify the urine below pH 5.5 in the setting of metabolic acidosis, which may be either spontaneous or induced by the administration of a systemic acid challenge. Associated clinical features may include hypokalemia, polyuria (increased urine output), hypercalciuria (high urinary calcium levels), nephrolithiasis, nephrocalcinosis (calcification of the renal parenchyma), nephrolithiasis/uroolithiasis (renal and urinary tract stones), growth retardation, and abnormalities of bone (rickets in children, osteomalacia in adults) (145, 431). Inherited forms of this type of RTA have been shown to be caused by mutations in the ATP6V1B1 (B1) or ATP6V0A4 (a4) subunits of the vacuolar H^+ -ATPase subunits that commonly underlie this trait, ATP6N1B, were revised by the HUGO Gene Nomenclature Committee in May 2002 (268, 474).

Other types of RTA include type II (proximal renal tubular acidosis, pRTA) characterized by an increased urinary fractional bicarbonate excretion due to defective proximal tubular bicarbonate reabsorption. Type III RTA is a mixed dRTA and pRTA. Type IV RTA is caused by aldosterone deficiency or unresponsiveness and shows a clinical picture similar to type I RTA except for hypokalemia (for review of RTA, see Ref. 145). The following paragraphs will focus on type I RTA (dRTA) because of the genetic and functional evidence for an important role of vacuolar H^+ -ATPases in its pathophysiology and etiology.

1. Primary dRTA

In children, dRTA almost always occurs as a primary condition (i.e., not associated with a systemic disease). Both autosomal dominant (100) and autosomal recessive (290) inheritance patterns have been reported. Patients with recessive dRTA are typically severely affected, presenting either with acute illness or with growth failure in the early years of life. In contrast, dominant dRTA is typically a milder disease (220, 269). In a subset of kindreds carrying mutations in the gene encoding for the ATP6V1B1 (B1) subunit, sensorineural hearing loss (SNHL) cosegregates with the renal phenotype (137). This hearing loss is progressive in nature (87), with the age at diagnosis of hearing loss quite variable, with reports as late as early adolescence (489). Severity ranges from mild-moderate impairment (87) to profound deafness (119).

2. SCL4A1 (AE-1/band 3) mutation in autosomal dominant dRTA

Several groups have shown that dRTA with dominant transmission is caused by mutations in SCL4A1, the gene encoding the band 3 Cl^-/HCO_3^- exchanger AE-1 (88, 89, 259, 269). In the renal type A intercalated cell, the Cl^- /HCO_3^- exchanger identified at the basolateral membranes represents a band 3 splicing isoform lacking the amino terminal 65 amino acids present in the erythrocyte band 3 protein (kAE-1) (72). The mutation in the AE-1 protein, therefore, affects the ability of A-intercalated cells to transport protons into the lumen of the collecting duct, and net acid secretion is reduced.

The majority of SCL4A1 dRTA mutations reported to date affect the codon R589 (8 of the 10 reported kindreds), raising the possibility that a dominant negative mechanism underlies the pathogenesis of dRTA in these patients; however, in vitro studies have suggested that the pathogenesis of dominant dRTA may be more complex than a simple loss of transporter activity (10, 88).

3. Recessive dRTA

While SCL4A1 is to date the only gene in which mutations have been reported in dominant forms of dRTA, the genetic basis of recessive dRTA (rdRTA) has proven more complex. SCL4A1 mutations have been occasionally reported in kindreds supporting recessive transmission of dRTA with hemolytic anemia (503, 529). However, examination of a larger cohort of kindreds excluded SCL4A1 mutation as a common cause of rdRTA by both linkage analysis and direct mutational screening (269). Subsequent genome-wide linkage analysis of kindreds supporting recessive transmission of dRTA has led to the identification of two genes encoding vacuolar H^+ -ATPase subunits that commonly underlie this trait, ATP6V1B1 and ATP6V0A4 (the original gene symbols, ATP6B1 and ATP6N1B, were revised by the HUGO Gene Nomenclature Committee in May 2002) (268, 474).

