Functional Role and Immunocytochemical Localization of the \( \gamma_a \) and \( \gamma_b \) Forms of the Na,K-ATPase \( \gamma \) Subunit*

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The \( \gamma \) subunit of the Na,K-ATPase is a member of the FXYD family of type 2 transmembrane proteins that probably function as regulators of ion transport. Rat \( \gamma \) is present primarily in the kidney as two main splice variants, \( \gamma_a \) and \( \gamma_b \), which differ only at their extracellular N termini (TELSANH and MDRWYL, respectively; Kuster, B., Shainskaya, A., Pu, H. X., Goldshleger, R., Blostein, R., Mann, M., and Karlshis, S. J. D. (2000) J. Biol. Chem. 275, 18441–18446). Expression in cultured cells indicates that both variants affect catalytic properties, without a detectable difference between \( \gamma_a \) and \( \gamma_b \). At least two singular effects are seen, irrespective of whether the variants are expressed in HeLa or rat \( a1 \)-transfected HeLa cells, i.e. (i) an increase in apparent affinity for ATP, probably secondary to a left shift in \( E_1 \leftrightarrow E_2 \) conformational equilibrium and (ii) an increase in \( K^+ \) antagonism of cytoplasmic Na* activation. Antibodies against the C terminus common to both variants (anti-\( \gamma \)) abrogate the first effect but not the second. In contrast, \( \gamma_a \) and \( \gamma_b \) show differences in their localization along the kidney tubule. Using anti-\( \gamma \) (C-terminal) and antibodies to the rat \( \alpha \) subunit as well as antibodies to identify cell types, double immunofluorescence showed \( \gamma \) in the basolateral membrane of several tubular segments. Highest expression is in the medullary portion of the thick ascending limb (TAL), which contains both \( \gamma_a \) and \( \gamma_b \). In fact, TAL is the only positive tubular segment in the medulla. In the cortex, most tubules express \( \gamma \) but at lower levels. Antibodies specific for \( \gamma_a \) and \( \gamma_b \) showed differences in their cortical location; \( \gamma_a \) is specific for cells in the macula densa and principal cells of the cortical collecting duct but not cortical TAL. In contrast, \( \gamma_b \) but not \( \gamma_a \) is present in the cortical TAL only. Thus, the importance of \( \gamma_a \) and \( \gamma_b \) may be related to their partially overlapping but distinct expression patterns and tissue-specific functions of the pump that these serve.

A small membrane protein, \( \gamma \), first described over 20 years ago in purified kidney Na,K-ATPase preparations (1, 2) associates, in approximately equimolar amounts, with the \( \alpha \) and \( \beta \) subunits (3, 4). Molecular cloning of the \( \gamma \) subunits of rat, mouse, cow, and sheep indicated a molecular weight of ~6500 (5). Cloning and sequencing of the human (6) and Xenopus laevis (7) \( \gamma \) subunits have also been reported. Comparison of sequences shows ~75% homology among \( \gamma \) subunits of the aforementioned different species but is much higher (93%) for only mammalian sequences. Further structural analysis has shown that \( \gamma \) comprises a single transmembrane domain and has an N-terminus-out, C terminus-in topology (7, 8). In addition, two major forms have been recently identified at the molecular level as described below.

On SDS-polyacrylamide gel electrophoresis, the \( \gamma \) subunit runs as a doublet (apparent molecular masses of ~8 and ~9 kDa) (5, 8), and a doublet is observed following expression in tissue culture cells (8, 9) and in vitro expression in the presence (5) but not absence of pancreatic microsomes (5, 7). Recent mass spectrometry of the \( \gamma \) chains of rat kidney Na,K-ATPase showed that \( \gamma_u \) (upper band on SDS-polyacrylamide gel electrophoresis) has a mass of 7184.0 ± 1 Da (carbamidomethyl cysteine) (10), corresponding closely to that for the published sequence without the initiator methionine (11), while \( \gamma_l \) (lower band) has a mass of 7337.9 ± 1 Da. Tryptic peptide mapping and sequencing by mass spectrometry reveals that the seven N-terminal residues of \( \gamma_u \), TELSANH, are replaced by Ac-MDRWYL in \( \gamma_l \), but otherwise the two chains are identical. These sequences are identical to those obtained by searching the expressed sequence tag data base (12). Expression of \( \gamma_u \) or \( \gamma_l \) cDNAs in human embryonic kidney (HEK) as well as HeLa cells was analyzed by Western blotting using antisera raised against a peptide representing the C-terminal 10 residues of the \( \gamma \) subunit. The results showed clearly that the major bands expressed correspond to \( \gamma_u \) or \( \gamma_l \) of the renal Na,K-ATPase. Additional minor bands seen after transfection, namely \( \gamma_u^* \) in HEK and \( \gamma_l^* \) in HeLa cells imply that these are cell-specific posttranslational modifications (10).

Although earlier studies showed that the \( \gamma \) subunit is co-expressed with \( \alpha \) and \( \beta \) ATPase subunits in kidney and not at the surface of Xenopus oocytes in the absence of \( \alpha \) and \( \beta \) subunits (7), Jones et al. (13) have reported expression of \( \gamma \) in the absence of the sodium pump on the apical surface of mouse blastocysts. In contrast with the ubiquitous localization of \( \alpha \) and \( \beta \) subunits, however, the \( \gamma \) subunit is expressed in a limited number of organs (8). Earlier studies showed identical expression patterns of \( \alpha \) and \( \gamma \) in renal proximal tubules and collect-

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1 The abbreviations used are: HEK, human embryonic kidney; TAL, thick ascending limb of Henle’s loop; cTAL and mTAL, cortical and medullary portions, respectively, of the thick ascending limb of Henle’s loop; MD, macula densa; AQP2, aquaporin 2.
ing ducts as well as co-immunoprecipitation of the γ subunit with both the α and β subunits in kidney membranes (5).

