A functional interaction between CHIF and Na-K-ATPase: implication for regulation by FXYD proteins

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CHIF has been expressed in oocytes and shown to play a role in the regulation or mediation of ion transport (24). Together, they are termed the FXYD family, after the invariant motif FXYD located in their extracellular domains. One member of this group is the corticosteroid hormone-induced factor (CHIF; FXYD 4). CHIF was cloned as an aldosterone-induced gene and is expressed only in the kidney and colon (3, 9, 23). In addition to its regulation by corticosteroids, CHIF mRNA and protein are induced by Na deprivation and K loading (23, 27, 28). Suppression of CHIF by low-K intake is independent of aldosterone-induced gene expression only in the kidney and colon (3, 9, 23). In addition to its regulation by corticosteroids, CHIF mRNA and protein are induced by Na deprivation and K loading (23, 27, 28). CHIF has been reported to be required for normal blastocyst formation, but the mechanism is unknown (13). A mutation in its transmembrane segment, which causes defective routing, is associated with primary hypomagnesemia (17).

The γ-subunit shares sequence and topological homology with six other proteins, some of which were shown to play a role in the regulation or mediation of ion transport (24). Together, they are termed the FXYD family, after the invariant motif FXYD located in their extracellular domains. One member of this group is the corticosteroid hormone-induced factor (CHIF; FXYD 4). CHIF was cloned as an aldosterone-induced gene and is expressed only in the kidney and colon (3, 9, 23). In addition to its regulation by corticosteroids, CHIF mRNA and protein are induced by Na deprivation and K loading (23, 27, 28). Suppression of CHIF by low-K intake is independent of aldosterone-induced gene expression only in the kidney and colon (3, 9, 23). In addition to its regulation by corticosteroids, CHIF mRNA and protein are induced by Na deprivation and K loading (23, 27, 28). CHIF has been reported to be required for normal blastocyst formation, but the mechanism is unknown (13). A mutation in its transmembrane segment, which causes defective routing, is associated with primary hypomagnesemia (17).

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α in X. laevis oocytes (5). Taken together, the above data suggest that CHIF could be another epithelial-specific auxiliary subunit of the pump with functional effects that are different from those of γ. The objective of the present study has been to test this hypothesis and elaborate on it using native epithelia and transfected mammalian cells. Structural and functional interactions between a mammalian modulator and the pump detected in the native environment or mammalian cells are likely to have physiological significance.

MATERIALS AND METHODS

Membrane preparation. Rats were killed by cervical dislo-
cation, and distal colons and kidneys were excised and rinsed in ice-cold PBS. Kidneys were dissected into cortices, inner medulla, and outer medulla, and microsomal membranes were prepared as described before (14). Distal colonic surface cells (colonocytes) were isolated by a modification of the procedure described elsewhere (22). In brief, colonic tubes were flushed three times with 10 ml of ice-cold PBS plus 2 mM DTT and inverted (lumen out). The inverted colons were tied at one end, filled with DMEM plus 10% FCS, 2 mM DTT, and 2 mM EDTA, and then tied at the other end as well. The filled colons were suspended in 25 ml of the above DMEM- EDTA medium and incubated at 37°C for 40 min with shak-
ing at 140 rpm. Colonocytes were collected from the medium by centrifugation and stored at −70°C. HeLa cell membranes were prepared as described before (12).

Antibodies and immunocytochemistry. A polyclonal antibody to the COOH tail of rat CHIF was raised against the synthetic peptide CKVPTLITPGAST as detailed elsewhere (23). Anti-
body to the COOH tail of γ and the NH2 terminus of γα have been described before (15). Two antibodies to the α1-subunit of Na-K-ATPase have been used. The first (6H) is a monoclonal antibody directed against the NH2-terminal segment of the α-subunit, kindly provided by Dr. M. J. Caplan (Yale University School of Medicine). The second antibody, raised against the COOH-terminal sequence KETYY, was kindly provided by Dr. J. Kyte (University of California at San Diego, La Jolla, CA). Colocalization of CHIF and γ in kidney segments was done as described previously (21, 23).