Given the well-established role of the H^+ -ATPase in H^+ secretion by intercalated cells in the collecting duct,
mutations in subunits can thus explain the failure of appropriate urinary acidification in these dRTA patients. Both ATP6V1B1 and ATP6V0A4 encode vacuolar H^+-ATPase subunits in the renal intercalated cell. ATP6V1B1 encodes the B1 isoform of the B subunit of the peripheral membrane (V_p) domain of the H^+-ATPase, while ATP6V0A4 encodes the a4 isoform of the a subunit of the transmembrane (V_o) domain. Both B1 and a4 subunit immunoreactivity has been detected at the apical plasma membrane of type A intercalated cells (386, 472, 474), establishing these subunits as components of the apical vacuolar proton pump that mediates proton secretion in the renal collecting duct.

B. Mutations in the ATP6V1B1 (B1) Subunit of the Vacuolar H^+-ATPase

1. Genome-wide linkage studies in rdRTA with kindreds

To investigate the genetic basis of recessive dRTA, Karet et al. (268) recruited 31 kindreds supporting recessive transmission of this trait. All index cases were diagnosed by the finding of an inappropriately alkaline urine (pH > 5.5) in the setting of a normal anion gap metabolic acidosis and renal potassium wasting; no evidence for secondary dRTA was present. Index cases came to clinical attention either because of severe dehydration and vomiting or because of failure to thrive and/or growth retardation. All were diagnosed by 6 years of age on average, with the majority presenting before 1 year of age. All cases had normal serum sodium, calcium, phosphate, creatinine, and magnesium. Nephrocalcinosis was present in all affected subjects, including infants; all patients for whom urine calcium was measured were also affected, one type of mutation, frameshift insertions or deletions occurring at position I386, has now been reported in six unrelated kindreds; it has been suggested that deletions were detected were notable for parental consanguinity, and as expected, affected individuals in these kindreds all harbor homozygous mutations. None of the identified mutations was found among a group of unrelated, unaffected individuals of similar ethnic origin, demonstrating that these mutations are specific for dRTA, rather than neutral polymorphisms present in the general population. While a variety of ATP6V1B1 mutations were identiﬁed, one type of mutation, frameshift insertions or deletions occurring at position I386, has now been reported in six unrelated kindreds; it has been suggested that this residue may represent a “mutation hotspot,” as the string of seven cytosine nucleotides at the site may provide a setting conducive to DNA polymerase slippage during replication. (268, 492).

2. ATP6V1B1 mutation in rdRTA

Screening of the entire ATP6V1B1 coding region as well as all intron-exon boundary sequences in those recessive kindreds supporting linkage to 2p13 as well as in additional kindreds classified as “sporadic” (i.e., kindreds in which a single affected individual is the offspring of unaffected, unrelated parents) led to the identiﬁcation of 15 different ATP6V1B1 mutations in 19 kindreds, which cosegregate with dRTA in each family (268). Several ATP6V1B mutations were identiﬁed that produce highly disruptive changes likely to result in loss of function of the encoded ATP6V1B1 protein; these mutations introduce premature termination codons, frameshift mutations, and splice site mutations. In other kindreds, missense mutations were detected; these missense and other mutations are dispersed along ATP6V1B1, rather than being clustered in a particular region of the protein, indicating that alterations in several regions of this protein led to a loss of B1 subunit, and by inference, vacuolar H^+-ATPase, function in intercalated cells. (268).

In all cases, the identiﬁed missense mutations alter amino acid residues that are conserved in evolutionary distant vacuolar H^+-ATPase B1 subunit homologs, suggesting crucial roles for these amino acid residues in H^+-ATPase function. All missense mutations identiﬁed alter residues conserved in B subunit homologs as phylogenetically distant as Neurospora and in some cases Archaeabacteria (495). All recessive kindreds in which mutations were detected were notable for parental consanguinity, and as expected, affected individuals in these kindreds all harbor homozygous mutations. None of the identiﬁed mutations was found among a group of unrelated, unaffected individuals of similar ethnic origin, demonstrat- ing that these mutations are specific for dRTA, rather than neutral polymorphisms present in the general population. While a variety of ATP6V1B1 mutations were identiﬁed, one type of mutation, frameshift insertions or deletions occurring at position I386, has now been reported in six unrelated kindreds; it has been suggested that this residue may represent a “mutation hotspot,” as the string of seven cytosine nucleotides at the site may provide a setting conducive to DNA polymerase slippage during replication. (268, 492).

