Regions of the Catalytic α Subunit of Na,K-ATPase Important for Functional Interactions with FXYD 2*

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The γ modulator (FXYD 2) is a member of the FXYD family of single transmembrane proteins that modulate the kinetic behavior of Na,K-ATPase. This study concerns the identification of regions in the α subunit that are important for its functional interaction with γ. An important effect of γ is to increase K + /H+ antagonism of cytoplasmic Na + activation apparent as an increase in K Na at high [K + ]. We show that although γ associates with α1, α2, and α3 isoforms, it increases the K Na of α1 and α3 but not α2. Accordingly, chimeras of α1 and α2 were used to identify regions of α critical for the increased K Na. As with α1 and α2, all chimeras associate with γ. Kinetic analysis of α2front/α1back chimeras indicate that the C-terminal (Lys907–Tyr1101) region of α1, which includes transmembrane (TM9) close to γ, is important for the increase in K Na. However, similar experiments with α1front/α2back chimeras indicate a modular role of the loop between TMs 7 and 8. Thus, as long as the α1 L7/8 loop is present, replacement of TM9 of α1 with that of α2 does not abrogate the γ effect on K Na. In contrast, as long as TM9 is that of α1, replacement of L7/8 of α1 with that of α2 does not abolish the effect. It is suggested that structural association of the TM regions of α and FXYD 2 is not the sole determinant of this effect of FXYD on K Na but is subject to long range modulation by the extramembranous L7/8 loop of α.

The Na,K-ATPase or sodium pump is an integral membrane protein found in the cells of virtually all higher eukaryotes. It couples the hydrolysis of one molecule of ATP to the electrogenic exchange of three intracellular Na + and a smaller, highly glycosylated β subunit whose role is to ensure the interaction with the catalytic subunit (18, 19). The sodium pump is an oligomer of two subunits, a large catalytic subunit (Ala replacement of Phe956 and Glu960) indicate the importance of the kidney. Other members include phospholemman (PLM or FXYD 1) MAT-8 (FXYD 3), CHIF (FXYD 4), RIC or Dysadherin (FXYD 5), Phosphohippolin (FXYD 6) and FXYD 7. A phospholemman-like protein, PLMS, is present in the shark rectal gland.

The γ modulator exists as two main variants, γa and γb, with distinct and overlapping localization along the nephron (8, 9). Mass spectrometry indicates that they differ only in the N terminus (10). In rat γa, TELSANH is replaced by Ac-MDRWYL in γb. Previous studies in our laboratory (8, 11–14) using membrane fragments isolated from γ-transfected rat α1-HeLa cells have shown that γ serves at least two distinct regulatory effects on pump kinetics and that these effects are the same for both variants. Thus, (i) γ increases the apparent affinity for ATP and (ii) increases K + /Na + competition at cytoplasmic Na + activation sites, as seen by an increase in K Na at high [K + ] concentration. In addition, in intact cells, γ increases the apparent K + affinity (15, 16).

Although the functional effects of γ on Na,K-ATPase have been extensively characterized, the structural basis of these effects is largely unknown. Earlier cryoelectron microscopy of the purified renal (α1B1) pump (17) suggested that the γ subunit is located in a pocket comprising TM9, TM6, TM2, and possibly TM4. Furthermore, recent homology modeling of the Na,K-ATPase based on the high resolution structure of the Ca-ATPase as well as cross-linking experiments have shown further that γ makes contacts with TM2, TM6, and TM9 of the α subunit (18, 19).

An important issue concerns the region(s) of α that interact(s) with the γ modulator, focusing on the interactions that are critical for producing the modulatory effects of γ. Mutagenesis of the α1 subunit (Ala replacement of Phe956 and Gln960) indicate the importance of TM9 in affecting the apparent affinity for extracellular K + (18). However, neither of these replacements abrogated the increase in K Na effected by γ.

The present study focuses on regions of the α subunit with which γ interacts to effect the increase in K Na at high [K + ] (K + /Na + antagonism). Kinetic studies using γ mutants (14, 20) and mimetic peptides comprising the transmembrane domain of γ, γ-TM (21), have clearly shown that the increase in K + /Na + antagonism is mediated by the TM region of γ. Here we show that although γ interacts with α1, α2, and α3 and increases K Na of α1 and α3, a significant change in the K Na of α2 could not be detected. Accordingly, an α1/α2 chimera approach was used to gain insight into regions of α that are relevant to its functional interaction with γ resulting in increased K + /Na + antagonism.