Detergent solubilization, immunoprecipitation, and immu-
noblotting of CHIF, α, and γ. Colonocytes, microsomal kidney membranes, and HeLa cell membranes were suspended in a buffer containing 25 mM imidazole and 1 mM EDTA, pH 7.5, and either 20 mM Tris-HCl, 20 mM NaCl plus 0.1 mg/ml oligomycin (incubated for 25 min at room temperature), or 10 mM RbCl plus 5 mM ouabain (incubated for 20 min at room temperature). Membranes were solubilized at 0°C by adding C12E10 to a final concentration of 1 mg/ml and a final protein concentration of ~0.5 mg/ml. The detergent-solubilized mem-
branes were centrifuged for 30 min at 100,000 g, the super-
natant was collected, and Tris-HCl, NaCl, or RbCl was added to a final concentration of 100 mM. An ~40-μg aliquot of protein was removed (total protein samples), and the rest was subjected to immunoprecipitation. Total protein samples were delipidated by adding 4 vol of methanol-ether (2:1) and incubation at −20°C overnight. The suspensions were spun down, and the pellets were dried and dissolved in SDS-PAGE sample buffer.

For immunoprecipitation, the detergent-solubilized pro-
teins (~300 μg) were incubated for 4 h at 4°C with an appropriate antibody (1:50). Protein A-Sepharose was added (50-μl volumes of washed and preinduced beads), and the suspensions were further incubated with swirling overnight at 4°C. The beads were sedimented and washed three times in the original imidazole-EDTA buffer plus Tris-HCl, NaCl, or RbCl plus 0.2 mg/ml C12E10. They were suspended in SDS-PAGE sample buffer and resolved on 10% Tricine gels together with 40-μg aliquots of the total delipidated membrane proteins. Proteins were transferred to polyvinylidene difluoride membrane filters in 3-(cyclohexylamino)-1-pro-
panesulfonic acid buffer plus 10% methanol (12 V, 1.4 h). The immunoblots were carried out as described previously (7, 11) using anti-KETTY (1:3,000), anti-CHIF (1:1,000), or anti-γ (1:1,000) antibodies and overlaid with horseradish peroxi-
dase-coupled goat anti-rabbit IgG (1:6,000).

Transfection and selection of HeLa cells. The coding region of rat CHIF was subcloned into the BamHI/BstXI site of the mammalian expression vector pIRESE hyg (Clontech). HeLa cells overexpressing the rat α1-subunit of Na-K-ATPase (HeLa-α1, kindly provided by Dr. J. B. Lingrel, University of Cincinnati College of Medicine) were transfected using lipofectamine (GIBCO BRL) according to the manufacturer’s instructions. Colonies overexpressing CHIF were selected in 400 μg/ml hygromycin B.

Membrane preparations and Na-K-ATPase assays. Membranes were prepared from transfected rat HeLa-α1 cells, and kinetic assays of Na-K-ATPase were carried out in triplicate as described previously (21). KA and VA values represent the least squares fit of the data of at least three separate paired experiments fitted to a simple Michaelis-Menten model. KA and VA, apparent affinities for Na and K, were similarly analyzed but with the data fit to the Gar-
Garrahnan noncooperative three-site (Na*) or two-site (K*) model for Na and K activation, respectively.

Results and Discussion

The objective of the present study has been to test this hypothesis and elaborate on it using native epithelia and transfected mammalian cells. Structural and functional interactions between a mammalian modulator and the pump detected in the native environment or mammalian cells are likely to have physiological significance.

Eighty-eight rubidium flux assays. 86Rb uptake was measured as described elsewhere (20). HeLa-α1 cells stably transfected with CHIF or empty vector were cultivated in 24-well plates. Confluent monolayers were washed twice at 37°C with 0.5 ml/well of a solution containing the desired amount of NaCl and KCl, a complementary amount of choline chloride to achieve a total salt concentration of 145 mM, 5 mM glucose, and 5 mM HEPES-Tris (pH 7.4). The final medium consisted of 465 μl of the above buffer plus 0.5 mM CaCl2, 0.5 mM MgCl2, 1 mM BaCl2, RbCl, and KCl as indicated (see results) and 0.1 mM furosemide. To block endogenous Na-K-ATPase activity, 10 μM ouabain was added to one-half of the wells, and 5 mM ouabain was added to the other half to block the transfected Na-K-ATPase. Monensin was added to a final concentration of 10 μM, and the cells were incubated for 15 min at 37°C under 95% O2-5% CO2. Gassing was then stopped, and uptake was initiated by the addition of 25 μl 86RbCl (100 μCi/ml) to each well. The reaction was stopped 15 min later by aspirating the radioactive medium and rinsing the wells four times with 0.5 ml of an ice-cold PBS buffer containing 5 mM BaCl2. The cells were lysed with two 0.4-ml volumes of 1 M NaOH, the cell lysates were collected, and lystate radioactive and protein contents were determined. Data were expressed as nanomoles Rb taken up per milligram protein per minute, and data from three wells on the same plate were averaged.