The functional role of the γ subunit has only recently begun to be investigated. Although it was previously shown that the γ peptide is not necessary for function (4, 14, 15) and γ subunit mRNA could not be detected in many tissues in both mammals (5, 6) and amphibia (7), recent experiments have shown that γ has an important functional role in some systems. Thus, treatment of mouse blastocysts with γ subunit antisense oligodeoxynucleotide reduced the amount of expressed γ subunit and caused a reduction in ouabain-sensitive 86Rb⁺ transport as well as delayed blastocoele formation (13). A recent report describing a mutation in γ in a family with dominant renal hypomagnesemia suggests a role of γ in magnesium reabsorption (16).

Studies in our laboratory (8, 11) have shown that γ is a tissue-specific regulator of the Na,K-ATPase and that it causes an increase in the apparent affinity of the enzyme for ATP in a manner that is reversible by anti-γ antisera. The specific effect of anti-γ on ATP affinity implies a specific structural interaction whereby the γ subunit counteracts short term changes in ATP concentration in kidney cells in which ATP utilization is high. A role of the γ subunit in interactions of the Na,K-ATPase with K⁺ was suggested on the basis of findings that the γ subunit is a component of the protein complex found in so-called “19-kDa membranes,” the product of tryptic purification. Anti-

of the last 10 residues and used for enzyme assays without further purification. Anti-γ and anti-α antibodies were raised against N-terminal peptides TELSANHC and MDRWLYLC, respectively, after coupling to keyhole limpet hemocyanin. Anti-γ was purified on an affinity column of TELSANHC. Attempts to affinity-purify anti-γ were unsuccessful. For affinity purification, peptides were coupled to Poros activated affinity chromatography columns (epoxide or amino), and the antibodies were purified on a BioCad Perfusion Chromatography appa-ratus. The antibody was eluted off the column in a solution of 0.2 M Tris-HCl, pH 2, neutralized immediately with Tris base, and diluted 1:1 with glycerol, and 0.02% sodium azide was added for preservation.

Immunocytochemical experiments were performed with affinity-purified anti-γ (C-terminal) diluted 1:200 and anti-γ, antibodies (1:100) and γ subunit antisera (1:100). In addition, the following polyclonal antibodies (as described in Ref. 20) have been used for co-localization: (i) anti-aquaporin 2 (AQP2) antisierum, (1:100); (ii) anti-Tamm-Horsfall protein antibody (sheep anti-romucoid from Biodiagnostic International, Kennebunk, ME; 1:50); (iii) antibody against the alpha subunit of Na,K-ATPase (1:50).

Immunolocalization of the γ Subunit of Na,K-ATPase in the Kidney—Rat kidneys were frozen in liquid N₂. Immunolocalization was performed as previously described (20) by incubating cryostat sections with the different anti-γ subunit antibodies (γ-C-terminal, γα, and γβ) and with a secondary antibody (goat anti-rabbit Fab fraction, Jackson; 1:200) coupled to the fluorochrome CY3 (red fluorescence). For colocalization experiments (20), the CY3-labeled sections were incubated with antibodies against different protein markers (see above) and then overlaid with fluorescein isothiocyanate-labeled (green fluorescence) goat anti-rabbit IgG Fab fraction or donkey anti-sheep antibody. The specificity of the anti-γ antibodies in immunofluorescence studies was established in competition experiments by including their respective peptides in the treatment of the kidney sections (data not shown).

Cloning and Transfection of Rat γα and γβ cDNAs—The cloning and transfection of rat γα and γβ cDNAs were carried out exactly as described for wild-type HeLa cells (10), except that a stable cell line expressing rat γα was used (α1-HeLa cells obtained as a gift from E. Jewell and J. B Lingrel; see Ref. 21). After transfection, the cells were cultured for 3 weeks in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and selected by including 400 μg/ml hygromycin B and 1 μM ouabain (10) in the medium. Western blot analysis was carried out as described previously (8) except using antibodies against the C terminus (γC35) and against the N termini of γα and γβ. Quantitative PhosphorImaging was carried out using a Storm PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Enzyme Assays and Kinetic Analysis of Data—Membranes were prepared from HeLa cells as described in Ref. 21. Unless indicated otherwise, Na,K-ATPase and Na-ATPase assays were carried out as described previously (11). Na-ATPase activity refers to activity measured at low (1 or 10 μM) ATP concentration, in the absence of K⁺. For experiments with HeLa cells expressing the ouabain-resistant rat α1 catalytic subunit (α1-HeLa), the cells are grown in 1 μM ouabain and assayed in medium containing a low (5 μM) ouabain concentration. Activities shown are the difference between ATP hydrolysis measured in the presence of low (5 μM) and high (5 mM) ouabain concentrations. For studies with wild-type HeLa cells, activities shown are differences in measured activity in the absence and presence of 5 μM ouabain. For studies of sensitivity to vanadate, at each concentration of vanadate, Na-ATPase assays were carried out in sets of triplicates, without and with vanadate, with low and high oua-bain added to one set of each (cf. Ref. 22). For practical purposes, either γα- or γβ-transfected α1-HeLa cells were compared, on the same day, with control α1-HeLa membranes. Data for the vanadate sensitivity of Na-ATPase activity, expressed as a percentage of that obtained in the absence of vanadate, were analyzed by fitting the data to a one-site model using a nonlinear least squares fit (Kaleidagraph computer program) to a general logistic function as referred to previously (23).

