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Running head: an apical $K_{Na}1.1$ channel in the TAL

Abstract

Apical potassium channels are crucial for thick ascending limb (TAL) of Henle's loop transport function. The ROMK (*KNCJ1*) gene encodes a 30-pS K channel whose loss of function causes the reduced NaCl reabsorption in the TAL associated with Type 2 Bartter's syndrome. In contrast, the molecular basis of a functionally ROMK-related 70-pS K channel is still unclear. The aim of this study was to highlight new specific channel properties that may give insights on its molecular identity. Using the patch-clamp technique on the apical membrane of mouse split-open TAL tubules, we observed that 70-pS K channel activity, but not ROMK channel activity, increases with the internal Na^+ and Cl^- concentrations, with relative 50 % effective concentrations (EC_{50}) and Hill coefficients (n_H) of 40.6 mM (SD 1.65) and 2.4 (SD 0.28) for Na^+, and of 29.3 mM (SD 2.35) and 2.2 (SD 0.39) for Cl^-. Conversely, 70-pS K channel activity was inhibited by internal K^+ with a relative EC_{50} of 64 mM (SD 13.5) and a n_H of 3.5 (SD 2.3), and by internal NH_4^+ and Ca^{2+}. The reevaluation of channel conductive properties revealed an actual inward conductance of ~ 170 pS, with multiple subconductance levels and an inward rectification, and a substantial permeability to NH_4^+ (P_{NH4}/P_K = 0.2). We conclude that the apical 70-pS K channel in TAL cells is a large-conductance Na^+- and Cl^--activated potassium channel functionally resembling a $K_{Na}1.1$ channel and propose that ROMK determines its functional expression possibly at the level of channel protein synthesis or trafficking.

New & Noteworthy: this study highlights new regulatory and conductive properties of the as yet poorly molecularly defined 70-pS K channel in the apical membrane of thick ascending limb of Henle's loop cells of the mouse kidney that functionally identify it as a large-conductance, Na^+- and Cl^--activated $K_{Na}1.1$-like channel.

Key words: mouse kidney; thick ascending limb; 70-pS K channel; $K_{Na}1.1$ channel; ROMK channel

Introduction

The thick ascending limb (TAL) of Henle's loop of the mammalian kidney reabsorbs approximately 30% of the filtered NaCl load in excess of water, hence plays a pivotal role in the maintainance of the extracellular fluid volume and blood pressure, and participates to ammonia cycle, acid-base metabolism and divalent cations homeostasis (1). The TAL transport function involves an active transcellular process by which Na^+ and Cl^- enter the cell by the apical Na^+-K^+-$2Cl^-$ cotransporter NKCC2 and leave the cell by the basolateral Na^+/K^+ ATPase and ClCK-Barttin chloride channels, and a passive paracellular reabsorption of Na^+, Ca^{2+} and Mg^{2+} driven by a lumen-positive transepithelial potential difference. It is now well established that a dominant apical membrane potassium conductance is the major pathway for the exit of K^+ into the tubular lumen necessary to maintaining steady-state intracellular K^+ concentration and to the generation of the transepithelial potential difference (1).

It is in general agreement that of the three types of potassium channels, a low-conductance (30 pS), an intermediate-conductance (70 pS) and a large-conductance (100-200 pS) Ca^{2+}-activated Maxi-K channels, functionally identified in the apical membrane of TAL cells (2) (3) (4) (5) (6), the 30-pS and 70-pS K channels are the main contributors to the apical membrane potassium conductance in native cells. During the past years, a great deal of effort has been put into their molecular identification, a prerequisite for understanding their role in the physiology and pathophysiology of renal salt reabsorption. There is now compelling evidence that the 30-pS K channel is formed by the homomeric assembly of inwardly rectifying channel ROMK (Kir1.1; *KCNJ1*) subunits and is critical for TAL function (7): the native 30-pS K channel shares many biophysical and pharmacological properties with the recombinant ROMK channel, is functionally detected in ROMK-expressing cells, and is absent in mice lacking *Kcnj1* and exhibiting the strongly reduced reabsorption of NaCl in the TAL associated with the Type 2 Bartter's syndrome caused by loss-of-function mutations in *KCNJ1*. In contrast, the molecular basis of the 70-pS K channel, a major contributor to the apical membrane potassium conductance in the rat (8), is still not clear. On the one hand, that the ROMK and 70-pS K

channels differ by their unitary conductances, kinetics and sensitivities to inhibitors early suggested two distinct molecular entities. On the other hand, studies in which the complete absence of 70-pS K channel activity in *Kcnj1*-null mice clearly established the requirement of *Kcnj1* expression for its functional expression (9) (10). From that emerged the paradigm of ROMK subunits forming part of 70-pS K channel structure, a whole apical membrane potassium conductance underlied by two channels types involving the ROMK subunit thereby providing a satisfactory explanation for the impaired NaCl reabsorption in the TAL of *Kcnj1*-null mice. It was then legitimately postulated that the properties shared by the ROMK and 70-pS K channels are confered by the ROMK subunit, those specific to the 70-pS K channel being accounted by the contribution of a yet-to-be-identified associated subunit. In either case, however, none of the currently known functional properties of the 70-pS K channel, largely established in the rat, is sufficiently distinctive to correlate with either one of the potassium channels subunits molecularly expressed in TAL cells (11).

The aim of this study was therefore to identify new biophysical and regulatory properties of the 70-pS K channel that may bring clues on its molecular identity. To this end, we considered the briefly mentioned absence of channel opening in the rat TAL unless its inner face was exposed to millimolar concentrations of Na^+ (3), a feature not shared by the ROMK channel in the rabbit TAL (5). We hypothesized that such a requirement of internal Na^+ for 70-pS K channel activity might have reflected a sensitivity to intracellular Na^+ reminiscent that exhibited by few potassium channels, namely the small-conductance G protein-coupled inwardly-rectifying (GIRK) Kir3.2 (GIRK2; *KCNJ6*) and Kir3.4 (GIRK4; *KNCJ5*) channels, and the large-conductance Na^+- and Cl^--activated $K_{Na}1$ channels $K_{Na}1.1$ (previously listed as Slack or Slo2.2; *KCNT1*) and $K_{Na}1.2$ (previously listed as Slick or Slo2.1; *KCNT2*) (12) (13). However, only *KCNT1* is expressed in TAL cells where it very likely encodes for a potassium channel functionally resembling a $K_{Na}1.1$ channel in the basolateral membrane (14) (15) (16) (17) (18) (19). Here, we examine whether the 70-pS K channel in the apical membrane of mouse TAL cells also exhibits the ions sensitivity and the conductive properties of a $K_{Na}1.1$ channel with the use of the patch-clamp technique on split-open tubules.