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3 The abbreviations used are: TM, transmembrane; WT, wild type; α1/α2α, α1-front/α2-back chimeras; α2/α1α, α2-front/α1-back chimeras.
**Experimental Procedures**

Mutagenesis, Transfection, and Cell Culture—All of the chimeric αs used in this study were derived from the ouabain-insensitive rat α1 and α2 cDNAs (kindly provided by E. A. Jewell and J. B. Lingrel) introduced into the HindIII site of a modified pBlues vector. Using Stratagene’s QuikChange site-directed mutagenesis kit, silent mutations encoding for novel restriction sites were introduced into identical positions of rat α1 and α2 cDNAs to create interchangeable restriction cassettes as described below. Chimeras α(1–311)/α2 and α(1–309)/α1 were prepared as described previously (22). Chimeras α(1–786)/α2 and α(1–783)/α1 were prepared by introducing a MunI site, which splits the codons for Ser752 of α1 and the corresponding Ser749 of α2 (the amino acid sequence is identical up to Leu746 of α1 and Leu783 of α2) by exchanging the 5’-Sph1(2343)-Mun1(2491) restriction cassettes of α1 with the 5’-Sph1(2344)-Mun1(2343) cassette of α2. Chimeras α(1–875)/α2 and α(1–872)/α1 and α1(1–907)/α2 and α2(1–904)/α1 were prepared as above, by introducing a Ndel site, which splits the codon for Ala848 of α1, corresponding to Ala845 of α2. The amino acid sequence is identical up to Pro872 of α1 (Pro877 of α2). BsiWI was introduced as well, by splitting codons Thr902 of α1 (Thr907 of α2) and the 5’-Sph1(3340)-Ndel2(780) cassette of α1 was exchanged with the 5’-Sph1(3344)-Ndel1(2630) cassette of α2 to create α(1–785)/α2 and α(1–782)/α1. Similarly, the 5’-Sph1(3340)-BsiW1(2944) cassette of α1 and the 5’-Sph1(3344)-BsiW1(2792) cassette of α2 were exchanged to generate α(1–907)/α2 and α(1–904)/α1. The full-length cDNAs for all chimeric constructs were then excised from the shuttle vector using HindIII and ligated into pcDNA3.1. Orientation was confirmed by restriction analysis and full-length cDNA sequences were verified. HeLa cells containing either the empty pRMS vector (HeLa-mock) or expressing βγ protein (HeLa-βγ-piRES) (10) were transfected with the pcDNA chimeric α constructs using the Lipofectamine technique (Invitrogen). Cells expressing the relatively ouabain-resistant rat α enzymes were then selected and maintained in culture as previously described (14).

Membrane Preparations—Na+/K+-treated microsomal membranes were prepared from the chimeric and WT cells as described earlier (23, 24). The protein concentration was determined using a detergent-containing modification (25) of the Lowry method (26).

Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-PAGE and Western blotting were carried out as described previously (8). Following transfer of the SDS-PAGE gels to polyvinylidene difluoride membranes, a section of the membrane comprising the lower molecular mass proteins (≤ 28 kDa) was analyzed with a polyclonal γ antibody (γC32) raised against the C-terminal KHRQV/NEDEL peptide that is essentially the same as γC33 used previously (8). The remaining membrane was blotted with monoclonal antibodies to detect the α subunit as described in the figure legends.

Co-immunoprecipitation—The method used was a modification of Garty et al. (27) as described elsewhere (21). Band densities were quantified using Molecular Dynamics ImageQuant software.