Data for Na⁺ and K⁺ activation of Na,K-ATPase activity were expressed as percentages of Vmax using the Kaleidagraph computer program (Synergy Software) with the noninteractive model of cation binding described by Garay and Garrahan (24),

\[ v = \frac{V(1 + K^\text{cat}[\text{cat}])}{(1 + [\text{K}]K^\text{cat})} \]  

where v represents the rate of the reaction, V represents the maximal rate, K^\text{cat} represents either K_Na or K_K (the apparent affinity for Na⁺ or K⁺, respectively), [cat] represents the concentration of cation (either Na⁺ or K⁺), and n represents the number of binding sites (either three in the case of the Na⁺ activation experiments or two in the case of K⁺). Values of Vmax and K^\text{cat} were obtained from this fitting procedure.

Evaluation of K⁺ activation at cytoplasmic sites was determined by analyzing Na⁺ activation profiles as a function of K⁺ concentration. As in our previous study (26) and based on the Albers-Post model with the assumption that Na⁺ and K⁺ bind randomly at three equivalent (noninteractive) cytoplasmic sites, the data were ana-lyzed using the relationship described by Garay and Garrahan (24),

\[ v = \frac{V(1 + K^\text{max}[\text{Na}])}{(1 + [\text{K}]K^\text{max})} \]  

where v represents the rate of the reaction, V represents the maximal rate, K^\text{max} represents either K_Na or K_K (the apparent affinity for Na⁺ or K⁺, respectively), [Na] represents the concentration of cation (either Na⁺ or K⁺), and K^\text{max} was obtained from this fitting procedure.
Where [Na] and [K] represent the cytoplasmic concentrations of Na\(^+\) and K\(^+\), respectively; \(\nu\) and \(V\) have their usual meaning; \(K_{\text{Na}}\) is the affinity for Na\(^+\) binding to cytoplasmic activation sites in the absence of K\(^+\); and \(K_{\text{K}}\) is the affinity for K\(^+\) acting as an antagonist of Na\(^+\) binding at cytoplasmic sites. This equation predicts a linear relationship between the apparent affinity constant for Na\(^+\), \(K_{\text{Na},\text{app}}\), and K\(^+\) concentration according to the following relationship:

\[
K_{\text{Na}} = K_{\text{Na}}(1 + [K]/K_{\text{K}})
\]

(Eq 3)

All experiments shown or described are representative of at least three similar experiments, except for experiments summarized in the inset of Fig. 1 and those shown in Fig. 6, in which cases the means of at least three independent experiments are shown. For the representative experiments shown, each data point is the mean ± S.D. of three replicate samples.

RESULTS

Functional Studies

The experiments described below were carried out to extend previous investigations of the functional role of \(\gamma\) and to compare the effects of the two variants, \(\gamma_a\) and \(\gamma_b\). This was done primarily by comparing the kinetic behavior of \(\gamma_a\)- and \(\gamma_b\)-transfected cells with mock-transfected HeLa cells and, where indicated, by analyzing the effect of anti-\(\gamma\) antibodies on the renal enzyme. Experiments were initially carried out with \(\gamma\)-transfected wild-type HeLa cells and then repeated with \(\gamma\)-transfected \(\alpha_1\)-HeLa to assure that effects, or absence thereof, were not specific to the species of \(\alpha\), either rat or human. Similar findings were obtained with both cell lines. Only those from either wild-type or rat \(\alpha_1\)-transfected HeLa cells are shown. Furthermore, we noted that selection and growth of \(\alpha_1\)-HeLa cells in a low concentration of ouabain (cf. Ref. 21) down-regulates endogenous human \(\alpha_1\) such that expressed \(\alpha_1\) predominates (experiments not shown).

Expression in Cultured Cells

We showed recently that expression in cultured human cells (wild-type HeLa cells and HEK cells), of cDNA encoding the two individual \(\gamma\) variants revealed additional bands (see Fig. 5 of Ref. 10). That these additional bands, termed \(\gamma_a\) and \(\gamma_b\), are due to posttranslational modifications was evidenced by the cell-specific manner of their appearance following transfection into cultured cells. Thus, \(\gamma_a\) appears primarily in HEK cells, and \(\gamma_b\) appears primarily in HeLa cells. In the present study, identical results were obtained for \(\gamma_a\) and \(\gamma_b\) expressed in rat \(\alpha_1\)-transfected HeLa cells (not shown). As in the case of wild-type HeLa, a conspicuous \(\gamma_b\) is visible in \(\gamma_b\)-transfected \(\alpha_1\)-HeLa, and only one band is visible in \(\gamma_b\)-transfected \(\alpha_1\)-HeLa cells. Densitometry of the \(\alpha\) and \(\gamma\) subunits of membranes from clones of \(\gamma_a\)-HeLa, \(\gamma_a\)-1-HeLa, \(\gamma_a\)-2-HeLa, and \(\gamma_b\)-2-HeLa, in which expression is relatively high and used for functional analysis, showed that the \(\gamma\) to \(\alpha\) ratios are >50% that of rat kidney (density of both \(\gamma\) bands).

Effects of \(\gamma\) on Apparent Affinity for ATP

Fig. 1 confirms the earlier finding that \(\gamma\) decreases \(K_{\text{ATP}}\) for ATP (low affinity binding) and shows further that a similar (2-fold) decrease in \(K_{\text{ATP}}\) (low affinity binding) is effected by \(\gamma_b\). Furthermore, this effect appears to be independent of the presence (HEK cells; Ref. 11) or absence (HeLa cells; this study) of \(\gamma\). It was also noted earlier that this effect of \(\gamma\) is abrogated by anti-\(\gamma\) antibody as evidenced in the increase in \(K_{\text{ATP}}\) following preincubation of either \(\gamma_a\)-transfected HEK cells or the rat kidney enzyme with the antisemur \(\gamma\)C33 raised against the last 10 residues of the C terminus. Similar effects of \(\gamma_a\) and \(\gamma_b\) were observed in other experiments (not shown) in which the cDNAs of these variants were transfected into \(\alpha_1\)-HeLa cells. Although anti-\(\gamma\) (C-terminal) decreases the \(V_{\text{max}}\) of kidney enzyme (8), variation in specific activity among different membrane preparations from the HeLa cells (see the legend to Fig. 1) precluded the determination of \(\gamma\) effects on \(V_{\text{max}}\).