3. Putative dysfunction of mutated B1 subunits

The exact mechanism(s) by which the variety of identiﬁed ATP6V1B1 mutations leads to impaired renal proton secretion, and thus dRTA, remains to be established. The putative function of the B1 subunit in the context of normal vacuolar H^+-ATPase structure and function has been discussed above. Comparison of the B1 subunit sequence with that of the analogous a-subunit of the F_1F_0-ATPase, for which the crystal structure has been reported (1), suggests that two of the identiﬁed ATP6V1B1 missense mutations (L81P and T275P) likely disrupt secondary structural motifs predicted for the B1
subunit (268). However, whether these mutant B1 subunits assemble in the vacuolar H\(^+\)-ATPase complex and whether other components of the vacuolar H\(^+\)-ATPase complex retain their ability to assemble and traffic appropriately to the apical surface of type A intercalated cells in the absence of normal B1 subunit is not yet known. The limited B1 subunit tissue expression pattern and accessibility has precluded study of these questions in tissue from dRTA patients harboring ATP6V1B1 mutations.

4. Hearing loss in dRTA patients harboring ATP6V1B1 mutations

In the majority (13/15) of dRTA kindreds in which ATP6V1B1 mutations were found, bilateral sensorineural hearing loss cosegregates with the renal phenotype (268). Consistent with a local role for the B1 subunit in inner ear function, ATP6V1B1 mRNA has been detected in human and mouse cochlea (268). In mouse cochlea and endolymphatic sac, B1 subunit immunoreactivity was observed in the interdental cell layer of the spiral limbus, an epithelial cell layer which forms part of the lining of the scala media and which thus is in direct contact with the inner ear fluid endolymph (268, 481). B1 subunit immunoreactivity was also detected in the epithelial cells of the endolymphatic sac (268), a major site of acid secretion into endolymph and the region of the inner ear where endolymph pH is subsequently lowest (167). However, the mechanism by which mutations in the B1 subunit cause hearing impairment requires further clarification.

5. ATP6V1B1 expression in other tissues

In the male reproductive tract of rats, the B1 subunit expression has been detected at high levels on the luminal plasma membranes and intracellular vesicles of a subset of epididymal cells (80) as well as subset of epithelial cells of the vas deferens (68). A role for these H\(^+\)-ATPase subunits in luminal acidification of the male reproductive tract has been demonstrated by the finding that in rat vas deferens, the H\(^+\)-ATPase inhibitor bafilomycin blocks up to 80% of net proton secretion into the lumen (68). Along much of the male reproductive tract, the luminal fluid is acidic, and it has been proposed that this acidity, mediated in part by H\(^+\)-ATPases, plays a role in sperm maturation and storage (85). B1 subunit mRNA has also been demonstrated in human placenta (524), where a bafilomycin-sensitive H\(^+\)-ATPase activity has been demonstrated in brush-border membranes (468). Together, these findings raise the possibility that fertility might be altered in dRTA patients harboring H\(^+\)-ATPase B1 subunit mutations; long-term clinical follow-up of the young dRTA cohort in which ATP6V1B1 mutations were detected may prove informative in this regard.

C. Vacuolar H\(^+\)-ATPase B1 Subunit (Atp6v1b1)-Deficient Mice

The phenotype of dRTA patients harboring ATP6V1B1 mutations demonstrates that with loss of function of the B1 subunit, and by inference loss of function of the distal renal apical proton pump, normal systemic pH cannot be maintained on a normal diet in humans. However, the relative contribution to distal urinary acidification of B1 subunit containing H\(^+\)-ATPase compared with other mechanisms of distal proton secretion (such as H\(^+\)-K\(^-\)-ATPases) has not been firmly established in humans (37). In this regard, the recent generation of mice deficient in Atp6v1b1, the murine vacuolar H\(^+\)-ATPase B1 subunit homolog (Atp6v1b1 \(-/-\)) (170), may provide a useful tool for dissecting mechanisms of distal urinary acidification.

