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## Are There Several Isoforms of Na,K-ATPase $\alpha$ 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  $\alpha$  subunit of Na,K-ATPase, we used two monoclonal antibodies, IIC9 and IIE2 (G8), specific for the  $\alpha_1$  and  $\alpha_3$  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  $\alpha_3$ -like, in agreement with previous pharmacologic and kinetic observations, whereas other nephron segments would express the  $\alpha_1$  isoform.

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

ATPase consists of two polypeptides, a catalytic  $\alpha$  subunit and a  $\beta$  subunit which is a glycoprotein. It is now clearly established that at least three isoforms of Na,K-ATPase  $\alpha$  subunit exist (termed  $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$ ). 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  $\alpha_1$  isoform has been repeatedly reported (3–5). In fact, the kidney is considered as a model of  $\alpha_1$ -containing tissue. However, using specific antibodies, small amounts of  $\alpha_2$  and  $\alpha_3$  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  $\alpha_1$ , 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<sup>+</sup> 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  $\alpha_1$  or  $\alpha_3$  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  $\alpha$  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  $\alpha_3$  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  $\alpha_3$  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 MgSO<sub>4</sub>, 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.44 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, 1 mM CaCl<sub>2</sub>, 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 CaCl<sub>2</sub> was

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<sup>1</sup> 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.

0.25 mm and collagenase and BSA were omitted, segments of nephron were dissected under a stereomicroscope. The different portions of the nephron were characterized at the time of their microdissection according to topographical and anatomical criteria as follows. Segments of proximal convoluted tubule (PCT) were dissected in the superficial kidney cortex next to their attachment to the glomerulus, proximal straight tubules (PST) were dissected from the outer stripe of the outer medulla and characterized by their attachment to the thin limb of Henle's loop. Medullary and cortical portions of the thick ascending limb of Henle's loop (MTAL and CTAL) were obtained from the inner stripe of the outer medulla and from the cortex, next to the macula densa, respectively. Distal convoluted tubule (DCT) and connecting tubule (CNT) were microdissected between the macula densa and the first branch point with another tubule and were distinguished from one another by their respective bright and granular appearance under microscopic observation. Collecting ducts were obtained either from the cortex (cortical collecting duct: CCD) below the last branching or from the outer medulla (outer medullary collecting duct: OMCD). After microdissection, the length of each piece of nephron, which served as reference for ATPase activities, was determined by automatic image analysis (14).

**Determination of Na,K-ATPase Activity**—Unless indicated otherwise, each piece of nephron was permeabilized by hypoosmotic shock and by rapid freezing in 0.2  $\mu$ l of distilled water. After thawing, 0.2  $\mu$ l of PBS buffer without or with IIC9 or IIE2 (G8) antibody was added to each sample. The dilution of antibody was calculated so as to obtain an antigen/antibody molar ratio in the  $1/50$ – $1/200$  range (Na,K-ATPase content of rabbit nephron segments was estimated from [ $^3$ H]ouabain binding measurements; Ref. 15). After 1 h of incubation at 4  $^{\circ}$ C, the medium surrounding tubular samples was aspirated and Na,K-ATPase activity was assayed as previously described (9).

Briefly, ATPase activity was determined by a radiochemical microassay (1  $\mu$ l incubation volume) based on the measurement of  $^{32}$ P<sub>i</sub> released from [ $\gamma$ - $^{32}$ P]ATP. Na,K-ATPase activity was taken as the difference between the mean activities of total ATPase (determined in the presence of sodium and potassium) and Mg-ATPase (determined in the absence of sodium and potassium and in the presence of ouabain). Each activity was measured on 4–6 replicate samples and expressed as nmol of ATP hydrolyzed/mm of tubule/h.

In each experiment, we compared Na,K-ATPase activity in nephron segments isolated from the same rabbit and preincubated in the absence or the presence of either IIC9 or IIE2 (G8) antibody.

