Immunocytochemical Localization of Vacuolar H-ATPase in the Opossum (Monodelphis domestica) Kidney: Comparison With the Rat

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ABSTRACT

With two different antibodies (a monoclonal antibody to the C terminus of the 31-kd subunit of H-ATPase and a polyclonal rabbit antiserum to whole bovine H-ATPase) the vacuolar-type H-ATPase pump in the different nephron segments of the gray short-tailed (Monodelphis domestica) opossum kidney has been immunocytochemically localized. There was moderate staining of the brush border and subvillar invaginations in the proximal convoluted tubules (PCT) of the opossum kidney only with the rabbit antiserum and not with the monoclonal antibody. This was in contrast to the rat kidney, where both antibodies showed significant staining of the brush border and subvillar invaginations. There was very minimal staining in the apical region of the thick ascending limb cells of the opossum as compared with a mild degree present in the rat kidney. The pattern and intensity of staining were similar in the remaining distal nephron segments with the only difference being that type A intercalated cells in the outer and inner medullary collecting ducts were less polarized in the opossum kidney. These findings suggest that different isoforms of H-ATPase exist in the brush border of the PCT versus the intercalated cells of the collecting duct in the opossum and between the PCT of opossum and rat kidneys.

Key Words: Acid-base, H-ATPase, ATPase, proton-ATPase

The vacuolar-type (V-type) H-ATPase pump plays a major role in the maintenance of acid-base homeostasis by the kidney. In the proximal tubule, this pump is present in the apical membrane (brush border and subvillar invaginations) (1-3). It contributes up to 40% of the overall proton secretion in the proximal tubule of the rat (4,5). The final regulation of acid-base homeostasis is in the distal nephron, where proton transport is predominantly via the V-type H-ATPase pump (6-12). Acid secretion within the collecting duct (CD) is primarily regulated by the intercalated cells (IC). The IC are specialized CD cells that maintain dense arrays of the H-ATPase in a polarized distribution on the plasma membrane and mediate net acid or bicarbonate secretion depending on localization at the apical or basolateral membrane, respectively (1,12,13).

The gray short-tailed (Monodelphis domestica) opossum (14,15) is a marsupial and as such is born with primitive renal structures that develop while the pup is attached to the mother and eats in her pouch. We have used the North American opossum (Didelphis virginiana) to study normal and abnormal renal development in different models of renal dysplasia (16), obstruction (17), and fetal nephrotoxicity (18). An analysis of the structural and functional features of M. domestica opossum kidney may provide information useful for understanding normal and abnormal renal developmental physiology.

In this study, we have used two different antibodies to immunocytochemically localize the V-type H-ATPase in the opossum kidney. We describe the distribution of V-type H-ATPase in different nephron segments of the gray short-tailed (Monodelphis domestica) opossum kidney and compare it with the rat kidney. This is the first study relating to the V-type H-ATPase pump in this species.

METHODS

Animals

Two adult M. domestica opossums weighing 90 to 100 g and three Sprague-Dawley rats weighing 180 to 200 g were euthanized after an ip injection of pentobarbital (50 mg/kg body wt).

Antibodies

For immunocytochemical localization studies, two different antibodies were used. E11, a monoclonal...
antibody raised against a 10-amino-acid synthetic peptide derived from the predicted sequence of the carboxy terminus of the bovine H-ATPase 31-kd subunit (19,20), and R3, a polyclonal rabbit antiserum to whole bovine H-ATPase pump (12) (both were generous gifts from Dr. Stephen Gluck, Jewish Hospital, Washington University, St. Louis, MO). Bound primary antibodies were detected with fluorescein isothiocyanate-labeled goat anti-mouse and goat anti-rabbit secondary antibodies, respectively.

Immunocytochemistry

Kidney slices, 2 mm thick, were fixed in B5 (HgCl₂, 0.22 M; sodium acetate, 90 mM; formaldehyde, 3.7%) overnight. Tissue sections, 4-μm thick, were deparaffinized in xylene solution, dehydrated in decreasing concentrations of ethanol, and serially incubated in Lugol's iodine solution–5% sodium thiosulfate followed by phosphate-buffered saline (PBS, pH 7.4). To block nonspecific binding of the primary antibodies, sections were incubated in the blocking solution (10% calf serum, 10% goat serum, 1% polyethylene glycol [Mr, 20,000] in PBS) for 30 min. Sections were then incubated overnight with undiluted supernatant from hybridoma E11 or R3 rabbit antiserum diluted 1:100 (vol:vol, in blocking solution). Samples were subsequently rinsed in PBS, reblocked for 15 min, and incubated in appropriate secondary antibodies (diluted at 1:50 in blocking solution) for 20 min. Sections were rinsed in PBS and mounted in a fresh mixture of para-phenylene diamine (2 mg/mL) in 50% glycerol (vol:vol; in PBS). The slides were viewed with a Nikon Optiphot-2 mercury epifluorescent microscope (Nikon Incorporation, Instrument Group, New York, NY).

For the negative control, we replaced E11 monoclonal antibody and R3 rabbit antiserum with MOPC-21, a nonspecific murine monoclonal antibody, and nonimmune rabbit serum, respectively.