Screening of a murine genomic library with human ATP6V1B1 cDNA probes led to the identification of Atp6v1b1, a gene encoding a 513 amino acid H\(^+\)-ATPase B subunit homolog sharing 93% identity with human ATP6V1B1. Conservation of genomic organization and syntenic chromosomal localization indicate that Atp6v1b1 is the murine ortholog of human ATP6V1B1 (169). Homologous recombination-mediated gene targeting of the Atp6v1b1 genomic locus was achieved by replacing Atp6v1b1 exons 7–11 with a neomycin resistance gene (170). Atp6b1 \(-/-\) chimeric mice gave rise to Atp6v1b1 \(+/-\) mice, which like human carriers of ATP6V1B1 mutation, were healthy and phenotypically normal. Intercrossing of Atp6v1b1 \(+/-\) mice gave rise to Atp6v1b1 \(-/-\) progeny that were born in predicted Mendelian ratios, providing no evidence for increased prenatal or neonatal lethality in the absence of functional B1 subunits.

Interestingly, while human dRTA patients harboring ATP6V1B1 mutation present as infants with metabolic acidosis, dehydration, growth retardation, and failure to thrive, Atp6v1b1 \(-/-\) mice appear healthy and grow normally. Atp6v1b1 \(-/-\) mice fed a standard laboratory diet produce urine that is significantly more alkaline than that of wild-type littermates, yet Atpv16b1 \(-/-\) mice are not acidic. After oral acid challenge, however, Atp6v1b1 \(-/-\) mice develop a metabolic acidosis that is more severe than that seen in wild-type controls, while maintaining an inappropriately alkaline urine, thus demonstrating the ability of these animals to serve as a useful model of recessive human dRTA (168). Clearance studies in anesthetized mice infused with furosemide suggest that the urinary acidification defect resulting from loss of functional B1 subunit may localize to the distal nephron; increased sodium delivery from the thick ascending limb, sufficient to induce a significant urinary acidification in wild-type mice, was not associated with urinary acidification in Atp6v1b1 \(-/-\) mice (27).
Measurement of Na\(^+\)-independent H\(^+\) flux (i.e., intracellular alkalization) from individual intercalated cells of single dissected \(Atp6v1b1\) +/- cortical collecting duct fragments has allowed insight into the relative contribution of B1 subunit containing vacuolar H\(^+\)-ATPases to proton secretion in this nephron segment. After an acute intracellular acidification, Na\(^+\)-independent H\(^+\) flux was significantly reduced to ~30% of wild-type levels, was concanamycin insensitive, and was similar to the wild-type H\(^+\) flux recorded in the presence of concanamycin. Together, these results indicate that most plasma membrane vacuolar H\(^+\)-ATPase activity in intercalated cells of the murine cortical collecting duct is mediated by vacuolar H\(^+\)-ATPases containing the B1 subunit, rather than another B subunit isoform (168). Whether proton secretion by intercalated cells in the medullary collecting duct also depends critically on the B1 isoform or whether another isoform can compensate remains to be determined.

Polarized expression of certain proteins with known intercalated cell expression (the vacuolar H\(^+\)-ATPase subunit A4 subunit, kAE-1, and pendrin) persists in cortical collecting ducts of \(Atp6v1b1\) +/- mice, suggesting that intercalated cell subtype differentiation persists (to at least some degree) in the B1 subunit (168). The rate of the vacuolar H\(^+\)-ATPase complex in the absence of functional B1 subunit, however, remains unknown. Whether the remaining subunits of the \(V_1\) domain assemble, and whether the \(V_1\) and \(V_0\) domains coassemble to form a nonfunctional vacuolar H\(^+\)-ATPase complex which does or does not reach its normal subcellular location will require further investigation. A recent study has shown that the B2 isoform is expressed together with the B1 isoform in intercalated cells from rat and mouse kidney (411). Under control conditions, the B2 isoform is expressed at lower levels compared with the B1 isoform and is mainly located in subapical vesicles. After stimulation of intercalated cells by chronic acetazolamide treatment (25), a significant amount of B2 isoform was detected in the apical membrane of type A intercalated cells, indicating that this isoform can potentially act as a compensatory isoform for the vacuolar H\(^+\)-ATPase activity. Future studies of \(Atp6v1b1\) +/- mice may prove a useful tool in elucidating the mechanisms of mammalian vacuolar H\(^+\)-ATPase assembly and/or trafficking.