Materials and methods

Animals and tissue preparation

Animals were handled in full compliance with the French government welfare policy and with the recommendations for the care and use of animals for scientific purposes put forward by the European Council Directive 2010/63/EU. The experimental procedures were approved by the Charles Darwin Ethics Committee for animal experimentation (Permit 8569). Adult C57BL/6J male mice (Envigo, Gannat, France) were housed in the Centre d'Explorations Fonctionnelles (UMRS1138, Paris, France; Permit A1-0872) on a 12:12 light/dark cycle (Zeitgeber time, ZT) and under constant temperature and humidity, and fed with pellets of a complete 0.25 % Na^+ and 0.6 % K^+ SAFE-A04 maintainance diet (Augy, France) with free access to tap water.

At the day of experiment, mice were euthanized at ZT 4 (11:00) by cervical dislocation. TAL segments were isolated after an enzymatic treatment procedure derived from that routinely used in the laboratory for the study of renal basolateral ionic channels. Briefly, the left kidney was perfused with Leibovitz L-15 medium containing 150 U/ml Worthington CLS-2 collagenase (Coger SAS, Paris, France), removed and cut into thin slices. Small pieces of cortex were then incubated in the same medium for 25 min at 37 °C. Cortical TAL segments, identified as the tubular part upstream glomerulus attachment or transition into the wider early distal convoluted tubule, were microdissected in ice-cold, collagenase-free, L-15 medium under stereomicroscope, then transfered into a perfusion chamber on the stage of an Axiovert™ 100 microscope (Carl Zeiss S.A.S., Rueil-Malmaison, France). In order to gain access to the apical membrane, tubules were split-open with the broken tip of a patch-clamp pipette filled with bath medium (see below for composition) and operated by a 4MRE motorized micro-manipulator and a SM8 remote control unit (Luigs and Neuman, Ratingen, Germany). Eventual debris at the luminal cell surface were removed by applying a gentle suction to the pipette.

Single-channel recording and analysis

Solutions. Patch-clamp pipettes were filled with a solution containing (in mM) 145 KCl, 1 $MgCl_2$, 10 D-Glucose and 10 HEPES, and adjusted to pH 7.4 with KOH. Tubules were bathed in a medium containing (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-Glucose and 10 HEPES, and adjusted to pH 7.4 with NaOH. A high-Na^+ medium typically bathed the inner face of cell-excised inside-out membrane patches and was modified in order to obtain solutions with various internal Na^+, Cl^-, K^+, NH_4^+ or Ca^{2+} concentrations (see Supplemental Table S1 for composition). When appropriate, the osmolarity of solutions was measured by an Autocal 13DR freezing point osmometer (Hermann Roebling Messtechnik, Berlin, Germany). The exchange of internal solution was performed by gravity and controlled either manually or by a RSC-200 multi-channel rapid solution changer (BioLogic, Seyssinet-Pariset, France) driven by the Axon Instruments® pClamp™ 10 Clampex software (Molecular Devices S.A.S., Villepinte, France).

Current Recordings. Currents were amplified by a L/M-EPC 7 (List Electronics, Darmstadt, Germany) patch-clamp amplifier, filtered at 1 kHz by a LPBF-48DG 8-pole Bessel filter (NPI Electronics GmbH, Tamm, Germany) and digitized at 2 kHz by an Axon Instruments® Digidata® 1440A A/D converter (Molecular Devices) for online monitoring and recording by the Clampex software. The bath reference was 0.5 M KCl in a 4% agar bridge connected to an Ag/AgCl pellet. When appropriate, clamp voltage V_c (i.e. = - $V_{pipette}$) values were corrected for liquid junction potential difference as measured by the Clampex software routine. All experiments were conducted at room temperature (20-25 °C).

Data Analysis. Single-channel recordings were analyzed offline using the Clampex software. Patch activity was quantified according to the equation $NP_o = I/i$, where I is the mean current passing through N channels and i is the unitary current amplitude. Because of patch-to-patch variability in channels activity, the NP_o in test condition was normalized to the NP_o in control condition measured on the same membrane patch.

Ions sensitivity parameters were determined by the nonlinear curve fitting of a four-parameter Hill equation to the normalized NP_o data using

$$NP_o = A + \{(B - A)/[1 + C^{n_H}]\} \quad (1)$$

where A and B are the minimum and maximum normalized NP_o, respectively, and n_H is the Hill coefficient. C is the $K/[X]$ or $[X]/K$ ratio for channel activation or inhibition, respectively, $[X]$ being the concentration of the tested ion X and K the $[X]$ value giving a response halfway between A and B (relative EC_{50}).

The channel inward slope conductance in cell-excised inside-out membrane patches exposed to asymmetrical transmembrane K^+ concentrations was determined by first fitting the Goldman-Hodgkin-Katz (GHK) constant field current equation for K^+ and Na^+ to i/V_c relationships data over the -100 to +40 mV V_c range using

$$i = \frac{V_c F^2}{RT}\left[P_K\left(\frac{a_o^K - a_i^K e^{\left(\frac{V_c F}{RT}\right)}}{1 - e^{\left(\frac{V_c F}{RT}\right)}}\right) - P_{Na}\left(\frac{a_i^{Na} e^{\left(\frac{V_c F}{RT}\right)}}{1 - e^{\left(\frac{V_c F}{RT}\right)}}\right)\right] \quad (2)$$

where F, R and T have their conventional thermodynamic meanings, P_X is the permeability to the ion X, a_o^X and a_i^X are the respective external and internal ionic activities of X, and taking activity coefficients of K^+ and Na^+ as 0.755 and 0.775, respectively (20), then by fitting the Boltzmann equation the first derivative of GHK data using

$$g = g_{-\infty}^{in} + \frac{g_{+\infty}^{out} - g_{-\infty}^{in}}{1 + e^{(V_c - V_c^0)/k}} \quad (3)$$

where g is the conductance at a given V_c, $g_{-\infty}^{in}$ is the inward conductance at infinite negative V_c, $g_{+\infty}^{out}$ is the outward conductance at infinite positive V_c, V_c^0 is the V_c value at $g_{-\infty}^{in} - g_{+\infty}^{out}$ and k is the logarithmic sensitivity for an e-fold increase in V_c.