Effect of \(\gamma\) on the Steady-state Conformational Equilibrium: Studies with Vanadate

The question of whether the effect of \(\gamma\) on \(K_{\text{ATP}}\) is primarily on interaction of the enzyme with ATP and/or secondary to an alteration in the conformational equilibrium was tested by using inorganic orthovanadate as a probe of the \(E_c\) conformation. Accordingly, vanadate sensitivity of ATPase activity was tested under conditions in which its effect should not be secondary to an alteration in \(K_{\text{ext}}\) interaction with the enzyme. This was done by measuring the steady-state hydrolysis of the Na\,-ATPase activity with ATP added at low concentration (1 \(\mu\)M) to assess turnover of the enzyme in the absence of K\(^+\) as in Ref. 22 with modifications described in the legend to Fig. 1. In one series of experiments, we tested the effect of anti-\(\gamma\) (C-terminal) on the renal enzyme. As shown in Fig. 2A, the sensitivity to vanadate is increased; the \(I_{50}\) is decreased ~2-fold (see legend to Fig. 2). As predicted, the opposite effect was observed with the two \(\gamma\) variants. In the typical experiment shown for \(\gamma_a\) in Fig. 2B, it is evident that, compared with control (mock-transfected) \(\alpha_1\)-HeLa cells (see “Experimental Procedures”), the sensitivity to vanadate is decreased. The \(I_{50}\) for vanadate was increased ~2-fold. Similar results were obtained for \(\gamma_b\)-transfected \(\alpha_1\)-HeLa cells (not shown; \(p < 0.01\)). These results are consistent with the conclusion that both \(\gamma_a\) and \(\gamma_b\) shift the \(E_1 \leftrightarrow E_2\) equilibrium in favor of \(E_2\) form(s) as suggested in earlier studies (8, 11, 25). Those experiments showed that (i) inhibition of the kidney enzyme by anti-\(\gamma\) is greater at acidic pH (pH 6.2) and least at alkaline pH (pH 8.9), under which conditions the \(E_2\) (K) \(\rightarrow E_1\) transition is
either the major rate-limiting or a non-rate-limiting step of the reaction, respectively.

Effects on Cation Interactions

\( K^+ \) Interactions Relevant to the \( E_2P \rightarrow E_2(K) \rightarrow E_1 \) Pathway—Treatment of the rat kidney enzyme with anti-\( \gamma \) decreases the \( K'_K \) for \( K^+ \) activation by 25%, at least at suboptimal ATP concentration (25). Those results are consistent with the conclusion that effects on \( K'_K \) are secondary to effects of \( \gamma \) on the \( E_2/K \) conformational equilibrium. However, similar experiments aimed to show the presumably opposite effect (increase in \( K'_K \)) of \( \gamma_a \) and \( \gamma_b \) on \( \alpha_1 \)-transfected HeLa cells were equivocal due to the large variances in small increases (\( \leq 25\% \)) in \( K'_K \) values due, presumably, to much lower specific activity and higher nonspecific ATP hydrolysis of HeLa membranes. In other experiments (not shown) in which the ouabain-sensitive influx of \( ^{86}\text{Rb}^+ \) (K') was assayed as in Ref. 23, a significant difference in the apparent affinity for extracellular \( K^+ \) between \( \gamma_a \)- or \( \gamma_b \)-transfected and mock-transfected \( \alpha_1 \)-HeLa cells could not be detected (experiments not shown). Nonetheless, Fig. 3 shows clearly that when the ATP concentration was reduced to 10 \( \mu \)M so that the \( K^+ \) deoctolation reaction becomes strongly rate-limiting, both \( \gamma_a \) and \( \gamma_b \) increased the extent of \( K^+ \) activation of Na-ATPase in HeLa cells. Similar results were obtained with \( \alpha_1 \)-HeLa cells (experiments not shown). This result is consistent with the conclusion that both variants increase the rate of the rate-limiting \( E_2(K) \rightarrow E_1 \) reaction, an effect abrogated by anti-\( \gamma \).

\( K^+ \) Antagonism of Na\(^+\) Activation—One of the notable differences between \( \alpha_1 \beta_1 \) pumps of kidney compared with many other tissues is the notably lower apparent affinity for Na\(^+\) (increased \( K_{Na} \)) in kidney, which is readily seen at high K\(^+\) concentrations (\( \geq 20 \) mM; Ref. 26). In that study, we showed that this increase in \( K_{Na} \) is accounted for by the higher apparent affinity for K\(^+\) (\( K'_K \)) as an antagonist at cytoplasmic Na\(^+\) activation sites rather than a difference in Na\(^+\) affinity, per se. At face value, the notion that this effect is due to interaction with \( \gamma \) seems unlikely, since this \( K^+ /Na^+ \) antagonism is even more dramatic in heart tissue (26) that is devoid of \( \gamma \) (8).