RESULTS

Figure 1 provides evidence for a specific interaction between the α-subunit of Na-K-ATPase and CHIF by immunoprecipitation from colonocyte membranes. Because preliminary experiments failed to show immunoprecipitation of CHIF by anti-α-subunit antibo-
dies, we conducted a series of experiments to optimize coimmunoprecipitation of γ and α-subunits from renal
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Na-K-ATPase, assuming that similar conditions would hold for CHIF and α. Coimmunoprecipitation of α- and γ-subunits solubilized from renal microsomes with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate has been described (18), but the efficiency was low. We have now found that the efficiency of coimmunoprecipitation depends strongly on the nature of the antibody, the type of detergent used, and ionic conditions. Of several anti-α-subunit antibodies tested, only a monoclonal antibody (i.e., 6H) directed against the NH2-terminal segment of the α-subunit was effective, and with the following detergents the order of efficiency was found to be C12E10 > 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate > dodecyl maltoside. In unrelated work, we have found that when pig kidney Na-K-ATPase is solubilized with either C12E10 or dodecyl maltoside, in the presence of Rb ions plus ouabain or Na ions plus oligomycin, the solubilized protein retains the ability to occlude Rb or Na ions, respectively. Also, specific Fe-catalyzed oxidative cleavage is preserved, implying that native structure is retained in these conditions (9a). In contrast, on Na-K-ATPase solubilization in the absence of these ligand combinations, the ability to occlude cations is lost, implying that the native structure is not maintained.

Figure 1A shows that when the renal enzyme was solubilized with C12E10 in the presence of Rb ions plus ouabain or Na ions plus oligomycin, the γ-subunit (i.e., both γα- and γβ-subunit splice variants) was coimmunoprecipitated quite efficiently by 6H, whereas in a medium containing only Tris ions the efficiency was lower. Thus it appears that a native structure is required for the most efficient coimmunoprecipitation. In optimal conditions, the efficiency of immunoprecipitation of γ by anti-α was estimated to be 15–20%. (The poor efficiency of dodecyl maltoside in coimmunoprecipitation experiments implies that the detergent disrupts α-γ interaction despite maintenance of cation occlusion and the Fe-cleavage pattern.) The experiment in Fig. 1B shows that 6H effectively immunoprecipitates CHIF from colon membranes solubilized with C12E10 in media containing Rb ions plus ouabain or Na ions plus oligomycin, whereas immunoprecipitation in the Tris-only medium is barely detectable. In contrast, the α-subunit was effectively precipitated in all three media. The conclusion is that the interaction between α and CHIF is preserved in the presence of Rb ions plus ouabain or Na ions plus oligomycin and that CHIF can be immunoprecipitated but is largely disrupted in media lacking these ligands. Note that the effect of pump ligands is not indicative of conformational dependence as such but reflects the ability of Rb plus ouabain or Na plus oligomycin to protect against denaturation and preserve an intact protein structure. In optimal conditions, the efficiency of immunoprecipitation of CHIF by anti-α was estimated to be ~10%. Immunoprecipitation of CHIF by a specific anti-α antibody, with an efficiency approaching that of γ and dependence on ligand conditions, excludes the possibility that the detected association is adventitious. On the contrary, these features provide strong evidence for specificity of the α-CHIF interaction.