Study of \(Atp6v1b1\) +/- mice has yet to suggest evidence for any phenotypes resulting from loss of B1 subunit function in extrarenal tissues. \(Atp6v1b1\) +/- mice of both sexes are fully fertile, providing no evidence to suggest an essential role of the B1 subunit in the male or female rodent reproductive tissues (170). Here again, a potential compensatory upregulation of the B2 subunit isoform still remains to be investigated. Auditory brain stem responses are normal in \(Atp6v1b1\) +/- mice, and the inner ears of these mice develop normally without evidence of any morphological abnormalities (138). Because the hearing loss in dRTA patients has been shown to vary in severity and age of onset, it is possible that additional genetic or environmental factors contribute to the development of sensorineural deafness in humans, but not mice, lacking functional B1 subunit. We have recently shown that the B2 subunit of the vacuolar H\(^+\)-ATPase is expressed in renal intercalated cells and in epididymal clear cells and that it can be located on the apical plasma membrane under some conditions that “activate” A-intercalated cells (411). Whether the B2 subunit is able to substitute for the B1 subunit to preserve or partially restore vacuolar H\(^+\)-ATPase function in all proton-secreting cells in B1-deficient mice remains to be determined.

D. \(ATP6V0A4\) Mutations in Patients with Autosomal Recessive dRTA

After the identification of \(ATP6V1B1\) as the gene product mutated in a subset of cases of rdRTA, genome-wide linkage analysis of a cohort of dRTA kindreds with normal hearing led to the mapping of a second locus for rdRTA at chromosome 7q33–34 (267). Notably, other than in hearing status, these kindreds were not found to vary significantly in clinical parameters from those rdRTA patients harboring \(ATP6V1B1\) mutations. A positional cloning approach led to the identification of the disease-causing gene, a novel kidney-specific gene with homology to the previously reported H\(^+\)-ATPase 116-kDa a subunit \(ATP6V0A1\) (former gene symbol \(ATP6N1B\)) (474).

Characterization of the \(ATP6V0A4\) genomic locus revealed that it comprises 23 exons; 20 of these exons encode the 840-amino acid a4 subunit of the vacuolar H\(^+\)-ATPase. Analysis of the \(ATP6V0A4\) cDNA clones indicated that each of the first three exons may be alternatively spliced to exon 4, which houses the translation initiation site (474). \(ATP6V0A4\) transcript was demonstrated in human fetal and adult kidney, but not in several other major organs.

Screening of the entire \(ATP6V0A4\) coding region as well as all intron-exon boundary sequences in nine rdRTA kindreds supporting linkage to chromosome 7q33–34 led to the identification of different \(ATP6V0A4\) mutations, which cosegregate with rdRTA, in eight of the kindreds (474). Several \(ATP6V0A4\) mutations were identified that produce highly disruptive changes predicted to result in loss of function of the encoded ATP6V0A4 protein; these mutations include a premature termination codon, frameshift mutations, and splice site mutations. In other kindreds, three missense mutations were detected. Two of the identified missense mutations alter amino acid residues that are conserved in all eukaryotic vacuolar H\(^+\)-ATPase a subunit homologs, suggesting crucial roles for
these amino acid residues in H^+-ATPase function; the third mutation (M580T) alters a residue located within a predicted transmembrane region, and which is equally hydrophobic in other a subunit homologs. All recessive kindreds in which mutations were detected were notable for parental consanguinity, and as expected, affected individuals in these kindreds all harbor homozygous mutations. None of the identified mutations was found among a group of unrelated, unaffected individuals of similar ethnic origin, demonstrating the specificity of these mutations for rdRTA.

The finding of a variety of different types of mutations located at multiple sites along the predicted ATP6V0A4 protein provided proof that mutations in this subunit can cause rdRTA. Importantly, the renal phenotype of rdRTA patients harboring ATP6V0A4 mutations provides functional evidence to suggest that vacuolar H^+-ATPases in the kidney do indeed contain a 116-kDa a subunit, an issue that previously has been debated (204, 206).