Nevertheless, to determine whether the \( \gamma \) subunit is relevant to this phenomenon in the kidney, the following experiments were carried out: (i) an analysis of the sensitivity of the enzyme to inhibition by K\(^+\) at low (5 mM) Na\(^+\) concentration (Fig. 4) and (ii) an extensive kinetic analysis to determine \( K'_{Na} \) as a func-
above, should itself have the effect of lowering the apparent affinity of Na$^{+}$ antagonism of rat a1-HeLa cells. Lines shown are least squares fits of the values of $K^*_{Na}$ determined at varying Na$^{+}$ concentration as indicated in Fig. 5, with the K$^{+}$ concentration kept constant at either 5, 10, 20, 50, or 100 mM. For each concentration of K$^{+}$, the three types of membranes were assayed concurrently in the same experiment. Each point shown is the mean ± S.D. from at least three separate experiments. $K_{Na}$ and $K_{Na}/K_{K}$ obtained from the least squares fits of the data to Equation 3 and values of $K_{K}$ calculated from the ratio of $K_{Na}/K_{Na}/K_{K}$ (Table I) shown in Table I. X, control, mock-transfected a1-HeLa; ○, γα, a1-HeLa; ●, γα-1-HeLa.

The data were analyzed by fitting the Na$^{+}$ activation curves to a noncooperative model of Na$^{+}$ activation (cf. Ref. 24). As indicated in the legend to Fig. 5, both γα and γδ decrease the apparent Na$^{+}$ affinity, and the decrease is greater at the higher K$^{+}$ concentration. Fig. 6 shows the results of a large series of similar experiments carried out for both variants at varying K$^{+}$ concentration. The plots show the relationship between $K_{Na}$ and K$^{+}$ concentration for control (mock-transfected) and γα- and γδ-transfected a1-HeLa cells. As described under “Experimental Procedures,” the data were analyzed according to Equation 3, which predicts a linear relationship between $K_{Na}$ and cytoplasmic $[K^+]$ whereby $K_{Na}$ and $K_{K}$ are the affinity constants for Na$^{+}$ (extrapolated to $[K^+] = 0$) and for K$^{+}$ at cytoplasmic site(s), respectively. At each K$^{+}$ concentration, the data point shown is the mean of at least three separate determinations of $K_{Na}$. The linearity of the plots indicates that this relationship provides a valid basis for using our data to estimate $K_{Na}$ and $K_{Na}/K_{K}$. As shown by the data summarized in Table I, both γα and γδ have similar effects. Both increase $K_{Na}/K_{K}$ with no significant effect on $K_{Na}$, consistent with the conclusion that the effect of both variants is mainly due to a decrease in $K_{K}$, i.e. an increase in the affinity for K$^{+}$ acting as an antagonist of Na$^{+}$ binding at cytoplasmic sites. It is noteworthy that (i) the kinetic constants for control a1-HeLa taken from Fig. 6 are virtually identical to those reported earlier (26), indicating the high reproducibility of the assays, (ii) the same effects of the two variants were observed in a similar series of experiments carried out with wild-type HeLa cells (experiments not shown), and (iii) the magnitude of the change in $K_{Na}/K_{K}$ (~50% increase) is notably similar to the higher (50%) $K_{Na}/K_{K}$ of kidney compared with a1-HeLa cells reported earlier (26).

As summarized in Table II, antisera raised against the cytoplasmic C- and extracellular N-terminal regions of γ failed to abrogate the effect of γ on K$^{+}$/Na$^{+}$ antagonism. Thus, treatment of the rat kidney enzyme with antisera γC33 raised against the C terminus failed to change the magnitude of K$^{+}$ inhibition noted at low Na$^{+}$ concentration (75% inhibition in both the presence and absence of 100 mM K$^{+}$); the same holds true of anti-γα raised against the N terminus of γα (see below).
are particular, the subunit in medullary TAL (mTAL) with few positive cortical TAL (cTAL), and faint labeling of proximal tubules and cortical collecting ducts. Its expression is more restricted than that of the subunit of the sodium pump, indicating that both subunits are not systemat-
ically coexpressed.

The recent identification of a splice variant of the subunit of Na,K-ATPase leads to the notion that the two forms, and , may have different cellular expression and function. In order to establish whether and have distinct localizations, immunofluorescence experiments were performed using antibodies specific for each variant, namely anti-TELSANH, and anti-MDRWYL representing the two extracellular N-terminal sequences that differ in and , respectively. Fig. 8 documents the expression of and in the cortex (A–H) and medulla (I–P). These antibodies were used either alone (A, E, I, and M, in red) or together with antibodies (in green) raised against either the subunit, anti-AQP2, or anti-Tamm-Horsfall protein, as indicated in the legend to Fig. 8. In the cortex, is expressed in most cortical tubules (proximal tubules, cortical collecting ducts) together with the subunit of the pump (except in cTAL, appearing in green), while colocalization with is detectable only in few tubules (i.e., cTAL) in yellow. In particular, the form is clearly present together with the subunit in the macula densa (MD) cells (Fig. SB, arrow) (i.e. at the very end of the cTAL (labeled by an asterisk)); both subunits colocalize in the MD basolateral membrane lining the glomerulus. In contrast, the form was never found in MD cells. In the cortical collecting duct (labeled cd), as identified by

### Table I

| Cells/Tissue       | $K_{Na}/K_{K}$ | $K_{Na}$ | $K_{K}$ |
|--------------------|----------------|----------|---------|
| 1-HeLa-mock        | 0.045 ± 0.002  | 0.86 ± 0.10 | 19.0    |
| 1-HeLa-ya          | 0.071 ± 0.001a | 0.83 ± 0.07 | 11.7    |
| 1-HeLa-yb          | 0.074 ± 0.002a | 0.87 ± 0.08 | 11.8    |
| Kidney (a,b)       | 0.102 ± 0.005  | 1.02 ± 0.09 | 10.0    |
| a1-HeLa            | 0.046 ± 0.001  | 0.91 ± 0.02 | 19.9    |

* Data from Ref. 26.  
* Values for $K_{Na}$ and $K_{Na}/K_{K}$ are obtained from best fits to Equation 3 (see Fig. 6; errors shown are S.E. of the curve fit, and $K_{K}$ is the calculated ratio of the two values. The 95% confidence interval for the percentage increases in $K_{Na}/K_{K}$ is 49–67% and 51–78%, respectively.