Enzyme Assays—Na+/K+-dependent activation of Na,K-ATPase activity was measured as described previously (14). Briefly, Na,K-ATPase activity was determined at 100 mM KCl and varying concentrations of NaCl and with 10 mM ouabain present to inhibit endogenous pumps. Baseline ATPase activity was determined at 100 mM KCl and absence of NaCl and with 100 mM choline chloride added to maintain a constant (200 mM) chloride concentration. For determination of the effects of the

![TABLE 1](image)

| Membranes | α1 | α2 | α3 |
|-----------|----|----|----|
| Mock-transfected | 5.3 ± 0.3* | 9.3 ± 0.7 | 9.3 ± 1.4 |
| γγ-Transfected | 8.3 ± 0.1* | 9.5 ± 1.2 | 16.6 ± 1.1 |
| Fold increase (γγ/control) | 1.6 ± 0.2 | 1.0 | 1.8 |
| No addition | 4.8 ± 0.2 | 8.1 ± 0.7 | 6.9 ± 0.7 |
| Plus γ-TM | 15.4 ± 0.8* | 9.3 ± 0.7 | 6.2 ± 0.7 |
| Fold increase (γγ/control) | 3.2 ± 0.2 | 1.0 |

* a/b Data taken from Pu et al. (8).

p < 0.01 compared with control.

Data taken from Zouzoulas et al. (21).

γ-TM peptide on α2, the permeabilized membranes were assayed as previously described (21), except that the peptide concentration in the final assay mixture was 1.67 μM. All experiments were carried out at least three times on two clones each of mock- and γ-transfected cells, with assays performed in triplicates.

Data Analysis—The data were analyzed using the Kaleidagraph computer program (Synergy Software) with the non-interactive model of cation binding described by Garay and Garrahan (28), i.e.,

\[ V = V_{\max}/(1 + K'_{Na} [Na^+]^3) \]  

(Eq. 1)

where \( V \) represents the rate of the reaction, \( V_{\max} \) is the maximal rate, and \( K'_{Na} \) is the apparent affinity for Na⁺. Superscript 3 denotes the number of Na⁺ binding sites. Values of \( V_{\max} \) and \( K'_{Na} \) were obtained from this fitting procedure.

**Results**

Earlier studies with cRNA injected Xenopus oocytes showed that the γ subunit can associate with the three major isoforms of the α subunit (15). In the present study, this finding was confirmed with mammalian cells. For these experiments rat α1-, α2-, and α3-HeLa cells were transfected with γ (γb variant) and following isolation of stable transfectants, membranes were isolated, solubilized with detergent, and subjected to immunoprecipitation with anti-β antibodies using the conditions developed by Garty et al. (27) as outlined under “Experimental Procedures.” These authors showed that co-immunoprecipitation is efficient only in conditions (solubilization in C50, the presence of either Rb⁺ or ouabain as used here, or Na⁺ or l-glutamycin) that preserve native pump structure (27). Samples taken before and after immunoprecipitation-

4 For simplicity, asterisks normally used to denote the ouabain-resistant forms of α2 and α3, i.e. α2* and α3*, constructed by Jewell and Lingrel (23) are omitted.
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Figure 2. Schematic illustration of the α1 and α2 chimeras. Regions of greatest divergence are indicated by the brackets above α1 (α1 numbering beginning with the mature N terminus, GRDKY).

Effects of γ on the Na+ activation Kinetics of the α Isoforms — Although γ associates with all three isoforms of α, the question remains whether the γ-mediated increase in K+/Na+ antagonism seen previously for α1 also holds true for α2 and α3. In the experiments described below, Na,K-ATPase activity for α1 and α2 was measured with NaCl varied and KCl kept constant at 100 mM. α3 was assayed at 50 mM KCl because K_i of WT α3 is high (16.5 mM at 100 mM KCl, see Ref. 29) and even higher with α3-yb membranes, precluding satisfactory extrapolation to V_max. The results presented in Table 1 show that, whereas γ increases K_i of α1 and α3 by almost ~1.6-fold, a significant change in K_i of α2 could not be detected. It is noteworthy that whereas K_i modulation of α2 pumps by γ, despite the high sequence identity between α1 and α2, provided a unique opportunity to use an α1/α2 chimera approach to identify regions of α interaction with γ, which are functionally important for the K_i effect. Fig. 2 depicts a linear representation of the α1-front half/α2-back half (α1/α2) and the reverse α2-front half/α1-back half (α2/α1) chimeras used for these experiments. The high degree of amino acid identity (86%) among the isoforms notwithstanding, the regions of most diversity between α1 and α2 are within residues 1–311 and 429–565 (α1 numbering), which span the cytoplasmic N terminus and part of the large cytoplasmic loop between TM4 and TM5 (see top of Fig. 2).