1. Expression of a4 in the proximal tubule and collecting duct

ATP6V0A4 immunoreactivity was detected at the apical surface of intercalated cells of the human cortical collecting duct and appeared to colocalize with ATP6V1B1, as judged by the similar distributions of these subunits in serial tissue sections (474). However, while both B1 and a4 subunit expression has been detected in all subtypes of intercalated cells of the cortical and medullary collecting duct (386, 472, 474, 482), only a4 subunit immunoreactivity has also been detected in the brush-border and subapical compartment of human proximal tubule, as well as in an apical pattern in the distal convoluted tubule (482). A similar proximal tubule localization for the a4 isoform was also reported in mouse (471, 482). The finding is puzzling given that the biochemical properties of ATP6V0A4 are identical to those of ATP6V1B1 but not to those of ATP6V0A4 (471); however, this finding does not exclude the possibility that mutation in one or more of these genes might underlie rdRTA in additional kindreds.

2. Expression of the a4 isoform in male reproductive tract and ear

In the epididymis and vas deferens, a4 isoform immunoreactivity has been detected in the luminal surface of a subset of epithelial cells, the narrow and clear cells, in mice (472) and rat (Pietremont, Futi, and Breton, unpublished observations). ATP6V0A4 expression has not yet been reported in human male reproductive tissues, however. In addition, Stover et al. (492) reported audiometrical follow-up of rdRTA patients from the initial cohort reported by Smith et al. (474) found to harbor ATP6V0A4 mutations. Interestingly, multiple dRTA cases who had documented normal audiograms when referred for study have since been found to have mild to moderate hearing loss and/or hearing loss that became evident in the second to fourth decade of life. ATP6V0A4 mRNA has been detected in fetal cochlea as well as adult vestibular epithelium (492). Why the auditory phenotype should differ in severity of hearing loss in patients with ATP6V1B1 and ATP6V0A4 mutations, however, remains to be established.

E. Evidence for Further Genes

A small number of rdRTA kindreds remain that were not found to harbor mutations in either ATP6V1B1 or ATP6V0A4 (492). Genetic linkage analysis has identified a small number of kindreds (one with hearing loss, one with audiometrically normal hearing, and two with unknown hearing status) that do not support linkage to either chromosomal locus, suggesting the existence of additional, as yet unknown, loci underlying this trait (492).

Candidate gene approaches prove useful in the identification of additional genetic loci underlying rdRTA. Subunits of the apical vacuolar H^+-ATPase of the type A intercalated cell would naturally prove attractive candidates. Given the central importance of the H^+-ATPase in normal cellular maintenance functions, it might be expected that disease would be caused only by mutation in a H^+-ATPase subunit; which, like the B and a subunits, has multiple tissue-specific subunit isoforms. Mutations in subunits with more ubiquitous patterns of expression might lead to embryonic lethal phenotypes, as was in fact observed after targeted disruption of both the murine H^+-ATPase proteolipid subunit (254) and accessory Ac45 subunit (450).

Recently, Smith et al. (471) reported the cloning of three novel genes, ATP6V1C2, ATP6V1G3, and ATP6V0D2, which represent tissue-specific isoforms of the human vacuolar H^+-ATPase C, G, and d subunits, respectively, with renal expression. Screening of these genes in eight rdRTA kindreds that are unlinked to either ATP6V1B1 or ATP6V0A4 failed to identify any potential disease-causing mutations in these subunits (471); however, this finding does not exclude the possibility that mutation in one or more of these genes might underlie rdRTA in additional kindreds.

In addition to subunits of the vacuolar H^+-ATPase, defects in a variety of gene products might explain the inappropriate distal urinary acidification, and thus the inadequate regeneration of HCO\textsubscript{3}^- which results in the metabolic acidosis seen in rdRTA. The complex pathway
of H\(^+\) and HCO\(_3\)\(^-\) handling in the type A intercalated cell suggests multiple gene products in which mutation might be predicted to inhibit proton secretion or bicarbonate reabsorption in this cell type. Indeed, mutations in carbonic anhydrase II (CAII), have been demonstrated to underlie the autosomal recessive syndrome of renal tubular acidosis, osteopetrosis, cerebral calcification, and mental retardation (434); however, as CAII also mediates HCO\(_3\)\(^-\) reabsorption in the proximal tubule, both a pRTA and dRTA are present in these patients.