### Table II

| Parameter measured | Effect of anti-γ(C-term) | Effect of anti-γb |
|--------------------|--------------------------|------------------|
| for ATP            | Increased (~2-fold)      | No change        |
| K'Na antagonism    | No change                | No change        |
| I0 for ouabain     | No change                | No change        |
| I0 for vanadate    | Increased (2-fold)       | Not determined   |
| (Na-ATPase)        |                          |                  |

**Are Interactions Involving the N Terminus Relevant to the Effects of γ?**

This issue was addressed using anti-γ, antiserum raised against the extracellular N terminus. As summarized in Table II, anti-γ failed to affect the activity at either limiting or nonlimiting concentrations of either ATP, Na+, or K+ or affect the concentration dependence of inhibition by ouabain (experiments not shown).

**Immunolocalization**

The intrarenal pattern of expression of the subunit of Na,K-ATPase was examined by immunofluorescence in the renal cortex and outer and inner medulla (Fig. 7). The anti-γ (C-terminal) antibody alone (CT3, red fluorescence), decorates several tubules (Fig. 7, A–C). All regions show staining, but it is particularly strong in outer medulla. Double immunofluorescence experiments were performed using antibodies against the subunit of Na,K-ATPase (Fig. 7, D–F), against AQP2 (apical membrane of principal cells of the collecting duct) in G–I and against Tamm-Horsfall protein (apical membrane of the thick ascending limb of Henle’s loop) in J–L, all evidenced by their fluorescein isothiocyanate-green fluorescence. Colocalization with the subunit appears in yellow. The subunit of Na,K-ATPase (Fig. 7, D–F) is apparent in all tubular segments, as expected: proximal tubules, loop of Henle, and distal nephron. In outer and inner medulla, there is extensive overlap of α and γ subunits (E and F), but in the latter some nephron segments express α but no γ subunit (F). In cortex, some cells (Fig. 7D) clearly show colocalization (arrow). Since they lie in close vicinity to the glomerulus, they may be macula densa cells; this is documented further in Fig. 8. Colocalization of AQP2 and the γ subunit (Fig. 7, G–I) was apparent in the cortex only, indicating that the collecting duct, in its cortical portion only, expresses the γ subunit. Tamm-Horsfall protein (Fig. 7, J–L) colocalizes with the γ subunit in medullary TAL (mTAL) (K, in yellow), with respective apical and basolateral expression. Thus, these experiments show that the γ subunit of Na,K-ATPase has predominant expression, at the protein level, in the mTAL, with low positive cortical TAL (cTAL), and faint labeling of proximal tubules and cortical collecting ducts. Its expression is more restricted than that of the α subunit of the kidney. Cryostat sections from rat kidney cortex (A, D, G, and J), outer medulla (B, E, H, and K), or the junction of outer and inner medulla (C, F, I, and L) were incubated with the affinity-purified anti-γ raised against the whole γ subunit of Na,K-ATPase (in red), either alone (A–C) or in the presence of the following antibodies: against the α subunit of Na,K-ATPase (1:50) (D–F), against AQP2 (1:100; to identify collecting ducts) (G–I), or against Tamm-Horsfall protein (1/50; to identify the thick ascending limb of Henle’s loop) (J–L), all appearing in green. Colocalization generates yellow fluorescence, g, glomerulus; p, proximal tubule; cd, collecting duct; asterisk, thick ascending limb of Henle’s loop. The arrow in D shows macula densa cells. Bar, 50 μm.
its apical expression of AQP2 in principal cells (in green in Fig. 8C), the γa variant is in the opposite membrane (i.e. in the basolateral membrane of principal cells), while these cells have only background fluorescence for γb (Fig. 8G); proximal tubules (labeled p) express both γ forms at low levels. Of particular interest is the observation that the cortical portion of the thick ascending limb of the loop of Henle (cTAL) expresses only γb (Fig. 8H), not γa (Fig. 8D), as shown by selective colocalization of γb with Tamm-Horsfall protein. Panels I–P illustrate the immunofluorescence for similar immunocytochemistry carried out with sections at the junction of the outer medulla and inner medulla. Antibodies specific for γa and γb show that each has a restricted pattern of expression. This expression is superimposed on that of the α subunit of the Na,K-ATPase in some (mTAL), but not all tubules (Fig. 8, J and N), since medullary collecting tubules (mcd) are positive only for the α subunit, while others (mTAL, labeled by an asterisk) express α together with either γa (Fig. 8J) or γb (Fig. 8N) although with lower intensity. The absence of γa and γb in the medullary and papillary collecting duct is shown by the distinct localization of AQP2 and either γa or γb (Fig. 8, K and O). All along the medullary thick ascending loops (identified with anti-Tamm-Horsfall protein antibody) there is evidence of both γa (Fig. 8L) and γb (Fig. 8P).

It is relevant that other studies2 showed that (i) anti-α immunoprecipitated both γa and γb from C16E4-solubilized membranes and (ii) either anti-γa or anti-γb (C-terminal) immunoprecipitated the α subunit. However, anti-γa did not immunoprecipitate γb. This result suggests that α/β subunits are present either as the complex α/β/γa or α/β/γb, but not α/β/γa/γb, but does not, of course, exclude the possibility of α/β subunits without associated γ subunits.

