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Are There Several Isoforms of Na,K-ATPase α Subunit in the Rabbit Kidney?*

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Previous pharmacologic and kinetic studies have demonstrated the axial heterogeneity of the rabbit kidney tubule with regard to Na,K-ATPase. To evaluate whether this heterogeneity might reflect the presence of distinct isoforms of the α subunit of Na,K-ATPase, we used two monoclonal antibodies, IIC9 and IIE2 (G8), specific for the α_1 and α_3 isoforms, respectively, as probes for changes in the specific activity of Na,K-ATPase at the level of successive segments of the rabbit nephron. Single, well defined nephron segments were obtained by microdissection of collagenase-treated kidney. Results indicate that IIC9 antibody inhibited Na,K-ATPase activity by >90% in all the segments of the nephron except the collecting duct. Conversely, IIE2 (G8) antibody abolished Na,K-ATPase activity in the collecting duct, whereas it had no effect in other nephron segments. These findings suggest that the rabbit collecting duct preferentially expresses a distinct isoform of Na,K-ATPase catalytic subunit, which is presumably α_3 -like, in agreement with previous pharmacologic and kinetic observations, whereas other nephron segments would express the α_1 isoform.

Sodium-potassium-activated adenosine triphosphatase (Na,K-ATPase)¹ is a ubiquitous ion pump responsible for the generation of the Na⁺ and K⁺ gradients across the plasma cell membrane. Therefore, this protein plays a central role in the regulation of multiple physiological processes, especially in Na⁺-transporting epithelial cells such as kidney cells. Na,K-

ATPase consists of two polypeptides, a catalytic α subunit and a β subunit which is a glycoprotein. It is now clearly established that at least three isoforms of Na,K-ATPase α subunit exist (termed α_1 , α_2 , and α_3). These isoforms display distinct functional properties with regard to ouabain and sodium affinity (reviewed in Refs. 1 and 2) and are expressed in specific tissues.

In the mammalian kidney, expression of the α_1 isoform has been repeatedly reported (3–5). In fact, the kidney is considered as a model of α_1 -containing tissue. However, using specific antibodies, small amounts of α_2 and α_3 isoforms of Na,K-ATPase were detected in human, pig, and mink kidney (6, 7). This apparent discrepancy probably results from the heterogeneity of kidney tissue. Indeed, the nephron itself, which constitutes the anatomical and physiological unit of the kidney, consists of a succession of segments with specific cytological, biochemical, and physiological properties. If one considers the fraction of whole kidney tissue constituted by these different segments and their relative Na,K-ATPase content (8), it is clear that almost all the Na,K-ATPase activity determined in homogenates from kidney cortex originates from the proximal tubule, whereas that determined in homogenates from the medulla originates from the medullary thick ascending limb of Henle's loop. It is therefore possible that quantitatively minor segments such as the collecting duct might contain an isoform of Na,K-ATPase different from α_1 , which cannot be detected on whole kidney membrane preparations.

This problem was circumvented by studying the properties of Na,K-ATPase at the level of well defined, single pieces of nephron using a microassay for this enzymatic activity (9). Previously, we have reported that the sensitivity of renal Na,K-ATPase to its specific inhibitor ouabain and its affinity for Na⁺ increase from the proximal segments of the nephron to the collecting duct (10, 11). In the present study we evaluated whether these differences might reflect the presence of distinct isoforms by looking, on the level of the successive segments of the rabbit nephron, at the effects on Na,K-ATPase activity of two monoclonal antibodies specific for α_1 or α_3 isoforms.

MATERIALS AND METHODS

Antibodies—The monoclonal antibody IIC9 (subclass IgG 2a) was raised against pig kidney Na,K-ATPase and previously mapped to the region 887–904 of the α subunit (12). Purification of IIC9 antibody was performed on protein A-Sepharose (Pharmacia) according to the manufacturer's protocol. As indicated by immunofluorescence experiments, IIC9 binds to kidney epithelial cells (LLC-PK1 line) (12, 13).