The NH_4^+ to K^+ permeability ratio (P_{NH4}/P_K) was determined by fitting the GHK equation to i/V_c data over the -80 to +30 mV V_c range obtained in inside-out membrane patches under opposite K^+ and NH_4^+ gradients, and in the presence of internal Na^+, using

$$i = \frac{V_c F^2}{RT}\left[\left(\frac{a_o^K - a_i^K e^{\left(\frac{V_c F}{RT}\right)}}{1 - e^{\left(\frac{V_c F}{RT}\right)}}\right) - \frac{P_{Na}}{P_K}\left(\frac{a_i^{Na} e^{\left(\frac{V_c F}{RT}\right)}}{1 - e^{\left(\frac{V_c F}{RT}\right)}}\right) - \frac{P_{NH_4}}{P_K}\left(\frac{a_i^{NH_4} e^{\left(\frac{V_c F}{RT}\right)}}{1 - e^{\left(\frac{V_c F}{RT}\right)}}\right)\right] \quad (4)$$

taking a NH_4^+ activity coefficient of 0.77 (21).

The OriginLab Corporation Origin® software (Ritme Informatique, Paris, France) was used to perform linear and nonlinear regression analyses, to generate amplitude histograms and, when appropriate, to fit a multiple peak Gaussian function to histogram data.

Data presentation and statistics

Comparisons of parameters between groups were performed with the Student *t* test for two independent variables. Comparisons of the parameters of two related values were performed by using the Student *t* test for paired samples. Data are reported as individual values or as mean (SD) for the indicated number of experiments. *P* values < 0.05 were taken to represent statistically significant differences.

Results

With a detectable activity in only 16 % of the cell-attached membrane patches (Table 1), the incidence of potassium channels in the apical membrane of mouse TAL tubules was low but actually comparable to the ~ 15 % previously reported by others in mice and rats (2) (3). The ROMK and 70-pS K channels were the only potassium channels detected as distinguished by the well established high, rapidly flickering and voltage-independent ROMK channel activity and the lower, voltage-dependent and "box-like" bursting 70-pS K channel activity (Figure 1a), together with their respective unitary conductance at resting membrane voltage (Figure 1b and g_0 in Table 1). In the rat TAL, the 70-pS K channel may account for as much as 80 % of the whole apical membrane potassium conductance (8). Here, the 70-pS K channel was the most frequently observed in situ, and the ROMK and 70-pS K channels exhibited similar apparent numbers of active channels (N_{app}) and activities (NP_o) (Table 1). Thus, according to the mean conductance per membrane patch one may calculate for each channel type (g_{patch} in Table 1), the 70-pS K channel may mediate most of the apical membrane potassium conductance in the mouse TAL.

Cell-attached membrane patches were systematically excised into the high-Na^+ medium. In this condition, the incidence of the ROMK and 70-pS K channels remained low (~ 21 % of the membrane patches), with respective frequencies, N_{app} and NP_o values as in the cell-attached configuration (Supplemental Table S2). Because openings of the 70-pS K channel in the rat TAL were observed only in the presence of internal Na^+ (3), an absolute prerequisite for the activity of the cloned $K_{Na}1.1$ channel (22), we first determined whether internal Na^+ is also required for 70-pS K channel activity in the mouse TAL. Figure 2 shows a continuous recording illustrating the effect of the complete removal of internal Na^+ on 70-pS K channel activity. In this recording, the membrane patch was initially exposed to 140 mM Na^+ and current levels corresponding to the simultaneous opening of up to six channels were observed. Then, channels activity rapidly disappeared when all internal Na^+ was replaced by Li^+, a weak substitute for Na^+ in activating the native $K_{Na}1$ and cloned $K_{Na}1.1$channels (23) (24), and was readily restored by the return back to the 140 mM Na^+-containing

medium. On the average of seven results obtained according to this sequence, the complete removal of internal Na^+ reduced the normalized NP_o by 97 % (SD 3.5) (P = 0.096 vs 100 % inhibition). From the five patches where very rare and brief openings could be observed in the absence of Na^+, the single-channel current amplitude was not significantly different from that in the presence of 140 mM Na^+ (P = 0.45).

The dependence of 70-pS K channel activity on internal Na^+ concentration is summarized in Figure 3. The dose-response curve established in four separate membrane patches exposed to various Na^+ concentrations shows that channel activity increased with internal Na^+ in a sigmoidal fashion with a relative EC_{50} of 40.6 mM Na^+ (SD 1.65) and a n_H of 2.4 (SD 0.28).

Since the Na^+-dependent $K_{Na}1.1$ channel is also activated by internal Cl^- (22) (24), the effect of altering the internal Cl^- concentration on the activity of the 70-pS K channel was then investigated. Figure 4 shows that its activity in a membrane patch bathed throughout with 140 mM Na^+ was strongly reduced when the internal Cl^- concentration was lowered from 145 mM down to 5 mM but rapidly recovered upon the reintroduction of 145 mM Cl^-. On the average of nine separate measurements, the lowering of internal Cl^- concentration reduced the normalized NP_o by 79 % (SD 10.3) (P = 0.0002 vs 100 % inhibition), and did not affect the single-channel current amplitude (P = 0.4). Channel activity increased with the internal Cl^- concentration, cumulated data from five membrane patches yielding a relative EC_{50} of 29.3 mM Cl^- (SD 2.35), a n_H of 2.2 (SD 0.39) and a predicted normalized NP_o of 0.14 (SD 0.037) in the complete absence of Cl^- (Figure 5).