Immunoblots

Opossum and rat kidney microsomes were prepared as described previously (20). Microsomal proteins were resolved on sodium dodecyl sulfate–11.25% polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. Membrane strips were probed with E11 and R3 antibodies by use of the enhanced chemiluminescence technique (ECL Western blotting, Amersham Life Science, Amersham Co., Arlington Heights, IL), as per the manufacturer's recommended protocol.

RESULTS

In the rat kidney (Figure 1), the distribution of H-ATPase was similar whether E11 (Figure 1A) or R3 (Figure 1B) antibodies were used and was similar to the previous reports with E11 (20) or polyclonal antibodies affinity purified to three subunits (70, 56, and 31 kd) of the bovine kidney vacuolar H-ATPase.
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Staining was observed in the invaginations at the base of the microvilli (subvillar invaginations) in all proximal tubule segments and in the brush border (microvilli) of S1 and S2 segments (Figure 1A and B). Mild staining was present in the apical pole of the thick ascending limb cells. There was moderate luminal plasma membrane staining of the distal convoluted tubule (DCT) cells. In the connecting tubule (CNT), there was a faint staining in the apical region of the CNT cells, whereas IC showed an intense staining that was frequently in the apical distribution (type A).

In the opossum kidney (Figure 2), rabbit nonimmune serum resulted in no staining at all (Figure 2A), whereas R3 showed a moderate staining of the brush border and subvillar invaginations of the proximal convoluted tubule (PCT) (Figure 2B). E11, however, showed only a minimal staining in the apical region of the PCT (Figure 2C). The thick ascending limb cells showed only a very faint staining in the apical region. The staining in DCT and CNT (Figure 2C) was the same with both antibodies and was very similar to that in the rat kidney.

In the cortical collecting duct (CCD), IC comprised 47.5 and 45% of the cells in the opossum and rat kidneys, respectively, and were distinguished from principal cells by their heavy staining for H-ATPase (the principal cells had minimal or no staining). Several distinct immunocytochemical patterns of IC staining were identified in the CCD. The percentage of IC with well-polarized apical staining (WPA, stain limited to the apical region of the cell), poorly polarized apical staining (PPA, stain accentuated in the apical region although extending beyond the nucleus to the basolateral region), diffuse staining (D, diffuse homogenous staining), bipolar staining (BA, simultaneously distinct basolateral and apical staining), poorly polarized basolateral staining (PPB, stain accentuated in the basolateral region although extending beyond the nucleus to the apical region), and well-polarized basolateral staining (WPB, stain limited to the basolateral region of the cell) in the opossum and rat kidneys is shown in Table 1.

In the outer medullary collecting duct (OMCD), IC comprised 45.5 and 47% of the cells in the opossum and rat kidneys, respectively. The majority (>90%) of IC in the outer strip and all of the IC in the inner strip of the OMCD had a pattern of staining consistent with type A-IC, exhibiting a heavy staining at the apical pole in both species (Figures 1C and 3A).

**TABLE 1.** Comparison of the frequency of different subtypes of IC in the CCD

<table>
<thead>
<tr>
<th></th>
<th>%IC</th>
<th>%WPA</th>
<th>%PPA</th>
<th>%D</th>
<th>%BA</th>
<th>%PPB</th>
<th>%WPB</th>
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<tbody>
<tr>
<td>Opossum</td>
<td>47.5</td>
<td>9</td>
<td>69.5</td>
<td>7</td>
<td>5</td>
<td>9</td>
<td>0.5</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>45</td>
<td>13</td>
<td>55.5</td>
<td>7.5</td>
<td>8.5</td>
<td>14</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Rat

* WPA, PPA, D, BA, PPB, WPB are described in the Results.

Figure 2. Immunocytochemical staining of the cortex of opossum kidney with nonimmune rabbit serum (A), R3 rabbit polyclonal antiserum (B), and E11 monoclonal antibody (C). (A) Nonimmune rabbit serum resulted in no staining at all in the cortex. (B) R3 rabbit antiserum resulted in a pattern of staining similar to that described in Figures 1A and B. (C) E11 shows only a very minimal apical staining in the PCT, whereas the staining in the IC of the CCD, CNT, and DCT is very prominent. In the CNT, the IC show prominent staining throughout the cell, with accentuation in the apical pole, whereas CNT cells show only luminal membrane staining. In the DCT, cells show a prominent luminal membrane staining.
ever, the type A-IC in the OMCD of opossum kidney were less polarized when compared with that of the rat kidney. In the inner medullary collecting duct (IMCD), IC were present only in the initial third and exhibited apical staining consistent with type A-IC (Figure 3B). The IC, which comprised approximately one third of the cells at the junction of the IMCD and OMCD, rapidly declined to 0 by the end of the initial third of the IMCD. Again, it was noticed that the type A-IC were less polarized in the opossum than in the rat kidney.

MOPC-21 or nonspecific murine monoclonal antibody and nonimmune rabbit serum (replacing E11 and R3 antibodies, respectively) resulted in no staining at all in either opossum or rat kidney.

