Human FXYD2 G41R mutation responsible for renal hypomagnesemia behaves as an inward-rectifying cation channel

Qun Sha, Wade Pearson, Lauren C. Burcea, Darian A. Wigfall, Paul H. Schlesinger, Colin G. Nichols, and Robert W. Mercer

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri
Submitted 5 November 2007; accepted in final form 25 April 2008

Sha Q, Pearson W, Burcea LC, Wigfall DA, Schlesinger PH, Nichols CG, Mercer RW. Human FXYD2 G41R mutation responsible for renal hypomagnesemia behaves as an inward-rectifying cation channel. Am J Physiol Renal Physiol 295: F91–F99, 2008. First published April 30, 2008; doi:10.1152/ajprenal.00519.2007.—A mutation in the human FXYD2 polypeptide (Na-K-ATPase γ subunit) that changes a conserved transmembrane glycine to arginine is linked to dominant renal hypomagnesemia. Xenopus laevis oocytes injected with wild-type FXYD2 or the mutant G41R cRNAs expressed large nonselective ion currents. However, in contrast to the wild-type FXYD2 currents, inward rectifying cation currents were induced by hyperpolarization pulses in oocytes expressing the G41R mutant. Injection of EDTA into the oocyte removed inward rectification in the oocytes expressing the mutant, but did not alter the nonlinear current-voltage relationship of the wild-type FXYD2 pseudo-steady-state currents. Extracellular divalent ions, Ca$^{2+}$ and Ba$^{2+}$, and trivalent cations, La$^{3+}$, blocked both the wild-type and mutant FXYD2 currents. Site-directed mutagenesis of G41 demonstrated that a positive charge at this site is required for the inward rectification. When the wild-type FXYD2 was expressed in Madin-Darby canine kidney cells, the cells in the presence of a large apical-to-basolateral Mg$^{2+}$ gradient and at negative potentials had an increase in transepithelial current compared with cells expressing the G41R mutant or control transfected cells. Moreover, this current was inhibited by extracellular Ba$^{2+}$ at the basolateral surface. These results suggest that FXYD2 can mediate basolateral extrusion of magnesium from cultured renal epithelial cells and provide new insights into the understanding of the possible physiological roles of FXYD2 wild-type and mutant proteins.

FXYD2 (Na-K-ATPase γ subunit) is a small, hydrophobic protein of 10 kDa, which was originally identified in purified preparations of the Na-K-ATPase (11). Although it is not required for Na-K-ATPase function, FXYD2 can influence the characteristics of the Na-K-ATPase αβ heteromer. When associated with the Na-K-ATPase, the subunit can modify the voltage dependence of K$^{+}$ activation (4) and influence the apparent affinity of the enzyme for Na$^{+}$, K$^{+}$, and ATP (2, 12, 38). FXYD2 belongs to a family of homologous single transmembrane proteins named after an invariant extracellular, FXYD motif (36). In mammals the family consists of seven members numbered according to the order of their initial sequencing: phospholemman (PLM; FXYD1), Na-K-ATPase γ subunit (FXYD2), mammmary tumor protein of 8 kDa (Mat-8; FXYD3), corticosterone hormone-induced factor (CHIF; FXYD4), related to ion channel (RIC; FXYD5), phosphohippolin (PHP; FXYD6), and FXYD7 originally identified in the databases. All family members have been shown to interact with and modify the activity of the Na-K-ATPase in a tissue- and isoform-specific manner (10, 12). Although the FXYD proteins influence the kinetic parameters of the Na-K-ATPase to only a modest extent, these effects may have important physiological consequences in cation homeostasis. Because each of these proteins has a different tissue distribution and regulatory role in Na-K-ATPase function, FXYD proteins may also serve as tissue-specific modulators of the Na-K-ATPase to adjust its kinetic properties to the needs of a specific tissue, cell type, or physiological state.