Figure 6 shows that, in contrast to the 70-pS K channel, the ROMK channel in the mouse TAL was not sensitive to a change in internal Na^+ or Cl^- concentration. Indeed, the substitution of Li^+ for all Na^+ in the bathing medium reduced ROMK channel normalized NP_o by only 0.28 % (SD 4.9) (n = 4; P = 0.91 vs no inhibition). Because less than 3% of the cell-excised membrane patches exhibited ROMK channel activity (see Supplemental Table S2), only two of them could be tested for an alteration in internal Cl^- concentration. In these membrane patches, however, the lowering of internal Cl^- concentration from 145 mM down to 5 mM did not affect ROMK normalized NP_o.

Unlike the apical Maxi-K channel in cultured TAL cells whose activity is increased by a rise in cytosolic Ca^{2+} (6), the 70-pS K channel in the rat TAL is dramatically inhibited by millimolar internal Ca^{2+} concentrations (3), an unusual feature shared with the cloned $K_{Na}1.1$ channel (25). Supplemental Figure S1 shows that the switch from a Ca^{2+}-free to a 1 mM Ca^{2+}-containing internal solution also reduced 70-pS K channel activity in the mouse TAL by 91 % (SD 7.5) (P = 0.1 vs 100% inhibition).

An elevation in internal K^+ concentration inhibits the Na^+-induced activity of the native $K_{Na}1$ channels in guinea-pig ventricular myocytes and of the $K_{Na}1.1$-like channel in the basolateral membrane of TAL cells (Supplemental Figure S2) (26). Figure 7 shows that the 70-pS K channel is also negatively regulated by internal K^+. Here, the activity of at least five channels in a membrane patch clamped at V_c = -60 mV and bathed throughout with 140 mM Na^+ and 145 mM Cl^- rapidly decreased when the internal K^+ concentration was increased from 9 mM to 145 mM and fully recovered upon the return back to the 9 mM K^+-containing medium. On the average of eight measurements, the high internal K^+ concentration decreased the normalized NP_o by 89 % (SD 10.3) (P = 0.02 vs 100% inhibition). Of note, this maneuver also reduced the single-channel current amplitude (P = 0.003), but most likely by the slight effect on current size of the left-shifted equilibrium potential for K^+ in this condition (see Figure 10 for clarity). Since this experimental series was performed by adding 136 mM K-gluconate to the high-Na^+ medium (see Supplemental Table S1), the effect of raising internal K^+ concentration on channel activity could have been related to an increased ionic strength or osmolarity. However, Supplemental Figure S3 shows that the exposure to a high internal K^+ concentration under constant ionic strength and osmolarity still markedly inhibited channel activity (n = 3). As summarized in Figure 8, the combined results from eleven separate membrane patches showed that internal K^+ concentration-dependently decreased 70-pS K channel activity with a relative EC_{50} of 64 mM K^+ (SD 13.5) and a n_H of 3.5 (SD 2.3).

Given the inhibition of the 70-pS K channel by internal K^+, the effect of NH_4^+, a good surrogate for K^+ through ionic channels and transporters, was assessed in the presence of 140 mM Na^+. Figure 9 shows that the channel was also negatively regulated by internal NH_4^+, the normalized

NP_o being reduced by 25 % (SD 12.8) (P = 0.03 vs no inhibition) when 10 mM NH_4^+ were added to the bathing medium (n = 4), and by 98 % (SD 3.8) (P = 0.35 vs 100 % inhibition) with 145 mM NH_4^+ (n = 4).

The 70-pS K channel conductance of ~ 85 pS we determined in cell-attached membrane patches at V_c = 0 mV (g_0 in Table 1) was in reasonable agreement with the 60-75 pS range of conductance reported by others at this voltage and hitherto defining the channel as an intermediate-conductance channel (2) (3) (9), but did not conform to the large inward conductance of ~ 180 pS characterizing the cloned $K_{Na}1.1$ channel (22). However, the steepness of the i/V_c relationship at negative membrane potential differences shown in Figure 1b, as well as of previously published ones (2) (3) (9), strongly suggested a 70-pS K channel of larger inward conductance. Therefore, its conductive properties were reinvestigated in cell-excised inside-out membrane patches under precisely controlled ionic conditions (Figure 10). In the presence of 145 mM K^+ in the pipette, and of 9 mM K^+ and 140 mM Na^+ in the bath, the GHK equation (equation 2) fitted well the obtained i/V_c data with a P_K of 0.41 10^{-12} cm^3 s^{-1} (SD 0.012 10^{-12}) (n = 5) close to the values obtained for the channel in the rat TAL and for native $K_{Na}1$ channels (3) (26). The fit of the Boltzmann equation (equation 3) to the first derivative of GHK data then yielded an inward conductance at an infinite negative clamp potential difference of 172 pS (SD 5.2) (n = 5) (Figure 10 inset), indicating a 70-pS K channel of actual large conductance.

By taking advantage of the remaining activity when the channel was exposed to 145 mM internal K^+ or NH_4^+ in the conditions given in Figures 7 and 9, we were able to establish the first i/V_c curves under a high internal K^+ or NH_4^+ concentration (Figure 10). Under symmetrical 145 mM K^+ condition, the currents through the channel reversed at -0.1 mV (SD 1.2) and the i/V_c relationship for inward currents was linear with a slope (g_{in}) of 173 pS (SD 4.5) (n = 4) that further established the large channel inward conductance. In addition, the outward currents were smaller than expected from Ohm's law with an outward chord conductance ($g_{chord\text{-}out}$), as defined by the slope of the curve between the reversal potential and V_c +40 mV, of 101 pS (SD 3.4) (n = 4) that yielded a rectification coefficient (g_{in} / $g_{chord\text{-}out}$) of 1.7. With 145 mM NH_4^+ added to the high-Na^+ medium, the channel

inward conductance was 164 pS (SD 8.1) (n = 4) and the fit of the GHK equation (equation 4) to the mean inward current data yielded a P_{NH4}/P_K of around 0.2.

Lastly, the 70-pS K channel exhibited the multiple conductance substates described for the $K_{Na}1.1$ channel (22). An example is given in Supplemental Figure S4 where partial channel openings and closures during a burst of activity are visible. The corresponding all-points current amplitude histogram showed that the data points between the fully open and closed states exceeded the values predicted from a double-Gaussian curve by the contribution of several substates.