At least three FXYD proteins can be detected in kidney cells: γα, γβ, and CHIF. Thus the question arises, whether the complexes with α- and β-subunits contain only one or more than one member. The experiments in Figs. 2 and 3 examined this question. In Fig. 2 (top), microsomes from rat renal medulla were dissolved in C12E10 in a medium containing Rb ions plus ouabain, and immunoprecipitation was carried with 6H, an anti-γα-specific antibody raised against the sequence TELSANH at the NH2 terminus, and an anti-γ COOH-terminal antibody that recognizes both γα and γβ. The blot was then probed with the anti-γ COOH-terminal antibody. The result is clear-cut. While both anti-α and anti-γ COOH-terminal antibodies immunoprecipitate both γα and γβ (seen as a doublet at apparent Mf values of ~9 and 10), the anti-γα antibody immunoprecipitates only γα and not γβ. All three antibodies immunoprecipitated the α-subunit as detected with anti-KETYY (not shown). The experiment proves that γα and γβ cannot be present together in the same complex. In some cultured cells transfected with γα, a modified form, referred to as γα′, is detected in addition to γα (1, 15, 21). Modified forms of γα or γβ were not detected in γ-subunit extracted from rat kidney membranes, when analyzed by mass spectrometry (15). Because only one band of γ was detected after immunoprecipitation with anti-γα, the experiment depicted
in Fig. 2 supports the conclusion that modified γα-subunits are not present in significant amounts in rat kidney membranes (15). CHIF is expressed primarily in the collecting duct and can be detected readily in microsomes prepared from renal papilla (23), and γ is also detected in renal papilla microsomes. In the experiment depicted in Fig. 2 (bottom), microsomes from rat renal papilla were dissolved as described above and anti-CHIF or anti-γ COOH-terminal antibodies were used for immunoprecipitation. The blots were probed with anti-CHIF or anti-γ COOH-terminal antibodies or with anti-KETYY to detect the α-subunit. The answer again is clear-cut. The anti-CHIF antibody immunoprecipitates CHIF and the α- not the γ-subunit, and the anti-γ antibody immunoprecipitates γ and α but not CHIF. (The band running just above the α-subunit is a contaminant in the antibodies.) The conclusion from Fig. 2 is that no mixed complexes such as α/β/γα/γb, α/β/γα, or γα/CHIF can be detected in renal membranes. Thus the kidney cells contain only the complexes α/β/γα, α/β/γb, and α/β/CHIF (or possibly α/β complexes without γ or CHIF).

In Fig. 3, using immunofluorescence we examined the degree of overlap of expression of γ and CHIF in different sections of rat kidney. The striking finding is that there is absolutely no overlap of fluorescence deriving from anti-γ (green) or anti-CHIF (red), although both proteins are found in all sections except the inner zone of the inner medulla that stains for CHIF but not γ. Thus CHIF and γ are expressed in different subpopulations of cells, consistent with their distribution in different nephron segments (21, 23, 29) (see also DISCUSSION). The result shows that γ and CHIF cannot participate in mixed complexes (such as α/β/γ/CHIF), in agreement with the immunoprecipitation experiments. It also eliminates a remote possibility that we failed to detect an α/β/γ/CHIF complex by immunoprecipitation, based on the unlikely assumptions that CHIF hinders anti-γ binding and γ also hinders anti-CHIF binding.

HeLa cells expressing the rat Na-K-ATPase α1-subunit were stably transfected with CHIF, and cells were then selected by growth in a medium containing hygromycin B. Membranes were prepared from cells transfected with CHIF or empty vector. As seen in Fig. 4A, the latter express α but not CHIF whereas the former express both α and CHIF. The relative intensities of CHIF and α in HeLa cells were compared with those found in native colonocyte membranes. The signal intensities were proportional to the amount of applied protein up to 9 μg. From a comparison of the intensities of CHIF and α, we estimate that the CHIF/α ratio in HeLa cells is ~70% of that in colonocytes. This level of expression is sufficient to enable detection of functional effects of CHIF, as described below. Immunolabeling of the transfected cells shows that CHIF is directed to the plasma membrane (Fig. 4B). CHIF is immunoprecipitated by the monoclonal anti-α antibody as well as by the anti-CHIF antibody (Fig. 4C). Thus the HeLa cells expressing CHIF appeared to be a good model system by which to study the effects of CHIF on the functioning of Na-K-ATPase.
As one approach to the functional characterization of the effects of CHIF, we have compared active \(^{86}\text{Rb}\) uptake into the transfected and control cells. Preliminary transport experiments showed that in the normal growth medium containing ~5 mM K ions, a large fraction of K (\(^{86}\text{Rb}\)) uptake is inhibited by ouabain (50–90%) and the ouabain-inhibited \(^{86}\text{Rb}\) uptake is linear for at least 20 min. Thus the flux assay appeared to be suitable for looking at the cytoplasmic Na and extracellular K concentration dependencies of the active flux. The method used for altering the cytoplasmic Na concentration, normally \(\approx10\) mM, involves preincubation of the cells with the ionophore monensin in media containing different Na concentrations. Monensin catalyzes an exchange of Na for H, and thus equilibration of internal and external Na ion concentration should be associated with large fluxes of protons. Within certain limits of a changed cytoplasmic Na concentration, the cell’s own pH-regulating systems are assumed to be able to maintain the cellular pH near normal (see also footnote 1). Preliminary experiments showed that for all concentrations of monensin between 2.5 and 10 \(\mu\)M, the extracellular and cytoplasmic Na concentrations were equilibrated, as judged by the increase or decrease in ouabain-inhibited \(^{86}\text{Rb}\) uptake when the Na concentration in the medium was changed. Monensin at 20 \(\mu\)M produced some nonspecific inactivation. Figure 5A presents a representative experiment that examines the Na ion concentration dependence of the active K (\(^{86}\text{Rb}\)) uptake between 2 and 50 mM Na for control and CHIF-transfected cells. The inset shows that ouabain produced a large inhibition of the \(^{86}\text{Rb}\) uptake at all Na concentrations. The flux rate increased as Na was raised from 2 to 50 mM, and evidently the CHIF-transfected cells were activated at significantly lower concentrations of Na ion compared with the control cells. The Na activation curves were fitted to simple Michaelis-Menten functions, because the flux could not be measured at sufficiently low Na concentrations to detect the normal sigmoidal shape of such curves. Table 1 presents the derived \(V_{\text{max}}\) and \(K_{0.5}\) values for three full experiments, which show that the apparent affinity for Na ions was increased two- to threefold in the CHIF-transfected cells whereas \(V_{\text{max}}\) was not significantly affected. The average value of \(V_{\text{max}}(\text{CHIF})/V_{\text{max}}(\text{empty vector})\) for the three experiments is 1.05 \(\pm\) 0.14. The observation that \(V_{\text{max}}\) was not changed excludes the possibility that the expression of CHIF affected the cell surface expression of the pump. The data for the three Na activation