In addition to their ability to influence Na-K-ATPase activity, there is strong evidence that FXYD family members can also regulate other transporters (35) and either influence ion channels or form channels themselves. For example, PLM induces a taurine-selective ion channel, Mat-8 a hyperpolarization-activated Cl$^{-}$ current (22, 25), and CHIF evokes slowly activating, depolarization-induced K$^{+}$ currents (3). FXYD2 induces large nonselective currents in Xenopus laevis oocytes (21, 33). It is not clear whether these proteins activate endogenous currents or form the actual ion pathway (39). Support for the latter comes from reconstitution studies with recombinant PLM. In planar lipid bilayers, recombinant PLM forms a taurine-selective ion channel with properties similar to the current observed in oocytes expressing PLM, suggesting that channel activity is intrinsic to the protein (22). In the absence of taurine, PLM may function as a nonselective channel (7). Studies with hyposmotically stimulated human embryonic kidney (HEK) cells overexpressing PLM (23) or cerebellar astrocytes with reduced PLM expression (24) suggest PLM may participate in the osmopdependent taurine leak pathway. In contrast to these studies suggesting activity inherent to the polypeptide, others indicate that X. laevis oocytes have endogenous channels with properties similar to those induced by the FXYD proteins (18). Influenza B virus NB protein, an unrelated single membrane spanning protein, activates an endogenous oocyte conductance by shifting its voltage dependence to less hyperpolarized potentials (34). Consequently, expression of membrane proteins in X. laevis oocytes may induce or modify endogenous currents (34), and it remains unclear whether the FXYD proteins are themselves sufficient for channel activity, or whether they require unidentified, endogenous protein partners.

Immunohistochemistry of rat kidney sections shows that FXYD2 is highly expressed in the basolateral membrane of the

http://www.ajprenal.org 0363-6127/08 $8.00 Copyright © 2008 the American Physiological Society
thick ascending limb of the loop of Henle and the distal convoluted tubule (DCT) (2, 27). Although a distinct functional role for FXYD2 is unclear, recent results suggest an important role in renal function. A human mutation in the gene coding the polypeptide results in renal hypomagnesemia associated with hypocalciuria (20). The conversion of a conserved glycine within the transmembrane domain to arginine (G41R) caused misrouting of FXYD2 from the plasma membrane to an intracellular compartment in transfected COS cells. The hypothesis of Meij et al. (20) is that this misrouting of FXYD2 results in the diminution of Na-K-ATPase activity at the plasma membrane with consequent hypomagnesemia. However, expression of the G41R mutant in HeLa cells (28), or polarized Madin-Darby canine kidney (MDCK) cells (see Fig. 6), does not retard the trafficking of the Na-K-ATPase to the cell surface. Therefore, the involvement of FXYD2 could be more complex and dynamic in the pathophysiology of autosomal dominant renal Mg2+ loss.

The aim of the present study was to further examine the cation channel activities of FXYD2 and the G41R mutation in X. laevis oocytes and to identify the electrophysiological properties of whole cell currents induced by FXYD2 and the G41R mutant. We report here that the G41R mutant generated whole cell ion currents with a novel Mg2+-dependent gating on inward rectification. In addition, substitution of Gly41 with other residues demonstrates that a positive charge at the site is required for this inward rectification. Moreover, when wild-type FXYD2 is expressed in MDCK cells, the cells in the presence of a large apical-to-basolateral Mg2+ gradient exhibit an increased transepithelial current. This current is significantly reduced in MDCK cells expressing the G41R mutant. The results demonstrate that the FXYD2 G41R mutant induces a channel in X. laevis oocytes and MDCK cells that is distinct from the wild-type FXYD2 channels.

MATERIALS AND METHODS

Wild-type FXYD2 and G41R expression in X. laevis oocytes. cDNAs encoding FXYD2 were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). Transfection of the wild-type or G41R cDNAs subcloned into pcDNA3.1(-) (pcDNA3.1(-) was used as described previously (21). Cell culture and Ussing chamber experiments. Type I MDCK epithelial cells were cultured in MDCK media DMEM containing 10% FBS, 800 μg/ml genetin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml Fungizone (all from Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 5% CO2. Cells were transfected with the wild-type or G41R cDNAs subcloned into pcDNA3.1(-) (Invitrogen, Carlsbad, CA) (19). As a control, MDCK cells were also transfected with the empty vector. To grow MDCK cells as polarized epithelia, cells were seeded on supports (culture plate inserts, 0.4-