Discussion

In this study, we used the patch-clamp technique on mouse split-open TAL tubules in order to determine whether the apical 70-pS K channel exhibits the regulatory and conductive properties of a $K_{Na}1.1$ channel. Basically designed and widely used for accessing apical ionic channels all along the nephron, this approach also allowed the study of ionic channels in the lateral membrane of renal cells (27). Since a large-conductance Na^+- and Cl^--activated, $K_{Na}1.1$-like, potassium channel is already functionally present in the basolateral membrane of mouse TAL cells (17) (19), it was of prime importance to ensure the apical location of the channel studied here. However, by taking care to precisely position the patch-clamp pipette tip onto the exposed apical membrane of cells within an intact open area, none of the membrane patches exhibited the typical activity of Kir4.1/Kir5.1 potassium or ClC-K/Barttin chloride channels that could have revealed an accidental patch-clamping of the lateral cell membrane (28) (29).

Our results show that the 70-pS K channel recapitulates the salient functional properties of the basolateral $K_{Na}1.1$-like and cloned $K_{Na}1.1$ channels. First, its activity increases with the internal Na^+ concentration, with a relative EC_{50} of ~ 40 mM Na^+ and a n_H of ~ 2.5 that are within the ranges of EC_{50} (8 to 100 mM Na^+) and n_H (2.4 to 4) values reported for the cloned $K_{Na}1.1$ channel (24) (25) (30) (31) and in good agreement with the EC_{50} of 30 mM Na^+ and the n_H of ~ 4 we previously determined for the basolateral channel (17). Second, the channel is also activated by internal Cl^-, with a relative EC_{50} of ~ 30 mM Cl^- in the presence of an elevated internal Na^+ concentration that falls well within the ranges of EC_{50} (8-35 mM Cl^-) reported for the basolateral and the cloned channels under similar conditions (17) (24) (30), and a n_H of ~ 2 close to the n_H of ~ 1 reported for the basolateral channel and that can be deduced from data on the cloned $K_{Na}1.1$ channel (17) (30). Furthermore, as observed for the basolateral $K_{Na}1.1$-like and cloned $K_{Na}1.1$ channels which are inactive in the absence of internal Na^+ but exhibit a significant activity in a Cl^--free medium (17) (22) (30), the presence of internal Na^+, but not that of Cl^-, is an absolute prerequisite for 70-pS K channel activity. Third, its inhibition by a millimolar internal Ca^{2+} concentration, also observed for the basolateral channel (17),

315 is in accordance with that of the rat and *Drosophila* $K_{Na}1.1$ channels by a wide range of divalent
316 cations (25). And fourth, it exhibits the large conductance and multiple conductance substates that
317 biophysically characterize the cloned $K_{Na}1.1$ channels (22), and a rectification coefficient of 1.7 close
318 to the ratio of 1.8 we found for the basolateral channel (17).

319 The 70-pS K channel also exhibits much less documented properties of Na^+-activated $K_{Na}1$
320 channels. Thus, its activity is inhibited by internal K^+, a feature so far reported for only native $K_{Na}1$
321 channels in guinea-pig ventricular myocytes and in avian trigeminal ganglion neurons (26) (32), and
322 shared with the basolateral $K_{Na}1.1$-like channel (this study). Here, internal K^+ concentration-
323 dependently decreased 70-pS K channel activity in the presence of a high-Na^+ medium with a relative
324 EC_{50} of around 70 mM K^+ and a n_H of 3.5 of the same order of magnitude as the EC_{50} of ~ 100 mM K^+
325 and the n_H of ~ 5 that can be deduced from published data in cardiac cells under similar conditions
326 (26). Similarly, we found that internal NH_4^+, which is unable to replace Na^+ in activating the cloned
327 $K_{Na}1.1$ channel (24), inhibited 70-pS K channel activity. Although we did not establish a complete
328 dose-response curve for the inhibitory effect of internal NH_4^+, our results indicate that it could be at
329 least as potent as K^+. To our knowledge, this constitutes the first observation of a native $K_{Na}1$ channel
330 inhibited by both internal K^+ and NH_4^+. Incidentally, the absence of current through the rat TAL 70-pS
331 K channel upon the substitution of K^+ or NH_4^+ for all internal Na^+ at that time suggested that K^+ and
332 NH_4^+ are not conducted by possibly acting as internal blockers of their own conductance (3). Our
333 results in mice show that the channel, while inhibited by a high internal K^+ or NH_4^+ concentration, is
334 actually able to conduct current in this condition.

335 Structurally, it is well established that $K_{Na}1.1$ channels are formed by the homotetrameric
336 association of $K_{Na}1.1$-α subunits. Indeed, size-exclusion chromatography experiments showed that
337 the purified chicken $K_{Na}1.1$ channel extended C-terminal domain elutes as a very stable
338 homotetrameric complex (33). In addition, the assembly of the C-terminus regulator of K^+
339 conductance (RCK) domain pairs of four $K_{Na}1.1$-α subunits is required to form the octameric ring
340 regulating the channel gate within which resides the Na^+-binding site, and the crossing of the

transmembrane S6 domains of four $K_{Na}1.1$-α subunits forms part of the selectivity filter within the ion conduction pathway (34). Nevertheless, as mentioned in the introduction, it is currently acknowledged that the 70-pS K channel is formed by the heteromeric association of the ROMK subunit with an unidentified one, each confering specific properties (9). ROMK channel activity is not sensitive to changes in internal Na^+, Cl^-, K^+ or Ca^{2+} concentration in the rabbit TAL (5), and the present study shows that the same holds true at least for Na^+ and Cl^- in the mouse TAL. Therefore, one can reasonnably infer that the sensitivity to internal ions of a 70-pS K channel structurally involving the ROMK subunit would very likely have accounted for the contribution of the associated subunit. Na^+-activated potassium channels are endowed with a DxR/KxxH motif located in their C-terminal domain that confers Na^+-sensitivity (12). To our knowledge, TAL cells express only two types of potassium channel subunits that possess such a motif, namely the Kir5.1 and $K_{Na}1.1$-α subunits (12) (28). However, the observations that, among the renal cells expressing both ROMK and Kir5.1 subunits, only TAL cells exhibit functional 70-pS K channels, and that these two subunits already do not physically associate in vitro (35), make very unlikely the formation of a large-conductance Na^+-sensitive potassium channel by the association of ROMK and Kir5.1 subunits in native cells. We obviously attempted to directly determine whether the ROMK and $K_{Na}1.1$-α subunits may molecularly colocalize in the apical membrane of TAL cells and physically interact. Unfortunately, none of the two commercially available anti-*Kcnt1* antibodies (clone N3/26) we tested gave a reliable result (see the limitation section below). Nonetheless, indirect data from the literature argues against a ROMK/$K_{Na}1.1$-α subunits interaction. Indeed, supporting the notion that the heteromerization of potassium channels subunits generally occurs between members of the same family, the Kir-type subunit and the voltage-gated potassium channel Kv-α-type subunit, which exhibits an overall structural organization in the transmembrane domains similar to that of the $K_{Na}1.1$-α subunit but very distant from that of Kir-α subunits (36), do not coassemble with each other to form heteromeric channels (37). Therefore, the $K_{Na}1.1$-like 70-pS K channel in the apical membrane of mouse TAL cells is most likely a channel structurally not involving the ROMK subunit.