On the whole, these results indicate that the γ subunit of Na,K-ATPase has a restricted pattern of expression along the nephron, as compared with the α subunit, with prevalent expression in the mTAL, and, at lower levels, in the proximal tubule, and the collecting duct in its cortical part only. The γa form is in the basolateral membrane of MD cells and of cortical collecting duct principal cells as well as in the medullary portion of the thick ascending limb of Henle’s loop. The γb form is selectively expressed all along the thick ascending limb of Henle’s loop (Table III).

**DISCUSSION**

**Effects of γa and γb on Catalytic Functions**—In earlier studies we showed that anti-γ antiserum raised against the C terminus of γ inhibits Na,K-ATPase of the renal enzyme but not of tissues that do not express γ (8). Further analysis showed that anti-γ decreases the apparent affinity for ATP probably by stabilizing the E2 form(s) of the enzyme. Thus, the pH dependence of the anti-γ-mediated inhibition of activity is consistent

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**FIG. 8. Expression of the γa and γb forms of Na,K-ATPase within the kidney.** Cryostat sections from cortex (A–H) and medulla (I–P) were used; this kidney zone is at the junction of most inner part of outer medulla (upper part of each image) and of the inner medulla or papilla (lower part of each image). Sections were incubated with antibodies raised against the γa (A–D and I–L) or the γb (E–H and M–P) subunit (appearing in red). These antibodies were used either alone (A, E, I, and M) or in the presence of antibodies (in green) against the α subunit of Na,K-ATPase (B, F, J, and N), against AQP2 (to identify the apical membrane of collecting duct principal cells) (C, G, K, and O), or against Tamm-Horsfall protein (THP; to identify apical membrane of thick ascending limbs of Henle’s loop) (D, H, L, and P). Colocalization generates yellow fluorescence. Conditions were as follows: γa alone (A and I); γa alone (E and M); γa + α1 Na,K-ATPase (B and J); γa + AQP2 (C and K); γa + THP (D and L); γa + α1 Na,K-ATPase (F and N); γa + AQP2 (G and O); γa + THP (H and P). g, glomerulus; p, proximal tubule; cd, cortical collecting duct; mcd, medullary and papillary collecting ducts; asterisk, Henle’s loop. The arrow in B shows macula densa cells appearing at the very end of Henle’s loop. Bar, 50 μm except in C and G (20 μm).

### Table III

Expression of the γa and γb subunit along the nephron

| Proximal tubule | Henle’s loop | Collecting duct |
|-----------------|--------------|-----------------|
|                 | mTAL | cTAL | Macula densa | CCD | OMCD | IMCD |
| γa              | +    | +    | −              | +   | +    | −    |
| γb              | +    | +    | +/−            | −   | −    | −    |

* CCD, OMCD, and IMCD, cortical, medullary, and papillary portions of the collecting duct, respectively.

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2 S. J. D. Karlish and R. Goldshleger, unpublished results.
with a role of anti-γ in shifting the equilibrium of the K⁺ deoclusion reaction \(E_2(K) \leftrightarrow E_γ(K)\) toward \(E_γ(K)\). It was hypothesized that anti-γ mediates its effects by disrupting interactions between the Na,K-ATPase complex and the γ subunit, such that the role of the γ subunit is to shift the equilibrium toward \(E_1\). By transfecting one of the two γ variants (γ₁), into HEK cells, it was then shown that this is indeed the case, at least for γ₁ (11). The studies described in this paper show that the same holds true for the γ₂ variant. Thus, as we show here, both variants decrease \(K_{ATP}\) to an equal extent, and furthermore this effect is abrogated by anti-γ (C-terminal) for both variants (summarized in Table III). The present observation that anti-γ treatment of the renal enzyme increases sensitivity to vanadate and, conversely, that γ transfection decreases sensitivity to vanadate, strongly support the conclusion that the change in \(K_{ATP}\) reflects primarily an effect on the \(E_1/E_2\) conformational equilibrium. The result in Fig. 3 is consistent with the assumption that γ₁ and γ₂ stabilize \(E_1\) by accelerating the rate of the conformational transition \(E_γ(K) \rightarrow E_1\).

The other new finding concerning the function of γ is that it has not one but at least two distinct effects on the catalytic function of the Na,K-ATPase. In addition to an increase in apparent ATP affinity, γ induced an increase in \(K^+/Na^+\) antagonism, which results in a reduction in the effective affinity of \(Na^+\) ions for activating Na,K-ATPase. As mentioned above, the mechanism cannot be explained on the basis of stabilization of \(E_1\) and thus implies an additional effect of γ on intrinsic binding of K⁺ ions at cytoplasmic sites (perhaps on one of the two K⁺ sites). This effect of γ on \(K^+/Na^+\) antagonism is seen equally with both γ variants, and, interestingly, this function of γ is not altered by antibodies raised against either the C terminus or N terminus (summarized in Table II). Overall, it is evident from this study that both γ variants alter the kinetics similarly, with no evidence of a significant difference between the two on catalytic function. It may also be noted that the functional effects do not depend on tissue-specific posttranslational modifications of the γ subunit, although such modifications can be observed in HeLa cells (γ₁) or HEK cells (γ₂). Expression of γ₁ in NRK-52E kidney cells has been reported to modulate (decrease) \(Na^+\) and K⁺ affinities (9). Although the effect on \(K_{Na}^+\) appears similar to that described here for γ₁, there are puzzling experimental differences. First, the level of γ expression was much lower in their experiments (15-20%) than in the present ones carried out with the HeLa transfectants (>50%) despite the similar functional effect. Second, the increase in \(K_{Na}^+\) was only observed in γ₁-transfected NRK-52E cells expressing a doublet, and not in clones expressing a single species the identity of which, in light of later studies (10), is not clear. These issues are difficult to reconcile with the present work with γ₁-transfected HeLa cells that express only a single γ₁ species. As far as \(K_{K}^+\) is concerned, we could not detect a significant difference either with membrane fragments (ATPase assays at high ATP concentrations) or in \(^{86}Rb^+(K⁺)\) influx studies in ATP-replete (millimolar ATP) cells, despite the higher level of expression in the HeLa cells. The reason for these discrepancies remains enigmatic.