Experiments were performed at room temperature (20°C). Intracellular electrodes were filled with 3 M KCl and had tip resistances from 0.5 to 2 MΩ. Data were digitized on-line and stored on a computer, or were digitized at 2 kHz onto videotape (Neuro-corder DR-890, Neuro-Data Instruments) for off-line analysis. Currents were recorded either in ND96 solution without Ca2+, with additions as indicated, or in KD98 (98 mM KCl, 1 mM MgCl2, 5-HEPES, pH 7.5). Intracellular injections were made using a pneumatic pressure ejection device (PV800; WPI, Sarasota, FL). All experiments were done at room temperature.

Isolation of oocyte membranes. Plasma membranes from X. laevis oocytes were prepared as described before (14). Approximately 200 oocytes were homogenized in 1.5 ml of ice-cold buffer containing 150 mM NaCl, 25 mM Tris·HCl, pH 7.4, 10 mM Mg acetate, and 10% (wt/vol) sucrose using a glass-lass homogenizer. The homogenate was centrifuged at 500 g for 5 min. The supernatant was removed and the pellet was resuspended in 1.5 ml of homogenization buffer and rehomogenized. The homogenate was centrifuged as before and the supernatant was recovered and combined with the first. The combined supernatants were then overlaid on top of a discontinuous sucrose gradient composed of 20% (wt/vol) sucrose in 50 mM NaCl, 25 mM Tris·HCl, pH 7.4, 10 mM Mg acetate (4.5 ml), and 50% sucrose in the same solution (5 ml) and centrifuged at 30,000 g for 1 h in a swinging bucket rotor. Membranes at the interfaces between 20 and 50% sucrose (enriched in intracellular membranes and designated heavy membranes) and 10 and 20% sucrose (enriched in plasma membranes and designated light membranes) were carefully removed. Membranes were diluted threefold in homogenization buffer and centrifuged at 100,000 g for 30 min. The membrane pellet was resuspended in homogenization buffer and subjected to SDS-PAGE and immunoblotting as described previously (21).

Wild-type FXYD2 and G41R expression in X. laevis oocytes. cDNAs encoding FXYD2 were subcloned into pSP64 (Promega, Madison, WI) and contains promoter elements for X. laevis globin that promote high levels of expression in X. laevis oocytes. cRNAs were transcribed in vitro using SP6 RNA polymerase and capping from linearized cDNA (mMessage mMACHINE RNA transcription kit, Ambion, Austin, TX). Stage V-VI X. laevis oocytes were isolated by partial ovariectomy under tricaine (MS-222; Sigma, St. Louis, MO) for 1 h. From 2 to 24 h after defolliculation, oocytes were pressure injected with ~50–80 nl of cRNA (0.1–2 μg/μl). Oocytes were maintained at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM Na-HEPES, pH 7.5) containing 2 mM Ca2+ and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) for 1–2 days before recording. Amino acid substitutions at G41 were introduced using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Two-electrode voltage clamp. FXYD2 currents were measured using the two-electrode voltage clamp technique in a small chamber (100 μl) mounted on the stage of a binocular microscope (SMZ-1, Nikon Instruments). The chamber was connected through agar bridges to the current-sensing headstage of the voltage clamp amplifier (OC-725C Oocyte Clamp, Warner Instruments) and rapidly perfused with a laminar flow of bathing solution supplied by one of five reservoirs connected to a manifold at the inlet to the chamber. The exchange rate for the external solution surrounding the oocyte was less than 1–2 s.