The monoclonal antibody IIE2 (G8) was raised against the synthetic decapeptide GDKKDDKSSP corresponding to the N terminus of the α_3 isoform of the sodium pump. Free, uncoupled peptide was used for the production of the antibody. For some reason, this antibody does not recognize the whole α_3 molecule from rat axolemma on a Western blot, but does react with the native enzyme and the peptide itself in ELISA. IIE2 (G8) antibody (IgM class) was purified by gel permeation chromatography on Sephacryl S-300 in the presence of 0.5 M NaCl. Both antibodies were stored at 2 mg/ml 0.1% bovine serum albumin (BSA) and 50% glycerol.

Microdissection of Rabbit Nephron Segments—Male New Zealand rabbits weighing 1.0–1.5 kg were anesthetized with pentobarbital. The left kidneys were quickly removed and perfused with the renal artery with 20 ml of ice-cold solution containing 0.1% (w/v) collagenase in 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.33 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1 mM MgCl₂, 10 mM Tris-HCl, 1 mM CaCl₂, as well as 0.2% (w/v) BSA, pH 7.4. Thin pyramids were then cut out along the corticopapillary axis of the kidney and incubated for 25 min at 35 °C in the same collagenase-containing solution. After rinsing in a collagenase-free medium similar to the collagenase solution, except that CaCl₂ was

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¹ The abbreviations used are: Na,K-ATPase, sodium-potassium-activated adenosine triphosphatase; BSA, bovine serum albumin; PCT and PST, proximal convoluted and straight tubule, respectively; MTAL and CTAL, medullary and cortical thick ascending limb of Henle's loop, respectively; DCT, distal convoluted tubule; CNT, connecting tubule; CCD and OMCD, cortical and outer medullary collecting tubule, respectively; ELISA, enzyme-linked immunosorbent assay.

amount of the α_3 isoform in pig kidney. The N-terminal sequence of the protein purified by immunoaffinity chromatography was that of the α_3 isoform (6). Thus, we expected the IIE2 (G8) antibody to be a specific probe for the detection of α_3 isoform in nephron segments.

Both antibodies were shown to have effects on ATPase activity measured in a continuous spectrophotometric assay at 37 °C. Pig kidney Na,K-ATPase was inhibited by 25–40% following the addition of IIC9 antibody (1:5–1:20 ratio, w/w), whereas there was no significant change in the activity of the Na,K-ATPase from rat axolemma. These data confirm the previous conclusion that the IIC9 antibody is specific for the α_1 isoform. In contrast, IIE2 (G8) antibody consistently stimulated enzymatic activity of Na,K-ATPase from rat axolemma by 40% in comparison with control nonimmune mouse IgM used at same concentration. This effect was also reproduced when ascites fluids were used as the source of antibodies. Therefore, monitoring the effects of the specific antibodies on the activity

of Na,K-ATPase should allow us to get a valuable information about isoform distribution at the level of single nephron segments.

Since primary structures of catalytic subunits of Na,K-ATPase from rabbit tissues are not available yet, we tested first whether IIC9 and IIE2 (G8) antibodies can recognize Na,K-ATPase from rabbit kidney and brain, respectively, to exclude possible species-specific variations. In ELISA assay IIC9 antibody bound to the enzyme from rabbit, rat, or pig kidney with nearly equivalent affinity. Similarly, IIE2 (G8) reacted with microsomes from rabbit brain as well as from rat brain and axolemma (data not shown).

Fig. 3 summarizes the data of *in vitro* inhibition of tubule Na,K-ATPase from several rabbits. IIC9 antibody inhibited >90% Na,K-ATPase activity from the proximal tubule (PCT and PST), the thick ascending limb (MTAL and CTAL) and the distal tubule (DCT and CNT) confirming the predominance of the α_1 isoform in these nephron segments. Surprisingly, however, the antibody had no detectable effect on the ATPase from the collecting duct, which probably means that the epitope is absent or somehow modified.