RESULTS AND DISCUSSION

To characterize Na,K-ATPase in single nephron segments, two monoclonal antibodies were used as specific probes. As follows from Fig. 1, the IIC9 epitope is highly conserved between  $\alpha_1$  subunits from several mammalian species including human (16), rat (17), pig (18), sheep (19), and horse (20). Moreover, this region is very homologous among all three known isoforms of the rat  $\alpha$  subunit ( $\alpha_1$ ,  $\alpha_2$ , and  $\alpha_3$  isoforms) (17). However, ELISA done with Na,K-ATPase from pig kidney (containing mostly  $\alpha_1$  isoform) and rat axolemma (representing a mixture of  $\alpha_2$  and  $\alpha_3$  subunits along with an insignificant amount of  $\alpha_1$ ) revealed dramatic difference in the recognition of these enzymes by IIC9 antibody. As shown in Fig. 2A,  $\alpha_2$  and/or  $\alpha_3$  isoforms bound the antibody with substantially lower affinity, whereas  $\alpha_1$  isoform was recognized well in the same conditions. The same conclusion was reached by competition binding (data not shown). There are several substitutions in amino acid sequence of the IIC9 epitope among different isoforms (Fig. 1). Although most of them (two out of three in each case) are conservative, the N termini of the peptides look quite different.

FIG. 1. Alignment of partial sequences of  $\alpha_1$  from rat (17), pig (18), sheep (19), human (16), and horse (20) and rat  $\alpha_2$  and  $\alpha_3$  (17) isoforms of Na,K-ATPase in regions encoding the epitopes for IIE2 (G8) (2–11) and IIC9 (887–904). Dashes represent amino acids identical to those in the rat  $\alpha_1$  isoform.

M G K G V C R D K Y E P  
 - - - - -  
 - - - - -  
 - - R - - - - -  
 - - - - G - - - - -  
 - - R - A - - E - S -  
 - G D K K D D K S S P

IIE2 (G8)

IIC9

W	I	N	D	V	E	D	S	Y	G	Q	Q	W	T	Y	E	Q	R
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T	T	-	-	L	-	-	-	-	-	-	-	-	-	E	-	-	-
T	V	-	-	L	-	-	-	-	-	-	-	-	-	-	-	-	-

- $\alpha_1$  rat (17)
- $\alpha_1$  pig (18)
- $\alpha_1$  sheep (19)
- $\alpha_1$  human (16)
- $\alpha_1$  horse (20)
- $\alpha_2$  rat (17)
- $\alpha_3$  rat (17)

Therefore, we concluded that at suitable dilutions, the IIC9 antibody can be used as a specific probe for the  $\alpha_1$  isoform of the Na,K-ATPase.

The second antibody, IIE2 (G8), was chosen to evaluate specifically the presence of the  $\alpha_3$  isoform in nephron segments, since it was raised against a specific peptide belonging to the  $\alpha_3$  sequence and was found to recognize the enzyme from rat axolemma (the most enriched in  $\alpha_3$ ) in ELISA (Fig. 2B). It bound much less to pig kidney Na,K-ATPase than to rat brain axolemma containing  $\alpha_3$  and  $\alpha_2$ . A polyclonal antibody raised against the same peptide was previously used to detect a tiny