AJP-Renal Physiol • VOL 295 • JULY 2008 • www.ajprenal.org
HBS. Nonspecific sites were blocked for 1 h at 37°C in HBS containing 5% normal goat serum (NGS). Primary antibodies against the α subunit of the Na-K-ATPase (5α; diluted 1:100) and γ (γ969; diluted 1:50; preparation of antibody described in Ref. 21) were diluted in HBS containing 1% NGS and applied to cells overnight at 4°C. Following three washes in HBS, FITC- or Cy3-conjugated secondary antibodies (Molecular Probes, Eugene, OR) diluted 1:1,000 in HBS containing 0.1% NGS were applied for 1 h at room temperature. Samples were washed three times in HBS and mounted in ProLong Antifade (Molecular Probes). Samples were viewed with a Zeiss Axioskop microscope (×40 objective). Images were captured digitally with a Zeiss Axioskop using Axiovision 2.0 software.

RESULTS

Expression of wild-type FXYD2 and the G41R mutant generates distinct ion currents in X. laevis oocytes. X. laevis oocytes were injected with FXYD2 and FXYD2 G41R mutant mRNAs (cRNA). All oocyte currents evoked by both depolarization and hyperpolarization were examined 1–3 days post-injection and unless noted, all experiments were performed using the voltage protocol as described previously (33). Whole cell electrodes in response to voltage steps between −140 and +80 mV from a holding potential of −10 mV in ND96 solution with a pulse duration of 2 s, with a 50-s interval between each pulse to give sufficient time for recovery from activation. For oocytes injected with wild-type cRNA, hyperpolarization to −140 mV evoked a slowly activated inward current that attained an amplitude of −35 to −40 µA within a 2-s activating pulse; depolarization also elicited a slowly activated outward current that reached an amplitude of 37 to 48 µA at +80 mV (Fig. 1A). These currents did not inactivate, even when activating pulses lasting longer than 1 min were applied. As shown in Fig. 1B the current-voltage relationship (I-V) of the wild-type-induced currents is nearly symmetrical between depolarization and hyperpolarization voltage steps, suggesting that the channels are nonselective. Using an inverted protocol of the voltage pulses, starting instead with +80 mV down to −140 mV, resulted in nearly identical I-V plots as from the original voltage protocol (data not shown). In addition, the I-V plots recorded in high [Na] (ND96) or high [K] (KD98) solutions were identical (data not shown).

Expression of the G41R mutation also gave rise to large inward currents (5 to 16 µA; Fig. 1A). However, the G41R mutant outward currents at +80, +60, +40, and +20 mV were smaller compared with the wild-type currents, indicating that the G41R-induced channels exhibit significant inward rectification (Fig. 1A). In addition, ouabain, a Na-K-ATPase inhibitor, did not significantly affect the wild-type or mutant FXYD2 currents (Fig. 1A). The FXYD2 G41R mutant required the injection of nearly 100 times more cRNA than the wild-type to obtain comparable conductance levels (Fig. 1C). As shown before in insect and mammalian cells, the majority of G41R protein is retained within intracellular compartments (20, 28), therefore it is likely that only a small percentage of the expressed mutant protein is delivered to the oocyte plasma membrane. Moreover, when wild-type FXYD2 is expressed in oocytes in the absence of the Na-K-ATPase α and β subunits, the majority of FXYD2 polypeptide is found in intracellular compartments (4). To better characterize G41R mutant localization in oocytes, we isolated plasma membranes from oocytes expressing wild-type or G41R FXYD2 polypeptides. As shown in Fig. 1D, both wild-type and mutant FXYD2 polypeptides are found in the light membrane fractions that are enriched in plasma membranes (14). Proportionally, more wild-type FXYD2 polypeptides are present in the plasma membrane fraction than the G41R mutant. However, it is clear that both...
the wild-type FXYD2 and G41R polypeptides are delivered to the plasma membrane where they can induce channel activity. 

Wild-type and G41R mutant channels display similar sensitivities to extracellular divalent cations and trivalent cations. As previously shown, the wild-type FXYD2 conductances are inhibited by extracellular Ba\(^{2+}\) and Cr\(^{3+}\) (33). Given the inward rectification property of the G41R-activated conductance, we examined the influence of extracellular divalent cations on the FXYD2-induced currents. As shown in Fig. 3, 10 mM Ba\(^{2+}\) (Fig. 3A) and 10 mM Ca\(^{2+}\) (Fig. 3B) inhibited both the wild-type and G41R conductances. However, neither the wild-type nor G41R FXYD2 conductances were inhibited by 10 mM extracellular Mg\(^{2+}\) (Fig. 3C). Interestingly, in the presence of 10 mM extracellular La\(^{3+}\), a potent Mg\(^{2+}\)-channel blocker, both the wild-type and G41R conductances are inhibited.

