Ankyrin-3 is a novel binding partner of the voltage-gated potassium channel Kv1.1 implicated in renal magnesium handling

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The voltage-gated potassium channel, Kv1.1, was recently identified as a causative gene in isolated dominant hypomagnesemia. The channel is situated in the distal convoluted tubule, where it participates in maintaining a favorable electrical gradient for driving magnesium ion into the cell through the transient receptor potential melastatin 6 channel. Pull-down experiments coupled to mass spectrometry using the carboxy-terminal domain of Kv1.1 as bait were used in mouse kidney lysates. Ankyrin-3 (ANK3) was identified as a binding partner of Kv1.1 and was enriched in isolated distal convoluted tubules as compared to whole kidney. Electrophysiology studies performed in HEK293 cells expressing Kv1.1 showed that ANK3 significantly inhibited Kv1.1-mediated currents (267 compared to 125 pA/pF) for control and ANK3, respectively. Finally, to evaluate a potential role of ANK3 in magnesium handling, the intrarenal abundance of ANK3 was measured in mice fed a low-, normal-, or high-magnesium diet for 10 days. Mice maintained on high dietary magnesium significantly doubled their fractional urinary excretion of magnesium, which coincided with a 1.8-fold increase in the renal expression of ANK3 compared to mice on a normal- or low-magnesium diet. Thus, our observations demonstrate a novel role for ANK3 in modulating the biophysical properties of Kv1.1. Such regulation appears to be particularly important in conditions of high dietary magnesium.

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Control of plasma magnesium (Mg^{2+}) levels by the kidney is essential to maintain ample biochemical reactions and physiological functions. Dysregulation of systemic Mg^{2+} homeostasis leads to several clinical symptoms such as muscle stiffness and seizures.[1] The study of clinical and genetic conditions related to hypomagnesemic states have given a better understanding of how the Mg^{2+} balance is regulated by the human body, demonstrating an instrumental role of the kidney. The human kidney filters ~2400 mg of Mg^{2+} per day, and reclaims ~95% of the filtered Mg^{2+} load.[2,3] The regulation of Mg^{2+} reabsorption throughout the nephron allows the kidney to respond to changes in Mg^{2+} balance and thereby keeps plasma Mg^{2+} levels within the normal range.[1] The distal convoluted tubule (DCT) is the major site of active transcellular Mg^{2+} reabsorption.[4] The apical uptake of Mg^{2+} in this segment is mediated via the transient receptor potential melastatin 6 (TRPM6) channel[5–7] and the apical Shaker-like voltage-dependent K^+ channel member 1 (Kv1.1).[8]

The *KCNA1* gene encodes for Kv1.1, being expressed in several organs including heart and smooth muscle, kidney, or brain, where it has been associated with episodic ataxia type-1, a pathological condition in which patients suffer from constant muscle rippling movements and episodic attacks of ataxia.[9] In contrast to Na^+ (NaV) or Ca^{2+} (CaV) voltage-dependent channels, Kv channels are not arranged in a single contiguous polypeptide containing four repeated domains, but consist of four different subunits that are clustered to form the ion permeation pathway across the membrane.[10,11] Each of these subunits contains six α-helical transmembrane segments, where the fourth segment incorporates multiple positive charged amino acids (arginines) responsible for sensing changes in membrane potential, the so-called voltage sensor.[12]

Recently, we described a key role for Kv1.1 in renal Mg^{2+} transport.[8] In a large family with isolated autosomal dominant hypomagnesemia due to renal Mg^{2+} wasting, a mutation in Kv1.1 was identified as the causative factor. The $Kv1.1_{N255D}$ missense mutation resulted in the loss of channel function in a dominant manner. Immunohistochemical studies showed luminal expression of Kv1.1 in DCT segments, where it colocalized with TRPM6.[8] Its location and function in addition to the phenotype observed in

affected patients suggests a key role in epithelial Mg^{2+} transport. The Kv1.1 channel is involved in establishing a favorable electrical gradient to drive Mg^{2+} transport into the DCT cell via TRPM6. As the apical influx of Mg^{2+} in DCT is suggested to largely depend on this electrical gradient, the absence of Kv1.1 is likely to depolarize the apical membrane, and it, thereby, impairs the establishment of a favorable driving force for Mg^{2+} entry.

At present, little is known about the molecular mechanisms regulating the function of Kv1.1 within the kidney. In general, the cytoplasmic terminal domains have a critical role in regulating the biophysical properties of ion channels and transporters.[13,14] We, therefore, used the carboxy (C)-terminal domain of Kv1.1 to screen mouse kidney lysates for novel binding partners that could modulate Kv1.1 function in the kidney, and hence Mg^{2+} transport in the DCT. The identified binding partners were subsequently characterized by immunohistological, cell physiological, and patch-clamp analyses.