A proton secretory defect might also be caused by mutation in genes encoding subunits of the H\(^+\)-K\(^+\)-ATPases expressed in the apical membranes of the type A intercalated cell. Mutations in other proteins necessary for targeting any of the above transporters to their appropriate subcellular localization or for regulating the rates of activity of these transporters might be predicted to cause a distal renal acidification defect. However, no defect in renal acidification has been reported from mice with a targeted disruption of either the gastric or colonic isoforms of the \(\alpha\)-subunit of the H\(^+\)-K\(^+\)-ATPase (356, 480). Likewise, mutations in the cellular machinery mediating Cl\(^-\) recycling in the type A cell might also be expected to cause a dRTA phenotype (33, 88, 269). Indeed, a recent mouse model deficient for the Kcc-4 (Sclc12a7) K\(^+\)-Cl\(^-\) cotransporter displays distal renal tubular acidosis and deafness. Expression of Kcc-4 was found in type A intercalated cells, and Kcc-4 is thought to participate in basolateral Cl\(^-\) recycling (54).

F. Vacuolar H\(^+\)-ATPase Loss or Dysfunction in Acquired Diseases

A number of acquired diseases are associated with changes in expression and function of renal vacuolar H\(^+\)-ATPases as demonstrated for Sjogren disease, acute ureteral obstruction, or intoxications with toluene or amphotericin B.

1. Sjogren's disease

Sjogren's disease is a systemic autoimmune disease involving the lachrymal and salivary glands as well as the central nervous system and the kidneys. The most common symptoms are dry eye and dry mouth, rheumatoid symptoms, and nephritis, which may be combined with RTA (116, 128). Several patients have been reported with primary Sjogren's disease and dRTA. The dRTA in these patients has been classified as a secretory defect and led to the assumption that defects in vacuolar H\(^+\)-ATPase activity may be associated. The serum of some patients contained antibodies reactive with structures in intercalated cells of the collecting duct and showed reactivity with purified vacuolar H\(^+\)-ATPase complexes by Western blotting. In addition, a loss of vacuolar H\(^+\)-ATPases (E1, A, and B1 subunits) and in some cases a reduction or loss of AE-1 Cl\(^-\)/HCO\(_3\)\(^-\) exchanger expression was detected by immunohistochemistry in kidney biopsies from these patients (116, 128, 224, 263). It should be noted that loss of vacuolar H\(^+\)-ATPase expression or function has been shown only in a small subset of patients with Sjogren's disease, and this phenotype might be present only in a subset of patients with Sjogren's disease. Indeed, other reports indicate that other patient populations with dRTA and Sjogren's disease did not show alterations in the expression pattern of vacuolar H\(^+\)-ATPases in their kidneys (36). In addition, other autoantibodies may be directed against other components of the acid-secreting machinery as shown for one patient with autoantibodies against CA II (264).

2. Acute ureteral obstruction

Acute obstruction of urinary flow is often associated with a defect in urinary acidification, which may occur before as well as after the relief of obstruction. In patients, dRTA is combined with hyperkalemia and increased urinary Na\(^+\) loss (40). The defect is corrected in some patients by aldosterone application similar to type IV RTA due to aldosterone deficiency (40). In most patients dRTA persists even after aldosterone supplementation. In animal models, obstruction results in abnormally alkaline urine (142). A decrease in vacuolar H\(^+\)-ATPase activity in isolated perfused medullary collecting ducts and dissected IMCD but not cortical collecting ducts has been described (297, 437, 519). In an immunohistochemical study, a discontinuity of apical staining in intercalated cells of the cortical collecting duct in obstructed kidneys was noted as well as a reduction in number of intercalated cells with a clear apical "rim" staining (422).