Ion selectivity of both the wild-type and G41R-induced ion channel activities. As previously noted (20, 30), the FXYD2-activated currents (\(I_{\text{FXYD2}}\)) appear to be nonselective. Replacement of Cl\(^{-}\) by gluconate did not qualitatively alter the currents, nor shift the reversal potential, suggesting that the FXYD2 current is not a Cl\(^{-}\) current. Replacement of Na\(^{+}\) by choline\(^{+}\) did not significantly change the amplitude or time dependence of \(I_{\text{FXYD2}}\), indicating a high permeability to cations. Next we determined whether the G41R mutant channel activity observed in oocytes is also selective for cations. \(V_{\text{rev}}\) of the wild-type and G41R-activated channels is not significantly changed either by an increase in the [K\(^{+}\)] or by a reduction in the [Cl\(^{-}\)] (data not shown). In contrast, in both the wild-type and G41R-activated channels, \(V_{\text{rev}}\) was significantly shifted to more negative (wild-type: 2.4 ± 2.7 to −10.2 ± 1.9 mV, \(n = 14\); G41R: 4.3 ± 3.4 to −13.4 ± 2.4 mV, \(n = 16\)) by substitution of extracellular Na\(^{+}\) with the large cation N-methyl-D-glucosamine (NMDG) in the Na\(^{-}\)-free solution, indicating these channels are highly permeable to cations.

A positive charge at residue 41 is required for inward rectification. To determine the structural basis for inward rectification of the G41R current, we used site-directed mutagenesis to introduce other amino acid residues at G41. As shown in Fig. 4, substitution of G41 by a bulky hydrophobic residue (L or V) resulted in FXYD2 channel activity. However, G41L or V activity were both significantly reduced compared with the wild-type activity. In contrast, changing G41 to glutamine or proline, or to a negatively charged glutamate, did not significantly alter channel activity. However, substitution of G41 to the positively charged lysine again resulted in a channel that exhibited significant inward rectification.

To further characterize FXYD2 (and the mutants) conductance, a different voltage protocol using a brief ramp from −140 to +80 mV was used (Fig. 5A). As shown in Fig. 5B, the wild-type FXYD2 conductance responding to the ramp voltage is significantly different than the G41R or G41K currents. The outward \(I_{\text{FXYD2}}\) is clearly larger than the outward G41R or G41K currents. Thus it appears that inward rectification requires a positive charge at residue 41 of the polypeptide.

Effect of expression of wild-type FXYD2 and G41R on the electrical properties of MDCK cells. To determine whether FXYD2 expression influences the electrical properties of MDCK cells, cells were transfected with the wild-type or G41R FXYD2 cDNAs. As shown in Fig. 6, when expressed in MDCK cells the wild-type FXYD2 polypeptides are delivered to the basolateral membrane surface. In contrast, the G41R mutation causes the retention of the polypeptide within intra-
cellular compartments. Although there is substantial localization of the polypeptide to the plasma membrane, the majority of the FXYD2 mutant polypeptide is within the cytoplasm. In contrast, the Na-K-ATPase subunit is localized to the basolateral plasma membrane. It is apparent that the expression of the G41R mutant does not influence the trafficking of the Na-K-ATPase to the cell surface. This result is consistent with previous studies demonstrating that the expression of G41R in HeLa cells does not retard the trafficking of the Na-K-ATPase to the plasma membrane (28). To test whether FXYD2 expression influences the electrical properties of MDCK cells, cells were grown on permeable supports and subjected to analysis using an Ussing chamber. After 8 days in culture and with Ussing buffer (140 mM NaCl, 5 mM KCl, 0.36 mM K$_2$PO$_4$, 0.44 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, and 10 mM HEPES, pH 7.4) at both the apical and basolateral surfaces, the transepithelial potential and resistance were not significantly different between the control (cells transfected with the empty expression vector, pcDNA), wild-type, and G41R-transfected MDCK cells (Table 1). In the same buffer the transepithelial voltage