Association of γ with α1/α2, and α2/α1, Chimeras and Its Effects on Their Na+ activation Kinetics — The co-immunoprecipitation experiment shown in Fig. 3 indicates that similar to its association with α1 and α2 (see Fig. 1), yb associates with all α1/α2, and α2/α1, chimeras.

In the experiments depicted in Fig. 4, the effects of γ on the apparent K_i of α1/α2, and α2/α1, chimeras were measured at 100 mM K+ and compared with the effects of γ on WT α1 and α2 isoforms. Fig. 4 (top panel) shows representative graphs of α2 and α2/α1, chimeras, and Fig. 4 (bottom panel) shows chimeras in the reverse α1/α2, configuration. Fig. 5 summarizes the results of the averages of replicate experiments carried out as shown for the representative experiments in Fig. 4 (see absolute values presented in Table 2).

Fig. 4 (top panel), Fig. 5, and Table 2 indicate that γ causes a significant increase in the K_i of all chimeras in the α2/α1, configuration, similar to that reported for WT α1. It is notable that this holds true for chimera (α2,1–875/3–1004) comprising only TMs 8–10 of α1 and thus encompassing TM9, which has been shown to be close to (17) and interact with γ (18, 19).

As shown in Fig. 4 (bottom panel), Fig. 5, and Table 2, γ did not increase K_i of chimeras α1/α2 up to α1,1–311/3–1004/α2, similar to its lack of effect on the WT α2 enzyme. However, a notable γ-mediated increase in K_i of α1,1–311/2 was observed. This latter effect of γ was not seen at low (10 mM) K+ concentration (experiment not shown) indicating that, like WT α1 (14), the γ-mediated increase in K_i of the α1,1–311/2 chimera reflects increased cytoplasmic K+/Na+ antagonism. Taken together and as discussed below, these observations suggest that the
experiments as percentages of concentrations of NaCl as indicated under “Experimental Procedures.” Data points shown are means ± S.D. (triplicate determinations) of representative experiments and are expressed as percentages of $V_{\text{max}}$. Top panel, $\alpha_2$ and $\alpha_2/\alpha_1$ chimeras. Bottom panel, $\alpha_1$ and $\alpha_1/\alpha_2$, chimeras. Dashed line, mock-transfected cells. Solid line, $\gamma$-transfected cells.

**TABLE 2**

Summary of effects of $\gamma$ on $K_{\text{Na}}$

Na,K-ATPase assays were carried out as described in the legend to Fig. 4. Each value is the mean ± S.E. of the number of separate experiments shown in brackets. $V_{\text{max}}$ values (nmol/mg/min) for $\alpha_2$, $\alpha_1_{1-311}/\alpha_2$, $\alpha_1_{1-786}/\alpha_2$, $\alpha_1_{1-902}/\alpha_2$, and $\alpha_1_{1-907}/\alpha_2$ were 300 ± 20, 81 ± 7, 212 ± 48, 270 ± 22, and 224 ± 16, respectively, for mock-transfected cells and 277 ± 17, 120 ± 9, 121 ± 6, 298 ± 30, and 257 ± 46, respectively, for $\gamma_b$-transfected cells. $V_{\text{max}}$ values (nmol/mg/min) for $\alpha_1$, $\alpha_2$, $\alpha_1_{1-311}/\alpha_1$, $\alpha_2$, $\alpha_1_{1-786}/\alpha_1$, and $\alpha_1_{1-902}/\alpha_1$ were 375 ± 25, 177 ± 11, 253 ± 78, 250 ± 12, and 168 ± 6, respectively, for mock-transfected cells and 520 ± 196, 244 ± 26, 330 ± 26, 405 ± 17, and 192 ± 2, respectively, for $\gamma_b$-transfected cells.