RESULTS

ANK3 as new Kv1.1-associated protein

A proteomic approach was used to identify novel binding partners that can modulate Kv1.1 activity in the kidney and thereby modify Mg^{2+} transport in DCT. Using the C-terminal domain of Kv1.1 coupled to glutathione *S*-transferase (GST) pearls as bait, co-immunoprecipitation and pull-down assays were performed on mouse kidney lysates and subsequently analyzed by mass spectrometry to screen for potential candidates. A script developed in-house was used to perform protein validation. The taxonomy of protein identifications was based on the number of uniquely identified sequences of a specific peptide, and/or clusters of proteins sharing the same set of peptides. All the binding partners identified were validated. The identified proteins were aligned at the NCBI (National Center for Biotechnology Information) databank to generate accession numbers and names for each protein, and detailed information on the protein can be found in www.ncbi.nlm.nih.gov as stated. Figure 1a shows a summary of the identified potential binding partners of Kv1.1. Selection of candidates for further analysis was based on positive localization in DCT. DCT-enriched isolates from Complex Parametric Analyzer and Sorter (COPAS)–sorted tubules,[15] expressing enhanced green fluorescent protein (eGFP) after the parvalbumin promoter, showed increased mRNA expression of ankyrin-3 (ANK3) when compared with total kidney. Solute carrier family 2 and 3, which are ubiquitously expressed in kidney, were not enriched in these samples (Figure 1b). Known DCT-specific markers such as parvalbumin, thiazide-sensitive NaCl cotransporter, and TRPM6 were also enriched in DCT isolates.[15] Subsequently, the interaction between ANK3 and Kv1.1 was confirmed by co-immunoprecipitation experiments. Coexpression of GST-labeled Kv1.1 C-tail with hemagglutinin-tagged ANK3 showed strong interaction (Figure 1c).

ANK3 decreases Kv1.1 activity

To examine whether the Kv1.1-interacting candidate ANK3 could modulate the channel function, we used the whole-cell patch-clamp technique in human embryonic kidney 293 (HEK293) cells transiently transfected with both proteins. Activation of the ion channel via the application of a series of depolarizing voltage steps elicited outward rectifying currents resembling the main biophysical characteristics of Kv1.1, that is, a fast delayed activation and slowly inactivating kinetics. These currents were absent in mock-transfected HEK293 cells (Figure 2a). Cells were clamped at a holding potential of -80 mV, and currents were elicited by the application of a family of voltage steps in 200-ms intervals, from -80 mV to $+50$ mV in 10-mV increments. Tail currents were recorded at -80 mV by using a high K^+ extracellular solution. Coexpression of ANK3 produced a significant reduction of the outward currents mediated by Kv1.1, when depolarizing voltage steps were applied (Figure 2a). This was also apparent from the averaged I–V relation curves (Figure 2b and c). Averaged Kv1.1 current densities were significantly reduced by ANK3 (267 ± 28 and 125 ± 20 pA/pF in the absence and presence of ANK3, respectively, $P < 0.05$; Figure 2c). Because the voltage sensitivity of Kv1.1 was significantly affected by ANK3, we obtained the curve for the voltage dependence of activation (G-V). By using the steady-state ionic currents evoked with our voltage step protocol, we confirmed that coexpression of ANK3 was accompanied by a decrease in Kv1.1 conductance from 3.1 ± 0.4 to 1.2 ± 0.1 nS (Figure 2d). However, analysis of normalized conductance (G/G_{max}) did not reveal a significant difference in the voltage requirement for channel activation ($V_{0.5}$): -31.1 ± 0.6 compared with -32.3 ± 1.1 mV in the absence and presence of ANK3, respectively ($P > 0.2$; Figure 2e). These results suggest that ANK3 decreases the open probability of Kv1.1 by impeding the flow of ions through the channel, possibly by stabilizing a closed state of the functional unit in the plasma membrane. In addition, the reversal membrane potential in HEK293 cells expressing Kv1.1 potassium channels, as well as coexpressing ANK3, was measured to investigate the possible modulatory effect of ANK3 on channel activity. Expression of Kv1.1 resulted in a reversal potential of -36.8 ± 1.1 mV ($n = 51$) compared with $+16.4 \pm 8.7$ mV ($n = 10$) in mock-transfected HEK293 cells. A small, although significant ($P < 0.05$), shift to more depolarized membrane potential was demonstrated in the presence of ANK3 -32.8 ± 1.6 mV ($n = 30$).

We analyzed the Kv1.1 time course of activation and inactivation parameters to investigate whether additional biophysical properties of Kv1.1 were affected by the interaction with ANK3 (Figure 3). The time constants of both processes were determined from fitting exponential functions to the relevant currents. This revealed no difference in the activation kinetics of Kv1.1 when it was coexpressed with ANK3 (Figure 3a–d). Fit of the rising phase of currents yielded time constants of 16.4 ± 0.6 ms in the absence compared with 17 ± 1.1 ms in the presence of ANK3. When evaluating the deactivation kinetics of the channel in the presence of ANK3,