![Fig. 3. Effects of external divalent or trivalent cations on both FXYD2 WT and G41R-induced currents. The I-V relationships of the WT and mutant currents: A: 10 mM Ba$^{2+}$ (WT, $n = 6$; G41R, $n = 8$); B: 10 mM Ca$^{2+}$ (WT, $n = 6$; G41R, $n = 6$); C: 10 mM Mg$^{2+}$ (WT, $n = 10$; G41R, $n = 12$); or D: 10 mM La$^{3+}$ (WT, $n = 8$; G41R, $n = 8$). Divalent extracellular and trivalent cations had similar inhibitory effects on both WT and G41R currents. Bars show means ± SE. In all cases, filled and unfilled triangles represent FXYD2WT before and after cation treatment, respectively; filled and unfilled circles represent FXYD2G41R before and after cation treatment.](image)

![Fig. 4. Ion channel properties induced by WT and G41 FXYD2 mutants in oocytes. Using the step voltage clamp protocol, the peak outward current was determined at the end of the depolarizing pulse to +80 mV, and the peak inward was measured at the end of hyperpolarizing pulse to -140 mV. Bars show means ± SE. The numbers (n) are listed on the top for each group. Substitutions of G41 are indicated at the bottom of the figure. For all constructs, ~50 ng cRNA was injected into each oocyte.](image)

![Fig. 5. Different voltage protocol approach was used with a brief ramp protocol from -140 to +80 mV. $I_{FXYD2WT}$ traces responding to ramp voltage are different than the G41R currents or G41K traces. The outward $I_{FXYD2WT}$ is clearly larger than the outward $I_{FXYD2G41R}$ or $I_{FXYD2G41K}$ traces. Each ramp trace represents average data from original ramp recording from each cell (WT, $n = 10$; G41R, $n = 12$; G41K, $n = 6$; H$_2$O-injected control, $n = 7$).](image)
was clamped with a stepwise voltage clamp protocol (identical to the protocol used in the oocytes) and the corresponding currents were recorded. As shown in Fig. 7A, under these conditions there were no significant differences between the control, wild-type, and G41R-transfected MDCK cells. Apparently FXYD2 channel activity is obscured by the endogenously expressed channels present in the MDCK cells. However, when the cells were exposed to a large Mg²⁺ concentration gradient (116 mM apical, 10 mM basolateral), the wild-type FXYD2 MDCK cells, at negative potentials, had an increase in transepithelial current compared with the control transfected cells (Fig. 7B). On the other hand, the G41R-transfected MDCK cells had a slight increase in transepithelial current at the negative potentials compared with the control transfected cells. However, this current was substantially reduced compared with the wild-type FXYD2 currents. These results are summarized in Fig. 7C.

To further characterize the FXYD2-induced currents in MDCK cells, we tested whether the transepithelial currents were inhibited by extracellular Ba²⁺. In X. laevis oocytes, extracellular Ba²⁺ inhibits the FXYD2-induced currents (Fig. 3). As shown in Fig. 8, 10 mM extracellular Ba²⁺ at the basolateral surface inhibited Mg²⁺ currents in MDCK cells expressing either the wild-type FXYD2 or G41R mutant. In contrast, 10 mM extracellular Ba²⁺ at the apical surface increased the transepithelial current. The mechanism by which apical Ba²⁺ increases the transepithelial current is unknown.

### Table 1. MDCK transepithelial potential and resistance in Ussing buffer

<table>
<thead>
<tr>
<th></th>
<th>Mock Transfected Cells (n = 12)</th>
<th>FXYD2 WT Transfected Cells (n = 12)</th>
<th>FXYD2 G41R Transfected Cells (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Em, mV</td>
<td>−2.1 ± 0.6</td>
<td>−1.8 ± 0.8</td>
<td>−2.6 ± 0.9</td>
</tr>
<tr>
<td>Rm, kΩ/cm²</td>
<td>1.54 ± 0.36</td>
<td>1.32 ± 0.19</td>
<td>1.20 ± 0.08</td>
</tr>
</tbody>
</table>

MDCK, Madin-Darby canine kidney cells; WT, wild type.