Conversely, IIE2 (G8) antibody did not affect ATPase activity from any nephron segments other than the collecting duct. However, instead of stimulating the enzyme, as observed in the test tube when rat axolemma was tested, inhibition of Na,K-ATPase activity occurred in CCD and OMCD. This last finding was unexpected, as it suggests that collecting tubules exclusively express an unknown isoform of Na,K-ATPase catalytic subunit, presumably α_3 -like, that differs from the α_3 isoform detected in rat axolemma.

Inhibition on Na,K-ATPase activity by the IIC9 antibody in proximal convoluted tubule (PCT) and medullary thick ascending limb (MTAL) and by the IIE2 (G8) antibody in collecting duct (CCD and OMCD) did not occur in non-permeabilized tubules (in that case permeabilization was done after preincubation with antibodies, just before ATPase assay) (Table I). This finding provides evidence for the inaccessibility of both epitopes in intact nephron structures. The inhibitory effects were also totally abolished by heat denaturation of the antibodies (data not shown). Nonspecific IgG or IgM used at same concentrations as the IIC9 and IIE2 (G8) antibodies had no effect on Na,K-ATPase activity in either PCT, MTAL, or CCD (data not shown).

By studying the effect of specific antibodies on Na,K-ATPase activity at the level of single, well defined nephron segments, it was possible in this study to demonstrate the presence of two populations of Na,K-ATPase with different immunological properties along the rabbit nephron. Thus, the collecting duct was found to express a protein recognized by an antibody specific for the α_3 isoform of Na,K-ATPase catalytic subunit, whereas more proximal nephron segments contain a protein reacting with an antibody specific for the α_1 isoform. Combined with our previous pharmacological and kinetic data (10, 11)

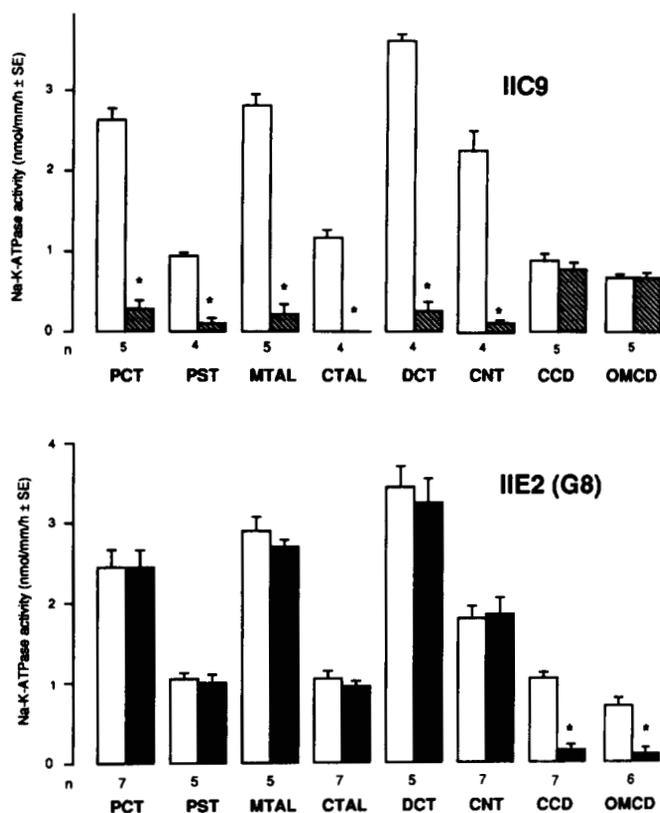


FIG. 3. *Top*, Na,K-ATPase activity was determined in the successive segments of rabbit nephron after preincubation in absence (open bars) or in presence of IIC9 antibody (hatched bars). *Bottom*, same as *top* using the IIE2 (G8) antibody. Values are means \pm S.E. from *n* animals. Values statistically different from controls (absence of antibody) were determined by Student's *t* test (*, $p < 0.001$).