\[V_{\text{max}} = \frac{V_{\text{max}}(\text{CHIF})}{V_{\text{max}}(\text{empty vector})} = 1.05 \pm 0.14\]

Footnote 1: In other experiments, the rate was measured at Na concentrations up to 75 mM and was constant between 30 and 75 mM. This finding provides indirect evidence that any cytoplasmic pH changes associated with equilibration of the Na by Na/H exchange are not great enough to affect the rate of pumping. If the increased rate of Na/H exchange required to equilibrate 75 mM Na compared with 30 mM Na had produced a significant increase in cellular pH, one could have expected the flux rate to fall between 30 and 75 mM Na.

Fig. 5. Ouabain-sensitive \(^{86}\text{Rb}\) fluxes in HeLa cells. Inset: \(^{86}\text{Rb}\) uptake into HeLa cells in the presence and absence of 5 mM ouabain. Measurements were done in the presence of 10 \(\mu\)M monensin and increasing concentrations of extracellular Na (substituting choline) as described in MATERIALS AND METHODS. Ouabain-sensitive \(^{86}\text{Rb}\) uptake in HeLa-\(\alpha\) cells transfected with CHIF (circles) or empty vector (triangles) is shown. Each data point is mean \(\pm\) SE of 3 plates. Solid lines, best fits to Michaelis-Menten kinetics. The best-fit parameters are CHIF-transfected \(K_{0.5} = 2.4 \pm 0.4\) mM; \(V_{\text{max}} = 10.3 \pm 0.4\) mmol-mg\(^{-1}\)-min\(^{-1}\). Empty vector-transfected \(K_{0.5} = 6.5 \pm 1.4\) mM; \(V_{\text{max}} = 9.5 \pm 0.7\) mmol-mg\(^{-1}\)-min\(^{-1}\).

Fig. 4. Expression CHIF in HeLa-\(\alpha\) cells. HeLa-\(\alpha\) cells were stably transfected with CHIF as described in MATERIALS AND METHODS. A: different amounts of protein extracts from colonocytes or HeLa cells transfected with CHIF or empty vector were blotted with the anti-CHIF or anti-\(\alpha\) antibody. B: immunolabeling of transfected cells with anti-CHIF antibody. C: cells stably transfected with CHIF or empty vector (Vec.) were dissolved in C\(_2\)E\(_{10}\) in the presence of RhCl\(_3\)-ouabain. Cell lysates were immunoprecipitated with the anti-\(\alpha\) antibody and then blotted with antibodies against CHIF or \(\alpha\) as described in the legend to Fig. 1.
Table 1. Effect of CHIF on kinetic parameters of active $^{86}$Rb uptake measured in whole cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$V_{\text{max}}$, nmol·mg$^{-1}$·min$^{-1}$</th>
<th>$K_{0.5}$, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Vector</td>
<td>1</td>
<td>10.2 ± 1.3</td>
</tr>
<tr>
<td>CHIF</td>
<td>2</td>
<td>9.9 ± 1.6</td>
</tr>
<tr>
<td>CHIF</td>
<td>3</td>
<td>9.5 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. CHIF, corticosteroid hormone-induced factor.

cells and 1.9 ± 0.4 mM for the CHIF-transfected cells. It may be noted that the effect of CHIF measured in the transport assay is distinctly greater than that measured in ATPase assays with broken membranes.