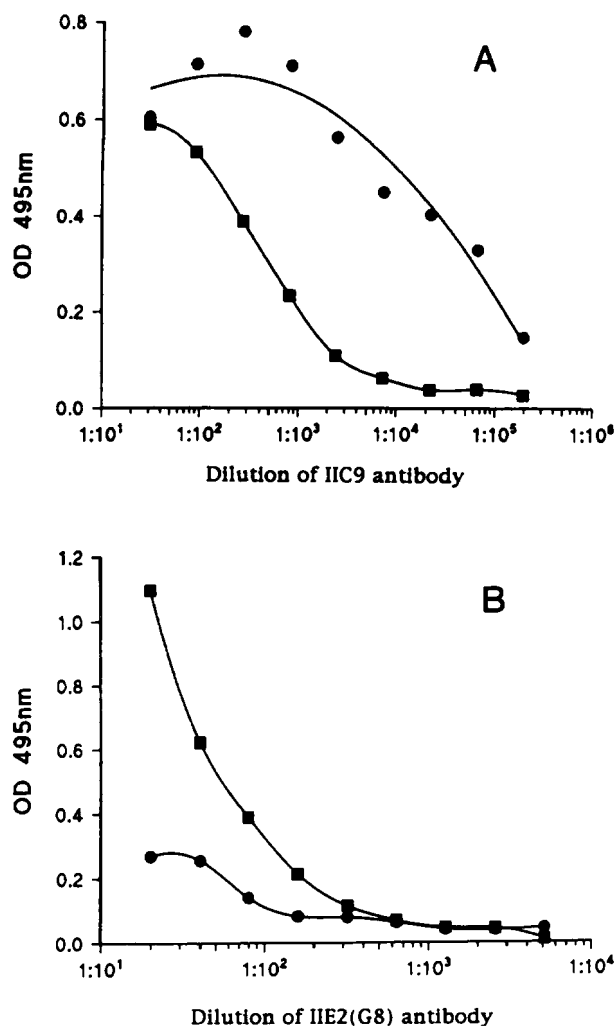


FIG. 2. Comparative binding of IIC9 and IIE2 (G8) antibodies to Na,K-ATPase by conventional ELISA assay. Crude microsomes (1  $\mu$ g/well) from pig kidney (●) and rat brain axolemma (■) were adsorbed to Nunc immunoplates and then incubated with different dilutions of IIC9 (A) and IIE2 (G8) (B) antibodies. The secondary antibodies were goat anti-mouse Ig, and color development was streptavidin-conjugated horseradish peroxidase (Bethesda Research Laboratories).

amount of the  $\alpha_3$  isoform in pig kidney. The N-terminal sequence of the protein purified by immunoaffinity chromatography was that of the  $\alpha_3$  isoform (6). Thus, we expected the IIE2 (G8) antibody to be a specific probe for the detection of  $\alpha_3$  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  $\alpha_1$  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  $\alpha_1$  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  $\alpha_3$ -like, that differs from the  $\alpha_3$  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  $\alpha_3$  isoform of Na,K-ATPase catalytic subunit, whereas more proximal nephron segments contain a protein reacting with an antibody specific for the  $\alpha_1$  isoform. Combined with our previous pharmacological and kinetic data (10, 11)

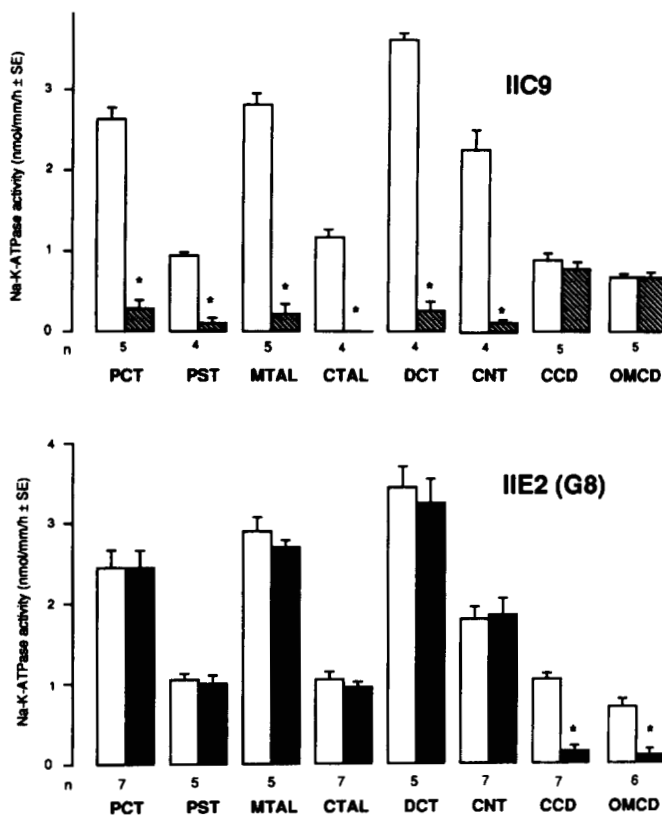


FIG. 3. *Top*, Na,K-ATPase activity was determined in the successive segments of rabbit nephron after preincubation in absence (open bars) or in presence of IIC9 antibody (hatched bars). *Bottom*, same as *top* using the IIE2 (G8) antibody. Values are means  $\pm$  S.E. from *n* animals. Values statistically 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  $\alpha_3$ -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  $\alpha_1$ -,  $\alpha_2$ -, and  $\alpha_3$ -ATPase cRNAs revealed the presence of  $\alpha_1$ -mRNA in all segments of the nephron and failed to demonstrate the presence of  $\alpha_3$ -mRNA in the collecting duct (21). This discrepancy might reflect species difference (rabbit *versus* rat). The relative proportion of  $\alpha_1$ -,  $\alpha_2$ -, and  $\alpha_3$ -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  $\alpha_3$  isoform, but rather an  $\alpha_3$ -like form structurally different from  $\alpha_1$  in the segment recognized by IIC9 antibody and similar to  $\alpha_3$  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  $\alpha_3$ -like form could resemble an  $\alpha_1$ - $\alpha_3$  chimera or be a new isoform.

The presence of an  $\alpha_3$ -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  $\alpha_1$ -related isoform, whereas the collecting

duct contains an  $\alpha_3$ -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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