| Cells          | Mock        | $\gamma_b$-Transfected |
|----------------|-------------|------------------------|
|                | $K_{\text{Na}}$ (mM) | $K_{\text{Na}}$ (mM) |
| $\alpha_2$     | 8.9 ± 0.4 (7)  | 9.7 ± 0.4 (7)  |
| $\alpha_1_{1-311}/\alpha_2$ | 9.3 ± 0.7 (4)  | 9.5 ± 1.2 (4)  |
| $\alpha_1_{1-786}/\alpha_2$ | 4.7 ± 0.2 (7)  | 5.4 ± 0.2 (9)  |
| $\alpha_1_{1-902}/\alpha_2$ | 5.2 ± 0.3 (5)  | 5.3 ± 0.1 (5)  |
| $\alpha_1/\alpha_2$ | 6.8 ± 0.4 (6)  | 9.4 ± 0.6” (6) |
| $\alpha_1$      | 5.3 ± 0.3b    | 8.3 ± 0.1” (6) |
| $\alpha_2_{1-309}/\alpha_1$ | 8.2 ± 0.4 (4)  | 11.6 ± 0.9” (4) |
| $\alpha_2_{1-786}/\alpha_1$ | 13.9 ± 0.4 (6) | 17.5 ± 0.8” (7) |
| $\alpha_2_{1-902}/\alpha_1$ | 12.7 ± 1.2 (5) | 18.8 ± 0.4” (5) |
| $\alpha_2_{1-904}/\alpha_1$ | 7.3 ± 0.4 (5)  | 12.2 ± 1.4” (4) |

$^a$ Data taken from Pu et al. (8).

**DISCUSSION**

Earlier studies showed that the $\gamma$ modulator has distinct kinetic effects on the Na,K-ATPase of cultured mammalian cells such as rat $\alpha_1$-transfected HeLa cells transfected with either $\gamma_a$ or $\gamma_b$. One effect is a $\gamma$-mediated increase in the apparent ATP affinity (11, 12). Another effect is a $\gamma$-mediated increase in $K_{\text{Na}}$ at high K$^+$ concentration (8, 14) because of an increase in $K^+$ antagonism of cytoplasmic Na$^+$ activation, which can have a significant effect under physiological conditions in which the intracellular Na$^+$ concentration limits the rate of pump activity. (For further discussion of the physiological importance of this $\gamma$ effect, see Ref. 7). It is noteworthy that these differences in apparent affinities are consistent with the differences in affinities seen with $\alpha_1\beta_1$ pumps in $\gamma$-rich kidney preparations compared with $\gamma$-free tissues (13). An additional distinct effect, observed only in intact HeLa cells, is an increase in apparent affinity for extracellular K$^+$ (16). This effect may be similar to a membrane potential-dependent decrease in $K_{\text{Na}}$ reported with Xenopus oocytes bathed in Na$^+$-containing medium (15, 30).

Regions of $\gamma$ Important for Modulation of Ligand Affinities—Distinct regions of $\gamma$ are important for its aforementioned distinct effects. The cytoplasmic C terminus is responsible for the $\gamma$-mediated decrease in $K_{\text{ATP}}$. This effect has been localized to its penultimate four residues (14), because their deletion abrogates the decrease in $K_{\text{ATP}}$. However, this $\gamma$
effects of γ are seen in the present study in which the addition of only the transmembrane region of γ increased $K_{Na}$ of α1 but not α2 pumps (see Table 1). It is noteworthy that, like γ, the functional effects of FXYD2 are also isoform-specific (37). Like γ, FXYD2 associates with α1, α2, and α3, but its effect on affinity for $K_{Na}$ (decrease in apparent affinity) is seen with α1 and α2, but not α3. Similarly to γ, FXYD2 resides in a groove made up of TMs 2, 6, and 9 of the α subunit (18).

**Conclusion**—Our findings support the view that the structural associations of transmembrane regions of the catalytic α subunit with FXYD proteins are not necessarily the sole determinants of the kinetic effects of these regulators on cation affinities. In the case of γ, the extramembranous L7/8 loop of α appears to modulate intramembranous α-γ interactions to effect the γ-mediated increase in $K^+$ antagonism of cytoplasmic Na$^+$ activation.

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*α* versus α1 isoform-distinct residues in the aforementioned peptides are underlined.
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