Immunoblot experiments with R3 antibody resulted in a high background, making accurate quantitation of the 31-kd bands very difficult. However, E11 antibody resulted in no background, and the data are presented in Figure 4 (Lanes 1 and 2 are rat, and Lanes 3 and 4 are opossum; cortex [c] or medulla [m]). With E11, the 31-kd immunoreactivity was always slightly lower in the opossum (both cortex and medulla) than in the rat kidney, indicating a slightly lower molecular weight of this subunit in the opossum. We also found that, although the 31-kd immunoreactive band was sharp and narrow in the opossum, it was broader with the appearance of being a composite of a few adjacent bands in the rat. The fact that E11 produces multiple bands clustered at ~31 kd, because of closely related isoforms, has been reported previously (19). Our results showing a broader 31-kd band in rat versus opossum suggests that E11 has recognized more isoforms in the former. This supports our immunocytochemical finding of brush border and IC versus only IC staining with E11 in the rat and opossum kidneys, respectively. The lower immunoreactivity noted with opossum medulla (Lane 4, Figure 4) is due to a larger contribution by an elongated papillary medulla, which is devoid of IC, in the opossum, resulting in a relative reduction of the 31-kd abundance per unit weight of the total tissue homogenate.

DISCUSSION

Although an epithelial cell line obtained from opossum kidney (OK cell line) has been well studied and found to be of proximal tubular origin (21), the opos-
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The opossum kidney itself has not been studied well, particularly in regard to acid-base regulation. In this study, we have used a monoclonal antibody (E11) to the C terminus of the 31-kd subunit and a rabbit polyclonal antiserum (R3) to the whole bovine H-ATPase for the immunocytochemical localization of the V-type H-ATPase in different nephron segments of the adult opossum kidney. We have also compared the distribution of the V-type H-ATPase in opossum and Sprague-Dawley rat kidneys. The main difference between the two species was that E11, which showed a moderately intense staining of the brush border and subvillar invaginations in the rat kidney (Figure 1A), produced only a minimal staining in the apical region of the PCT of the opossum kidney (Figure 2C). This was in contrast to a moderate staining generated by R3 antibody in both species (Figures 1B and 2B).

Because E11 monoclonal antibody is site specific (directed against a synthetic peptide derived from the predicted sequence of the C-terminal 10-amino-acid residues of the bovine kidney H-ATPase 31-kd subunit) whereas R3 antisem is against the whole bovine H-ATPase (recognizing 31-, 56-, and 70-kd subunits), the discrepancy of staining between E11 and R3 in the two species suggests that the 31-kd subunit in the PCT of opossum kidney has a different C-terminal amino acid composition than that of the Sprague-Dawley rat. Furthermore, the fact that E11 resulted in a very bright staining of the IC along the entire CD indicates that different isoforms of H-ATPase exist in the IC and brush border membrane of the opossum kidney and that they differ at least in their C terminus composition. Our immunoblots with E11 showing a slightly lower molecular weight of the 31-kd immunoreactive band in the opossum and a broader 31-kd immunoreactivity with the rat kidney also support the view that E11 reacts with different isoforms of this subunit in rat and opossum kidneys. However, the reduced anti-31-kd immunoreactivity observed with the opossum PCT may be due to a decreased reactivity of the E11 antibody (decreased sensitivity) with the opossum protein, and although we strongly speculate the presence of different isoforms, our results may not be considered conclusive at this time. The existence of different H-ATPase isoforms in the brush border membrane, kidney microsomes, and IC has been shown before (19,22). Furthermore, in a previous report of the kidney biopsy of a patient with Sjogren's syndrome and distal renal tubular acidosis, we showed the absence of PCT brush border membrane staining with an accentuation of subvillar invagination staining, suggesting that the loss of brush border membrane H-ATPase may be acquired in some pathologic states (23).

In both species, 45 to 47% of the cells in the CCD and OMCD were IC. In the CCD, around two thirds of the IC were of type A (apical staining) variety, and a majority (~85%) of them were poorly polarized. The remaining one third of the IC in the CCD were either poorly or well-polarized type B (basolateral staining) or had a diffuse or bipolar pattern of staining. In the inner stripe of the OMCD, all of the IC were of the type A variety, although the apical accentuation of staining was less prominent in the opossum kidney and at times they seemed to have a diffuse pattern of staining (Figure 3A). In the IMCD, both species showed prominent H-ATPase staining only in the IC, present in the initial third of the IMCD, and there was no H-ATPase staining detectable in either the principal cells or the IMCD cells (Figure 3C).

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REFERENCES

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Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature, as in dropsy. The course is the common one, namely, the kidneys and the bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease, then, is chronic, and it takes a long period to form; but the patient is short-lived. If the constitution of the disease be completely established, for the melting is rapid, the death speedy. Moreover, life is disgusting and painful; thirst unquenchable; excessive drinking, which, however is disproportionate to the large quantity of urine, for more urine is passed; and one cannot stop them either from drinking or making water.


(Aretaeus the Cappadocian probably lived in the 2nd century, A.D., and was a contemporary of Galen. He may have lived in Cappadocia, a region in Asia Minor near the river Euphrates.)