The ROMK-independent structure of the 70-pS K channel obviously questions the mechanism accounting for the requirement of *Kcnj1* for its functional expression leading to the complete absence of apical ROMK and 70-pS K channels activity in *Kcnj1*-null mice. An interesting possibility might be an indirect alteration in $K_{Na}1.1$-α protein synthesis or in the trafficking of the $K_{Na}1.1$ channel upon *Kcnj1* deletion. Indeed, the excretion of Uromodulin (*UMOD*), or Tamm-Horsfall glycoprotein, is significantly reduced in *Kcnj1*-null mice, after the pharmacological inhibition of the ROMK channel in primary mouse TAL cells and in patients with Type 2 Bartter's syndrome (38). In addition, *Umod* deficiency in mice decreases NKCC2 cotransporter, and ROMK and ClC/K channels mRNA levels in TAL cells (39), and NKCC2 and ROMK proteins expression at the apical membrane by their defective intracellular trafficking (39) (40) (41). Since a missense $Umod^{R186S/+}$ mutation was recently shown to cause a major retention of premature Uromodulin in the endoplasmic reticulum and to decrease the amount of *Kcnt1* mRNA transcripts in mouse TAL cells (42), further experiments are needed in order to determine whether the absence of functional apical 70-pS K channels in ROMK-deficient mice might have been accounted for a *Kcnj1*-dependent, Uromodulin-mediated, defect in $K_{Na}1.1$-α channel protein expression or trafficking to the apical membrane.

To be functionally relevant, the sensitivity of the apical 70-pS K channel to internal ions should occur in the ionic conditions that exist in TAL cells. However, channel sensitivity to internal ions in cell-free membrane patches predicts a very low activity at the respective resting intracellular Na^+, K^+ and Cl^- concentrations of 15, 155 and 35 mM prevailing in TAL cells (43) (44) (45). Furthermore, given the known complex interplay between internal Na^+, Cl^- and K^+ in regulating native $K_{Na}1$ channels in excitable cells or the cloned $K_{Na}1.1$ channel, it is very likely that the sensitivity of the 70-pS K channel to internal ions might has been underestimated. Indeed, the cloned $K_{Na}1.1$ channel is cooperatively activated by intracellular Na^+ and Cl^- ions so that an increase in the internal concentration of either of them increases the sensitivity to the other (30). Accordingly, the lowering of internal Cl^- concentration from 145 mM to 40 mM causes a ~ 2.5-fold decrease in the sensitivity to internal Na^+ of the basolateral $K_{Na}1.1$-like channel (17). Conversely, an increase in internal K^+

decreases the sensitivity to internal Na^+ of native $K_{Na}1$ channels in excitable cells and vice versa (26) (32). Therefore, one would expect a channel activity in situ even lower than that directly extrapolated from our measurements. Yet, the 70-pS K channel exhibits the significant activity in cell-attached membrane patches (NP_o of ~ 0.9, see Table 1) previously observed by others for the channel in mice and rats (2) (3) (4) (8) (9) (43). Such a paradox has been frequently reported regarding the sensitivity of $K_{Na}1$ channels in excitable cells to internal Na^+ and early proposed to reflect its modulation by an endogenous cytosolic factor(s) lost upon membrane patch excision (23). Very little is currently known on the modulation of the sensitivity of native and cloned $K_{Na}1$ channels to internal ions. Indeed, only one study reports a shift in the EC_{50} for Na^+ from 50 mM to 17 mM upon the direct binding of the oxidized form of nicotinamide dinucleotide (NAD^+) to $K_{Na}1$ channels in rat dorsal root ganglion neurons (46). Whether NAD^+ increases the sensitivity of the 70-pS K channel to internal Na^+ also in TAL cells, thereby making plausible its regulation by low cytosolic Na^+ concentrations in situ, remains to be established. More generally, the identification of factors modulating the sensitivity of the apical and basolateral $K_{Na}1.1$-like channels not only to internal Na^+ but also to Cl^-, K^+ and NH_4^+, whether they be a diffusible cytosolic factor or some other undefined process, should more precisely define the conditions under which physiologically relevant ion concentrations control channels activity in their native environment.

For now this uncertainty makes difficult to ascertain the role of intracellular ions as regulators of $K_{Na}1.1$-like channels activity in TAL cells. In neurons, the sensitivity of $K_{Na}1$ channels to internal Na^+ is crucial to the control of electrical activity where the change in membrane potential caused by the influx of Na^+ during action potentials is counteracted by the afterhyperpolarisation generated by channels activation (47). In the TAL, apical and basolateral potassium channels are essential to TAL transport function by establishing the potential differences across cell membranes and generating the transepithelial potential difference (1). In addition, as emphasized by others (48), the TAL is the only nephron segment where the large transcellular movements of Na^+ and Cl^- associated with NaCl reabsorption cause massive influxes of K^+ via the apical NKCC2 cotransporter