### DISCUSSION

Electrophysiological properties of the FXYD2-activated channels and the G41R mutant-activated conductance. When expressed in X. laevis oocytes, FXYD2 activates large nonselective ion channels (21). The FXYD2 currents exhibit unique activation kinetics. The voltage jump to the first test potential −140 mV initially induced a small conductance (instantaneously after the voltage step), followed by a progressive...
increase in conductance. Following activation, the deactivation kinetics at the holding potential of −10 mV were extremely slow (>5 min for full recovery), consequently with a subsequent interpulse interval of 30 s, the conductance was already partially activated at the holding potential. Therefore, an instantaneous current, followed by the time-dependent development of more complete activation, was seen during subsequent hyperpolarizing test pulses. Thus it seems that the shape of the I-V relationship with this protocol might be dependent on the time at which the current amplitudes are evaluated, and the order in which the voltage pulses are applied. To evaluate the original voltage clamping protocol, inverted voltage pulses, starting from +80 mV down to −140 mV, were also examined. Identical I-V plots from the two protocols demonstrated that the activated currents were not dependent on the order of the pulse protocol.

External divalent cations Ba\(^{2+}\), Ca\(^{2+}\), and the trivalent lanthanide inhibited the FXYD2 channels in a voltage-independent manner. La\(^{3+}\) ions are considered sensitive and potent blockers for Drosophila TRP, TRPL, human TRP1, TRP3 (26), and native Ca\(^{2+}\) channels (15) and I\(_{\text{CRAC}}\) (5). External Mg\(^{2+}\) did not inhibit the channels induced by the wild-type or the mutant G41R channel. The FXYD2 channel activities in oocytes are independent of exogenously expressed Na-K-ATPase α and β subunits (21) and extracellular ouabain does not alter the FXYD2 current amplitude or kinetics (Fig. 1). In Sf-9 insect cells, a cell line that does not express the Na-K-ATPase subunits (21) and extracellular ouabain does not alter Na-K-ATPase activity (13), there is strong evidence that members of the family can also influence the activity of other transporters or ion channels (1, 12). When reconstituted into planar lipid bilayers, recombinant PLM initiates ion conductances similar to those observed in oocytes expressing PLM, suggesting activity intrinsic to the protein (7). A recent report suggests that CHIF directly modulates the voltage sensitivity of the KCNQ channels, although there is no evidence for actual colocalization of CHIF and KCNQ1 channels in native tissues (16). The present study demonstrates that an inward rectifying cation channel was formed in the oocyte membrane by expression of a mutant FXYD2 that is associated with hereditary renal hypomagnesemia. In MDCK cells, wild-type FXYD2 leads to an increase in transepithelial current when there is a large Mg\(^{2+}\) concentration gradient across the epithelium. This transepithelial current is significantly reduced in MDCK cells expressing the G41R mutant. Similar to the oocyte currents, extracellular Ba\(^{2+}\), when present at the basolateral surface, inhibited the FXYD2-induced currents. These findings suggest that FXYD2 forms a cation channel within the basolateral membrane of MDCK cells that can mediate Mg\(^{2+}\) efflux.