TABLE I

Effect of tubule permeabilization on the inhibition of Na,K-ATPase by IIC9 and IIE2 (G8) antibodies

Before Na,K-ATPase measurement, nephron segments were preincubated for 1 h at 4 °C in the presence of either the IIC9 or the IIE2 (G8) antibody or of the solvent (PBS). Preincubation was carried out either on intact tubules (in that condition, tubules were permeabilized only before ATPase assay) or on permeabilized tubules (hypoosmotic shock plus freezing). Na,K-ATPase activity is expressed as $\text{nmol}\cdot\text{mm}^{-1}\cdot\text{h}^{-1} \pm \text{S.D.}$ The inhibitory effects of IIC9 (on PCT and MTAL) and of IIE2 (G8) (on CCD and OMCD) observed in permeabilized tubules were not seen in intact nephron segments.

	Intact tubules			Permeabilized tubules		
	PBS	IIC9	IIE2 (G8)	PBS	IIC9	IIE2 (G8)
PCT	2.73 \pm 0.16	2.92 \pm 0.31		2.81 \pm 0.38	0.16 \pm 0.14	
MTAL	2.92 \pm 0.36	3.04 \pm 0.54		3.01 \pm 0.62	0.37 \pm 0.22	
CCD	0.85 \pm 0.19		0.94 \pm 0.11	1.02 \pm 0.11		0.16 \pm 0.12
OMCD	0.51 \pm 0.83		0.48 \pm 0.10	0.46 \pm 0.09		0 \pm 0.06

indicating that rabbit collecting duct is more sensitive to ouabain and to sodium than more proximal nephron segments, these results strongly suggest that the collecting duct of the rabbit does preferentially express an α_3 -like isoform of Na,K-ATPase catalytic subunit. The fact that the population of Na,K-ATPase inhibited by the IIE2 (G8) antibody is detected exclusively in the collecting duct probably explains why it was not detected in most studies using whole kidney membrane fractions, since collecting ducts have low Na,K-ATPase activity (8) and only constitute a small fraction of the whole kidney tissue.

It should be mentioned, however, that the present results do conflict with recent data obtained at the level of specific segments of nephron. Indeed, *in situ* hybridization studies on rat kidney sections using rat α_1 -, α_2 -, and α_3 -ATPase cRNAs revealed the presence of α_1 -mRNA in all segments of the nephron and failed to demonstrate the presence of α_3 -mRNA in the collecting duct (21). This discrepancy might reflect species difference (rabbit *versus* rat). The relative proportion of α_1 -, α_2 -, and α_3 -mRNAs might also be different from that of the corresponding proteins if the specific half-lives of these mRNAs and/or proteins are different (22). Finally, and most probably, this apparent discrepancy may indicate that the collecting duct does not express the α_3 isoform, but rather an α_3 -like form structurally different from α_1 in the segment recognized by IIC9 antibody and similar to α_3 in the N-terminal region recognized by IIE2 (G8) antibody. The hypothesis is supported by our findings that the IIE2 (G8) antibody inhibits Na,K-ATPase activity from the collecting duct, whereas it stimulates that from rat axolemma. This α_3 -like form could resemble an α_1 - α_3 chimera or be a new isoform.

The presence of an α_3 -like isoform of the catalytic subunit of Na,K-ATPase in the collecting duct would make this nephron segment a preferential site for action of endogenous ouabain (23) in the kidney. This may be of physiological importance, since this nephron segment is the last one where urinary excretion of sodium and potassium, and thereby the whole body salt balance, is controlled.

In summary, the present study clearly reveals the presence of two populations of Na,K-ATPase with distinct immunological characteristics along the nephron of the rabbit. Proximal segments express an α_1 -related isoform, whereas the collecting

duct contains an α_3 -like isoform. Development of new methodological approaches such as polymerase chain reaction at the level of single nephron segments (24) will facilitate the molecular identification of this protein.

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