and the basolateral Na^+/K^+ ATPase. In order to maintain steady-state intracellular K^+ concentration, and hence the potential difference across apical and basolateral membranes, these K^+ influxes must be coordinately counterbalanced by appropriate K^+ effluxes (*i.e.* K^+ recycling) through apical and basolateral potassium channels. We previously proposed that the sensitivity of the basolateral $K_{Na}1.1$ channel to Na^+ and Cl^- may provide a signaling linkage between the entry and the basolateral exit of K^+ (17). The identification of internal Na^+ and Cl^- as new regulators of the apical 70-pS K channel suggests that a similar coupling between the entry of K^+ and its apical recycling through the channel may exist. On the other hand, the inhibitory effect of internal K^+ on the activity of the apical and basolateral channels in the context of NaCl reabsorption is puzzling since the associated increase in intracellular K^+ concentration would decrease channels activity and hence antagonize the apical and basolateral exits of K^+. Thus, as in cardiac cells, $K_{Na}1.1$ channels activation in TAL cells might also take place in specific pathological conditions, such as digitalis toxicity where the increase in internal Na^+ concentration is accompanied by a decrease in K^+ concentration in rat TAL cells (49), and protect from damage by maintaining hyperpolarized the cell membranes. Finally, regarding the apical 70-pS K channel, also intriguing is its inhibition by internal NH_4^+. The TAL is an important site of NH_4^+ reabsorption and numerous studies etablished that apical NH_4^+ entry into TAL cells is mediated predominantly by NKCC2 functioning as a Na^+-NH_4^+-$2Cl^-$ cotransporter (50). Our observations that the channel is substantially permeable to NH_4^+ and able to conduct current in the presence of internal NH_4^+ would make it in a position to also contribute to the luminal uptake of NH_4^+. However, the extent to which its inhibition by internal NH_4^+, which may partly account for its reduced activity, and lower apical membrane potassium conductance and voltage in intact rat TAL tubules luminally exposed to 10 mM NH_4^+ (51), may limit its contribution to the net NH_4^+ absorption by the TAL needs further investigation.

A limitation to this study is the lack of strong and definite conclusion on the precise molecular nature of the 70-pS K channel, since neither data from *Knct1*-null mice nor data using molecular biology tools was provided. When combined, these data should infirm or confirm the

445 expression of the $K_{Na}1.1$-α subunit protein in TAL cells apical membrane, its involvement in forming
446 the channel and the ROMK-independent channel structure.

447 In conclusion, this study highlights new regulatory and conductive properties of the 70-pS K
448 channel in the apical membrane of mouse TAL cells that are most consistent with those of a $K_{Na}1.1$
449 channel. This strongly indicates that the ROMK subunit is not a structural component of the channel
450 and we suggest that the ROMK-dependent 70-pS K channel functional expression, which accounts for
451 the absence of apical potassium channels activity in *Kcnj1*-null mice and their severe salt-wasting
452 Type 2 Bartter's syndrome phenotype, may involve a mechanism by which *Kcnj1* expression possibly
453 affects *Kcnt1* transcription, $K_{Na}1.1$-α subunit protein synthesis or channel trafficking.

Data availability: data will be made available upon reasonable request.

Supplemental material: Supplemental Tables S1 and S2, and Figures S1 to S4:

https://figshare.com/s/8f234b08cbf16ec42f02

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621 **Figures legends**

622 **Figure 1**. **In situ apical potassium channels in the mouse TAL**. ROMK and 70-pS K channels properties
623 in cell-attached membrane patches with 145 mM KCl in the patch pipette, and 140 mM NaCl and 5
624 mM KCl in the bath. (**a**) Left : single-channel recordings in two separate membrane patches at the
625 indicated clamp voltage. C- is the current level corresponding to the closure of all channels, and O_1
626 and O_2 are the current levels corresponding to the opening of one and two channels, respectively.
627 Right : voltage-dependence of ROMK and 70-pS K channels activities. For each channel type, data
628 were normalized to the NP_o at resting membrane potential difference (V_c = 0 mV). Each point is the
629 mean of three (□) or seven (■) measurements and SD is shown as error bars. * $P < 0.05$ vs NP_o at V_c =
630 0 mV. (**b**) ROMK and 70-pS K channels i/V_c relationships in the conditions given in **a**. Each point is the
631 mean of three (□) or thirteen (■) measurements and SD is shown as error bars.

632

633 **Figure 2. Effect of internal Na^+ removal on 70-pS K channel activity.** Continuous recording of 70-pS K
634 channel activity in a cell-excised inside-out membrane patch whose internal face is exposed to the
635 indicated Na^+ concentrations (in mM). Bath K^+ and Cl^- concentrations were kept at 9 and 145 mM,
636 respectively, and V_c was set at -40 mV. C- is the current level corresponding to the closure of all
637 channels. The lower left *a* and *b* traces are excerpts at an expanded time scale (*) taken from the
638 corresponding sections of the recording. The lower right graph summarizes NP_o data from seven
639 membrane patches, shown as individual values (□ and dashed lines) and as mean (■ and solid line)
640 with SD shown as error bars when larger than symbol. Data were normalized to the NP_o during the
641 initial exposure to 140 mM internal Na^+. * $P < 0.0001$ vs NP_o = 1.

642

643 **Figure 3. Dependence of 70-pS K channel activity on internal Na^+ concentration.** (**a**) Left : recordings
644 of channel activity in a cell-excised inside-out membrane patch at the indicated internal Na^+
645 concentrations (in mM) in the conditions given in Figure 2, except V_c set at 0 mV. C- is the current
646 level corresponding to the closure of all channels and O_1 to O_6 are the current levels corresponding to