The dependence of the flux on extracellular Rb ion concentration between 0.1 and 5 mM was measured in cells bathed in a medium containing 30 mM Na and treated with monensin. The monensin treatment raises the cytoplasmic Na to a saturating concentration (see Fig. 5). Two full experiments showed no significant difference in the $V_{\text{max}}$ and Rb activation curves of control and CHIF-transfected cells. The curves were fitted to the Hill equation to derive the Hill coefficient, $K$, and $V_{\text{max}}$. The average value of $V_{\text{max}}$ for CHIF-transfected cells is plotted against the Rb concentration in Fig. 6B. Clearly, the curves are superimposable. Values of the fitted parameters for either control or CHIF-transfected cells are $-0.25 ± 0.05$ mM ($K$); $-1.54 ± 0.13$ (Hill coefficient); and $0.41 ± 0.08$ mM (the corresponding value of $K_{0.5}$ for Rb ions).

As a complementary approach to the measurements of active $^{86}$Rb uptake into the intact cells at 37°C, we have measured the kinetics of ATP, Na, and K activation of Na-K-ATPase in isolated membranes (21). Results from ATPase assays summarized in Table 2 indicate that CHIF caused a modest but significant increase in $K_{\text{Na}}$ ($K_{\text{Na}}$ decreased 30%; $P < 0.01$) without affecting either $K_{\text{ATP}}$ or $K_{\text{K}}$.

DISCUSSION

This paper provides evidence for a specific structural and functional interaction of CHIF with the $\alpha$-subunit of Na-K-ATPase in colon and kidney membranes and in cultured HeLa cells transfected with CHIF. The demonstration of modulation of the Na affinity of the pump by CHIF in a mammalian cell at 37°C provides important support for the hypothesis that this functional effect of CHIF has physiological significance. In the kidney, CHIF and the $\gamma$-subunit are expressed in different nephron segments, and the two splice variants of $\gamma$ are also expressed differentially in different nephron segments in renal cortex (21). In kidney membranes, the coimmunoprecipitation experiments show the existence of $\alpha/\beta/\gamma_d$, $\alpha/\beta/\gamma_b$, and $\alpha/\beta/\text{CHIF}$ complexes but no mixed complexes containing $\gamma_b$ plus $\gamma_d$ or $\gamma$ plus CHIF. The segregation of CHIF and $\gamma$ to sepa-

Table 2. Effect of CHIF on kinetic parameters of Na-K-ATPase activity measured in isolated membranes

<table>
<thead>
<tr>
<th>Control</th>
<th>CHIF</th>
<th>CHIF/Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{\text{Na}}$, mM</td>
<td>5.37 ± 0.67</td>
<td>3.76 ± 0.93</td>
</tr>
<tr>
<td>$K_{\text{K}}$, mM</td>
<td>0.71 ± 0.16</td>
<td>0.71 ± 0.17</td>
</tr>
<tr>
<td>$K_{\text{ATP}}$, μM</td>
<td>441 ± 35</td>
<td>432 ± 41</td>
</tr>
<tr>
<td>$V_{\text{max}}$, nmol·mg$^{-1}$·min$^{-1}$</td>
<td>289 ± 20</td>
<td>357 ± 37</td>
</tr>
</tbody>
</table>

Values are means ± SE with no. of membranes. $K_{\text{Na}}$, $K_{\text{K}}$, $K_{\text{ATP}}$, apparent affinities for cytoplasmic Na, extracellular K, and ATP, respectively. *Significantly different from 1.0 ($P < 0.001$).
rate complexes is attributable to their different cell-specific expression. On the other hand, γα and γβ are expressed in the same cells, at least in the thick ascending limb of the loop of Henle. Therefore, the lack of mixed complexes indicates that the 1:1:1 α-to-β-to-γ ratio is the true form of the molecular complex (see also Ref. 15). In contrast to the unequivocal result in Fig. 2 excluding the possibility of mixed complexes such as α/β/γα/γβ, immunoprecipitation of γα with γβ and α was recently reported (2). Membranes from rat kidney medulla were dissolved with C12E8 at room temperature in a buffer containing 25 mM imidazole, pH 7.3, and 1 mM EDTA, before the immunoprecipitation reaction. As emphasized above, these conditions of detergent solubilization do not maintain the Rb occlusion or a native protein structure intact, as judged by the Fe-catalyzed cleavage. Thus complexes detected in these conditions should reflect the inactivated state. Membrane proteins denatured by excess detergent are known to undergo irreversible aggregation, particularly at raised temperatures. It cannot be excluded that individual soluble complexes α/β/γα and α/β/γβ underwent aggregation and were then immunoprecipitated.