3. Drugs

The use of several drugs as well as the ingestion or abuse of some toxic substances may result in secondary forms of dRTA. The substances described include lithium, toluene, or amphotericin B. Lithium is regularly used to treat bipolar mood disorders and may induce dRTA (38, 142, 380). In some studies a leak pathway has been postulated where protons leak back into the cell resulting in net loss of base equivalents (380). In contrast, other authors showed a reduction in proton secretion in the collecting duct in patients (38). In agreement with these studies, lithium did not induce bicarbonate leak but reduced the membrane potential in isolated turtle bladder resulting in a decrease of proton secretion (21). Proton secretion was restored when the membrane potential was clamped to initial values. Also, the effect of lithium on distal urinary acidification was blunted in rats receiving amiloride, which itself decreased urinary acidification (353). These results suggest that the effect of lithium is at
least partly due to interactions with the amiloride-sensitive epithelial Na\(^+\) channel ENaC, which shows a high permeability for lithium (190). Its inhibition may result in a less lumen-negative potential in the cortical collecting duct reducing vacuolar H\(^+\)-ATPase activity. Paradoxically, a recent study has reported a dramatic increase in collecting duct cells expressing apical H\(^+\)-ATPase in lithium-treated rats, which would be expected to increase urinary net acid excretion (281).

Exposure to toluene, a volatile organic solvent, may also result in hypokalemia and hyperchloremic metabolic acidosis due to impaired urinary acidification. Inhalation of toluene may occur during work or has been used by so-called “toluene-sniffers” (156, 410, 501). Studies in isolated turtle bladder, a model for the collecting duct epithelium, indicated a reduction in vacuolar H\(^+\)-ATPase activity and a proton back-leaf into tissue after toluene exposure (41). Further metabolic studies in patients indicated that the metabolic acidosis may be also the result of a more general metabolic disturbance with urinary loss of K\(^+\) and Na\(^+\) due to excessive excretion of hippurate, a toluene metabolite (99).

Treatment with amphotericin B, an antifungal drug, has been shown in human and several animal models to impair distal urinary acidification by a mechanism described as a gradient lesion leading to proton back-leaf into cells (349, 433). Animal experiments have confirmed that the rate of H\(^+\) secretion in the collecting duct is not reduced under amphotericin B treatment but that the proton gradient from urine to the cell cannot be maintained, suggesting a back-leaf of protons (142, 202, 433). The defect in tubular function, however, is not restricted to urinary acidification, and defects in water reabsorption and urinary concentration have also been found (582).

X. SUMMARY AND OPEN QUESTIONS

Even though great progress has been made in the past decade in elucidating molecular mechanisms underlying acid-base transport in the nephron, and the important role of vacuolar H\(^+\)-ATPases, highlighted by the identification of mutations in subunits of the pump in patients with dRTA, some important questions remain to be answered.

Due to the complete sequencing of the human genome and its ongoing refinement, it is expected that in the very near future, all human subunits of the vacuolar H\(^+\)-ATPase will be identified, their gene structure analyzed, and patients with primary dRTA will be investigated for mutations in additional subunits. In parallel, the tissue- and cell-specific expression of distinct isoforms of the many subunits of the pump should be clarified as well as isoform-specific functional features. Based on this new molecular information, it may be possible to elucidate mechanisms underlying the cell and cell organelle-specific regulation of vacuolar H\(^+\)-ATPases which may be linked to the expression of certain subunit isoforms.

Another important field in the regulation of vacuolar H\(^+\)-ATPases is the identification of the molecular nature of the Cl\(^-\) conductance that shunts Cl\(^-\) in parallel with transported protons and which may modulate proton pump function and acidification both of vesicular compartments and at the plasma membrane. As discussed above, none of the candidate proteins identified so far fulfills all requirements or meets the functional properties found in preparations from native tissues.

A third interesting question relates to the fact that vacuolar H\(^+\)-ATPases can be found in opposite membranes of polarized cells and that the trafficking is regulated by only poorly understood mechanisms. The essential role of the vacuolar H\(^+\)-ATPase in endo- and exocytosis has been revealed, but the regulation of these processes and the role of the pump is not fully understood. Thus the identification of associated proteins governing the polarized expression and trafficking of vacuolar H\(^+\)-ATPases will be important to understand the physiology of this pump and the cells in which it is expressed.

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