647 the opening of one to six channels. Right : relative amplitude histograms (0.2 pA bins) obtained at the
648 indicated internal Na^+ concentrations (in mM). (**b**) Dose-response relationship of the effect of
649 internal Na^+ concentration on channel activity in the conditions given in **a**. Data from four membrane
650 patches were normalized to the NP_o in the presence of 140 mM Na^+, and are shown as individual
651 values (□, n = 4 for each condition) and as mean (■) with SD shown as error bars when larger than
652 symbol. The dashed line is a nonlinear fit of the Hill equation to the mean data (adjusted R^2 = 0.999).
653 * $P < 0.05$ vs $NP_o = 1$.
654
655 **Figure 4**. **Effect of low internal Cl^- on 70-pS K channel activity**. Continuous recording of 70-pS K
656 channel activity in a cell-excised inside-out membrane patch whose internal face is exposed to the
657 indicated Cl^- concentrations (in mM). Bath Na^+ and K^+ concentrations were kept at 140 and 9 mM,
658 respectively, and V_c was set at -40 mV. C- is the current level corresponding to the closure of all
659 channels. The lower left *a* and *b* traces are excerpts at an expanded time scale (*) taken from the
660 corresponding sections of the recording. The lower right graph summarizes NP_o data from nine
661 membrane patches, shown as individual values (□ and dashed lines) and as mean (■ and solid line)
662 with SD shown as error bars. Data were normalized to the NP_o during the initial exposure to 145 mM
663 internal Cl^-. * $P < 0.0001$ vs $NP_o = 1$.
664
665 **Figure 5**. **Dependence of 70-pS K channel activity on internal Cl^- concentration.** (**a**) Left : recordings
666 of channel activity in a cell-excised inside-out membrane patch at the indicated internal Cl^-
667 concentrations (in mM) in the conditions given in Figure 4. C- is the current level corresponding to
668 the closure of all channels, and O_1 is the current level corresponding to the opening of one channel.
669 Right : relative amplitude histograms (0.2 pA bins) obtained at the indicated internal Cl^-
670 concentrations (in mM). (**b**) Dose-response relationship of the effect of internal Cl^- concentration on
671 channel activity in the conditions given in **a**. Data from five membrane patches were normalized to
672 the NP_o in the presence of 145 mM Cl^-, and are shown as individual values (□) and as mean (■) with

673 SD shown as error bars. The number of observations in each condition is given in parentheses. The
674 dashed line is a nonlinear fit of the Hill equation to the mean data (adjusted R^2 = 0.998). * P < 0.001
675 vs NP_o = 1.
676
677 **Figure 6. Effect of internal Na^+ or Cl^- removal on ROMK channel activity.** Continuous recordings of
678 ROMK channel activity in two separate cell-excised inside-out membrane patches whose internal
679 face is exposed to the indicated Na^+ (**a**) or Cl^- (**b**) concentration (in mM). In **a**, bath K^+ and Cl^-
680 concentrations were kept at 9 and 145 mM, respectively, and V_c was set at -60 mV. In **b**, bath Na^+ and
681 K^+ concentrations were kept at 140 and 9 mM, respectively, and V_c was set at -40 mV. C- is the
682 current level corresponding to the closure of all channels. In **a** and **b**, the lower *a* and *b* traces are
683 excerpts at an expanded time scale (*) taken from the corresponding sections of the recording.
684
685 **Figure 7. Effect of elevated internal K^+ concentration on 70-pS K channel activity.** (**a**) Continuous
686 recording of channel activity in a cell-excised inside-out membrane patch whose internal face is
687 exposed to the indicated K^+ concentrations (in mM). Bath Na^+ and Cl^- concentrations were kept at
688 140 and 145 mM, respectively, and V_c was set at -60 mV. C- is the current level corresponding to the
689 closure of all channels. *a* and *b* are excerpts at an expanded time scale (*) taken from the indicated
690 sections of the recording. (**b**) Summary of NP_o data from eight membrane patches in the conditions
691 given in **a**, shown as individual values (□ and dashed lines) and as mean (■ and solid line) with SD
692 shown as error bars. Data were normalized to the NP_o during the initial exposure to 9 mM internal K^+.
693 * P < 0.0001 vs NP_o = 1.
694
695 **Figure 8. Dependence of 70-pS K channel activity on internal K^+ concentration.** (**a**) Left : recordings
696 of channel activity in a cell-excised inside-out membrane patch at the indicated internal K^+
697 concentrations (in mM) in the conditions given in Figure 7. C- is the current level corresponding to
698 the closure of all channels and O_1 to O_5 are the current levels corresponding to the opening of one to

five channels. Right : relative amplitude histograms (0.2 pA bins) obtained at the indicated internal K^+ concentrations (in mM). (**b**) Dose-response relationship of the effect of internal K^+ concentration on channel activity in the conditions given in **a**. Data from eight membrane patches were normalized to the NP_o in the presence of 9 mM K^+, and are shown as individual values (□) and as mean (■) with SD shown as error bars. The number of observations in each condition is given in parentheses. The dashed line is a nonlinear fit of the Hill equation to the mean data (adjusted R^2 = 0.98). * $P < 0.001$ vs $NP_o = 1$.

Figure 9. 70-pS K channel sensitivity to NH_4^+. Effect of internal NH_4^+ concentration on 70-pS K channel activity. (**a**) Recordings of channel activity in two separate cell-excised inside-out membrane patches with 145 mM K^+ in the pipette and 140 mM Na^+ in the bath and exposed to the indicated NH_4^+ concentrations (in mM), at V_c = 0 mV. C- is the current level corresponding to the closure of all channels. (**b**) Summary of the corresponding NP_o data, shown as individual values (□ and dashed lines) and as mean (■ and solid line) with SD shown as error bars. Data were normalized to the NP_o during the initial control (0 NH_4^+) condition. * $P < 0.05$ vs $NP_o = 1$, ** $P < 0.0001$ vs $NP_o = 0$.

Figure 10. 70-pS K channel conductive properties. 70-pS K channel i/V_c relationships in cell-excised inside-out membrane patches with 145 mM K^+ in the patch pipette and 140 mM Na^+ in the bath, in the presence of 9 mM internal K^+ (■), 145 mM internal K^+ (□) or 145 mM internal NH_4^+ (○). Each point is the mean of five (■) or four (□, ○) measurements and SD is shown as error bars when larger than symbol. Under asymmetric K^+ concentration condition (■), the solid line is the average of five non-linear fits of the GHK equation to i/V_c curves (adjusted R^2 = 0.942 - 0.98). The inset shows the inward slope conductance determined by the first derivative of the individual GHK data as function of V_c. For clarity, only points at the applied clamp voltages are shown, with SD shown as error bars. The continuous line is a non-linear fit of the Boltzmann equation to the mean first derivative data (adjusted R^2 = 0.998). Under symmetric 145 mM K^+ concentration condition (□), the dashed line is a

linear regression fit to inward currents data (adjusted R^2 = 0.997). With 145 mM NH_4^+ in the bath (○), the dotted line is a fit of the GHK equation to the mean data (adjusted R^2 = 0.998)**.**