Role of cation channels in renal hypomagnesemia. Like calcium, magnesium plays an important role in many physiological and biochemical processes. Homeostasis of Mg\(^{2+}\) is precisely regulated and depends on the balance between intestinal absorption and renal excretion. Generally, management of total body Mg\(^{2+}\) resides mainly in the nephron segments of the kidney. From the glomerular filtrate, the proximal tubule reabsorbs 10%, the thick ascending limb of the loop of Henle absorbs 70–80%, and the DCT reabsorbs 10% of the filtered magnesium. Beyond the DCT, there is little Mg\(^{2+}\) reabsorption, therefore, the DCT plays an important role in regulating the final urinary excretion of Mg\(^{2+}\) (29). Current understanding of the mechanisms involved in Mg\(^{2+}\) transport across the DCT suggests that an apical Mg\(^{2+}\) channel and a basolateral Mg\(^{2+}\) extrusion system are involved in transepithelial Mg\(^{2+}\) transport (8). More recently, members of the transient receptor potential (TRP) channel family have been identified as Mg\(^{2+}\)-permeable channels that play important roles in magnesium homeostasis. TRP channel melastatin 7, TRPM7, is ubiquitously expressed and has been characterized as an ion channel that is permeable to a variety of divalent cations, including Zn\(^{2+}\), Ca\(^{2+}\), and Mg\(^{2+}\). Under physiological conditions, TRPM7 facilitates the influx of primarily Mg\(^{2+}\) and thus plays a crucial role in cellular Mg\(^{2+}\) homeostasis (32). A closely
related family member, TRPM6 is a Mg\(^{2+}\)-permeable channel predominantly expressed along the apical membrane of the DCT, the small intestine, and colon. Mutations in TRPM6 cause autosomal recessive hypomagnesemia with secondary hypocalcemia (HSH) (31, 40). A missense mutation, P1071R, in the putative pore-forming region of TRPM6 causes the dominant negative inhibition in TRPM6/7 heteromeric channel complexes (9). The functional defect in the putative pore of TRPM6/7 channel appears to be responsible for the impaired body Mg\(^{2+}\) homeostasis. Although the mechanisms for the apical transport of Mg\(^{2+}\) are relatively well characterized, the exact mechanisms for active Mg\(^{2+}\) efflux at the basolateral membrane are unknown. Moreover, the function of FXYD2 in magnesium transport and the role that the FXYD2 G41R mutation plays in hypomagnesemia are not clear. Surprisingly, the FXYD2 knockout mouse has a phenotype indistinguishable from the wild-type mouse (17). These mice do not exhibit hypomagnesemia and have normal Mg\(^{2+}\) urine concentrations.

This would suggest that in mice FXYD2 does not play a direct role in magnesium transport or that other pathways can compensate for the loss of FXYD2.

Using peptides derived from the transmembrane (TM) domain, Therien and Deber (37) found that the FXYD2 TM region oligomerizes in the mild detergent perfluorooctanoate. This association is blocked in peptides that contain the G41R or the more conservative G41L substitutions. This is consistent with the observation that glycine, with its small size, is often found at the interface of TM helices where it maximizes van der Waals forces (30). These results suggest that native FXYD2 polypeptides exist as oligomers and that the G41R mutation may abrogate this association. More recently, it was found using X. laevis oocytes that the complete wild-type FXYD2 oligomerizes (6). However, in contrast to studies using the TM domain, the FXYD2 G41R polypeptides also self-oligomerize and oligomerize with wild-type FXYD2. When associated with the wild-type FXYD2, the G41R mutant reduces the trafficking of wild-type FXYD2 to the plasma membrane (6). Thus the dominant nature of the G41R mutation may be mediated through its association with wild-type FXYD2. This dominant nature of the disorder is supported by the observation that individuals with a 11q23.3-ter deletion, a breakpoint within the FXYD2 gene, have normal serum Mg\(^{2+}\) levels suggesting that the presence of the FXYD2 G41R mutant polypeptide rather than hypoinsufficiency causes the hypomagnesemia (20).

Understanding the etiology of the FXYD2-mediated hypomagnesemia will require a more thorough characterization of the functional properties of the protein. Physiological stress studies of the FXYD2-deficient mouse along with the creation of FXYD2 G41R mutant mice may provide important insights into the physiological function of native and mutant FXYD2 proteins. In addition, elucidating the distinct functional properties of the FXYD2 G41R polypeptide may provide valuable clues to understanding the elements of the FXYD2-mediated hypomagnesemia.

REFERENCES


GRANTS

This work was supported by National Institutes of Health Grants GM-39746 and DK-064704 to R. W. Mercer.

AJP-Renal Physiol • VOL 295 • JULY 2008 • www.ajprenal.org