The recognition that the γ subunit induces at least two functional effects, only one of which is abrogated by anti-γ (C-terminal), suggests that there must be more than one region of interaction between the γ and α subunits on which the functional sites reside. Presumably, the effect on the \(E_1/E_2\) equilibrium and apparent ATP affinity involves the C-terminal sequence KHRSQVNEDEL, and the \(K^+/Na^+\) antagonism is mediated by other sequences in the molecule. Our observations that antibodies raised against the N-terminal sequence of γ₁, TELSANH, do not affect any kinetic function (summarized in Table II) imply that this sequence is not responsible for functional interactions. (Antibodies raised against the N terminus of γ₂ are reactive only with SDS-denatured enzyme, which precludes meaningful interpretation of its failure to alter the effects of γ₂ on αβ1 pumps.) Nevertheless, the distinctness of the two effects is underscored by the observation (26) that polyethylene glycol-mediated fusion of kidney pumps into cells devoid of pumps (dog erythrocytes) abrogates the kidney-specific increase in \(K^+/Na^+\) antagonism but not anti-γ-mediated inhibition of overall activity. Definition of the regions of interaction of γ and α subunits is a question to be addressed in the future.

**Physiological Role**—The physiological significance of the γ subunit could be that it provides a self-regulatory mechanism for maintaining the steady-state activity of the pump in the kidney. This notion is underscored by its abundance in mTAL (see below), suggesting that its functional effects are tailored to meet the requirement of \(Na^+\) and \(K^+\) homeostasis in the prevailing environmental conditions and in particular by the observations of the dual effects on the kinetic properties, the one on \(K_{ATP}\) and the other on \(K_K\), the affinity of the pump for \(K^+\) acting as an antagonist of cytoplasmic \(Na^+\). The effect of γ on \(K_{ATP}\) was discussed recently in terms of its importance in maintaining pump activity under putative anoxic parts of the medulla, i.e., to increase ATP utilization and maintain optimally high intracellular \(K^+\) and low \(Na^+\) under energy-compromised conditions as discussed previously (11, 25, 27). Such a regulator of \(K_{ATP}\) should alter the pump’s affinity for the nucleotide only moderately, for an excessive increase would affect even greater decreases in ATP concentration, thus leading to compromised cell viability. mTAL is characterized by a rapid transcellular \(Na^+\) flux, and the cellular \(Na^+\) concentration should reflect the balance of rates of passive \(Na^+\) entry and active \(Na^+\) efflux. The ability of γ to increase \(K^+/Na^+\) antagonism at the cytoplasmic surface as shown in this study may provide a means of acute regulation of the steady-state \(Na^+\) concentration. A lowered effective cytoplasmic \(Na^+\) affinity for activating the pump, due to a regulatory interaction, may be tailored to fit cells in which the steady-state \(Na^+\) concentration is higher than in cells that lack the regulator, but that, nevertheless, must respond to changes in \(Na^+\) entry. Thus, the optimal affinity for cytoplasmic \(Na^+\) ions should be one at which there is plenty of reserve capacity for responding to changes in cell \(Na^+\) at the prevailing set point of \(Na^+\) concentration. The recent report of a putative dominant-negative mutation (G41R) in the γ subunit of the Na,K-ATPase may be relevant to a role of γ in maintaining intracellular \(Mg^{2+}\) secondary to elevating intracellular \(Na^+\). Such a relationship between intracellular \(Na^+\) and \(Mg^{2+}\) was not seen in sublingual mucous acini (28), but also in renal tubular cells.³

³ G. Quamme, unpublished observations.
ing information about γ expression in medulla and papilla, in addition to cortex, with identification of tubular segments) and in terms of specific expression of each γ form in tubules. They also show that both forms of the γ subunit are in the basolateral membrane, i.e., in close vicinity to the pump.

The γ subunits of Na,K-ATPase share homologies with other small molecules, such as CHIF (channel-inducing factor), phospholemman, and phospholamban, which are thought to be regulators of ion transporters or channels. Among these, CHIF also has a restricted pattern of expression, in the surface cells of the distal colon epithelium and in the terminal portions of the nephron (20, 29). More specifically, CHIF is found to some extent in the cortical collecting duct but essentially in its medullary and papillary portions. As for the γ subunit of Na,K-ATPase, CHIF is located essentially at the basolateral membrane. A specific interaction between CHIF and Na,K-ATPase in colon membranes has been demonstrated recently (30).

Although we cannot absolutely exclude the possibility of different functional effects of γa and γb, the lack of a notable difference between γa and γb with respect to their effects on pump kinetics may not be surprising. The two variants differ with respect to only the six or seven residues at the extracellular amino terminus. Accordingly, the two variants may influence differentially such properties as membrane targeting, pump turnover, or basolateral signaling and affect the rate of active Na+ and K+ transport by altering the density of pumps in the basolateral membrane. It is also plausible that each of these variants may influence differentially, in a cell-specific manner, some interactions of the pump with the extracellular matrix. Of particular relevance is the proposal that extracellular hensin influences directly polarity of collecting duct intercalated cells (31).

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