After transfection of CHIF into the HeLa cells expressing the rat α1-subunit to a level comparable to that in native colon membranes, the apparent affinity of cytoplasmic Na for activating the pump-mediated active K (Rb) uptake was raised two- to threefold compared with that in control cells (K0.5 6.3 ± 2.0 mM for control cells and 1.9 ± 0.4 for the CHIF-transfected cells). In contrast, the Vmax values and apparent affinity for activation by extracellular Rb were unaffected by expression of CHIF (K0.5 for Rb ions, −0.41 ± 0.08 mM; Hill coefficient, −1.54 ± 0.13 for both sets of cells). The raised affinity for cytoplasmic Na ions is similar to the effect reported recently after expression of CHIF in oocytes, although the magnitude of the effect is larger in the HeLa cells (5). In the oocyte study, CHIF was also found to decrease by about twofold the apparent affinity for extracellular K ions at large negative membrane potentials (up to −150 mV) in the presence of extracellular Na ions (90 mM) but not in the absence of extracellular Na ions. The lack of effect of CHIF on the apparent affinity for extracellular Rb ions in the HeLa cells is different from the oocyte result. However, this is most likely due to different conditions of the measurements. The membrane potential in the cultured cells is unlikely to exceed −50 mV, a potential at which the effect on K affinity was quite small in the oocytes (see Fig. 7B in Ref. 16). Furthermore, the extracellular Na concentration was only 30 mM in our study compared with 90 mM in the oocyte experiments. In oocytes, the effect of CHIF on K0.5 for K ions was shown to depend on extracellular Na concentration and was interpreted as an effect of the membrane potential on the affinity of extracellular Na ions for competition at the K sites (5). The effect could be expected to be reduced even more at the lower Na used in our experiments (30 mM) compared with the 90 mM used in the oocyte experiments, and, as mentioned before, it disappeared altogether in the absence of extracellular Na ions. Overall, the effects in oocytes and HeLa cells are not inconsistent with each other.

In assays of Na-K-ATPase activity using isolated membranes, CHIF significantly reduced KNa from 5.37 ± 0.67 to 3.76 ± 0.93 mM with no significant change in KKr or KATP. Thus qualitatively the effect of CHIF in reducing KNa with no effect on KKr is the same in isolated membranes and whole cells. However, the magnitude of the effect on KNa in isolated membranes (CHIF/control 0.69 ± 0.12) is distinctly lower than in whole cells (CHIF/control 0.3 ± 0.12). This finding could imply that CHIF-pump interactions are relatively weak and are partially disrupted by the experimental manipulations involved in isolating membranes. Other evidence that suggests that CHIF-pump interactions in isolated HeLa cell membranes are relatively weak compared with γ-pump interaction may reflect a greater sensitivity to disruption by raised C12E10 concentrations and greater dependence on the ionic conditions of solubilization in immunoprecipitation assays (results not shown).

The CHIF-induced increase in affinity for cytoplasmic Na ions, with no change in Vmax or apparent affinity for extracellular Rb (K) ions in transport assays, and similar if smaller effects on KNa, with no effects on KKr or KATP observed in ATPase assays, has an interesting mechanistic implication. In principal, a change of two- to threefold in apparent cytoplasmic Na affinity could be the result of an effect on either the intrinsic binding affinity of Na sites or the rate constants of the catalytic cycle that stabilize the E1 conformation of the protein to which the cytoplasmic Na ions bind. In the latter case, one could also expect to observe an effect on Vmax and, in particular, a reduced apparent affinity for extracellular Rb (K) ions due to a reduced steady-state level of the E2P conformation to which the Rb (K) ions bind and, conversely, an increased KATP. Because none of the latter three effects was observed, the simple explanation is that CHIF does not affect the E1-E2 conformational equilibrium but raises the intrinsic affinity for cytoplasmic Na sites. Of the three cytoplasmic sites for Na ions, the third site, which does not bind K ions and is selective for Na ions, could be a good candidate for such an effect.

The functional effects of CHIF, described here and elsewhere (5), including the increased apparent cytoplasmic Na affinity and decreased extracellular K affinity at high negative membrane potentials in Na-containing media, are quite different from those of the γ-subunit described previously (1, 19, 21, 26). The effects of γ include 1) an increased ATP affinity, due to stabilization of the E1 conformation; 2) an increased K vs. Na antagonism at the cytoplasmic surface, leading to a reduced apparent cytoplasmic Na affinity at the physiological cytosolic K concentration; 3) a raised apparent affinity for extracellular K at high negative membrane potential in a Na-containing medium; and 4) induction of unselective cation channels. In all cases, the kinetic effects on the pump are of moderate mag-
nitude, at most two- to threefold changes in apparent affinities of ligands. The physiological significance of the kinetic effects of CHIF and γ on pump kinetics must focus on the cytosolic Na concentration, which is limiting in normal physiological conditions, or on the ATP concentration, which may fall in anoxia. As mentioned above, whether changes in extracellular K affinity, observed in some conditions, are physiologically significant is unclear because the extracellular K concentration is normally close to saturating. Similarly, the physiological significance of γ-induced channels is not clear.

Presumably, the different functional modulation of the Na-K pump by γ or CHIF serves different physiological needs of the cells in which they are expressed. In the kidney, the γ-subunit is expressed in the medullary thick ascending limb of Henle’s loop at a high concentration, in the distal convoluted tubules, and at lower levels in proximal tubules and macula densa (21). The medullary thick ascending limb of Henle’s loop segments are characterized by a very high rate of Na pumping and transepithelial Na reabsorption, which serve their role in generating renal salt gradients. We have argued previously that an increased ATP affinity allows maintenance of Na-K pump rates in response to rapid falls in ATP levels accompanying anoxic episodes, whereas a decreased cytoplasmic Na affinity may allow the pump to respond sensitively to increases in cytoplasmic Na at higher set point levels of cytosolic Na (21, 26). In contrast to γ, CHIF is expressed exclusively in cortical and medullary collecting ducts and could be expected to serve the different special needs of these segments, particularly the crucial role of the cortical collecting duct in K homeostasis and its regulation by mineralocorticoids. A physiological rationale for the observed functional effects of CHIF must, of course, fit the evidence for a physiological role of CHIF in K homeostasis, based on the raised expression induced by high serum K and a low serum Na concentration (28). The Na-K pump rate both responds to changes in the cytosolic Na concentration and is responsible for setting the normal set point cytosolic Na concentration, in conjunction with the passive Na entry systems. A higher Na affinity of the Na-K pump could lead to a reduced cytosolic set point Na concentration and thus maintain the driving force for Na entry and transepithelial potential, which drives transepithelial Na reabsorption from the luminal fluid, already depleted of Na. A CHIF-induced increased Na affinity should adapt the pumping rate to the low cellular Na resulting from the low luminal Na and allow the rate, and thus net Na reabsorption, to respond effectively to a rapid increase in cytosolic Na associated with mineralocorticoid-induced Na permeability at the luminal surface. It has been noted previously that the affinity of the Na-K pump for cytosolic Na in cortical collecting ducts is higher than in other segments of the nephron (4). This may now be attributed to the regulatory interaction of CHIF with the pump.

An obvious implication of the findings in this paper and related results (5) is that other FXYD proteins may serve as regulators of the Na-K pump in different tissues in which they are specifically expressed. The different proteins of the FXYD family may modulate Na-K pump function so as to optimally adapt it to the requirements and environment of the cells in question. The distinct functional effects on pump kinetics of γ and CHIF fit well with the evidence for their complementary expression pattern along the nephron, which implies distinct physiological roles. Of interest in this respect is the recent finding that expression of the γ-subunit is induced in kidney cells adapted to grow in 600- or 900-mosmol/kgH2O solutions (8). Another example of a specific FXYD protein-pump interaction concerns a phospholemman-like protein in the shark rectal gland Na-K-ATPase, which modulates the enzyme activity in a manner regulated by phosphorylation by PKC (16). We have also observed coimmunoprecipitation of phospholemman and the α-subunit from bovine cardiac sarcolemma vesicles (Füzesi M, Garty H, and Karlish SJD, unpublished observations). Thus the investigation of possible regulatory roles of the different FXYD proteins is an attractive subject for future investigation. The experimental systems described here can now be exploited for further analysis and comparison of the structural and functional interactions between the α-subunit and different FXYD proteins.

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A portion of the data has appeared before in abstract form (Bio

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