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# The apical 70-pS K<sup>+</sup> channel in the thick ascending limb of Henle's loop is a large-conductance Na<sup>+</sup>- and Cl<sup>-</sup>-activated, K<sup>+</sup> Na<sup>+</sup> 1.1-like, channel

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**The apical 70-pS K channel in the thick ascending limb of Henle's loop is a large-conductance Na<sup>+</sup>- and Cl<sup>-</sup>-activated, K<sub>Na</sub>1.1-like, channel**

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21 **Running head:** an apical K<sub>Na</sub>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 (*KCNJ1*) 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<sup>+</sup> and Cl<sup>-</sup> 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<sup>+</sup>, and of 29.3 mM (SD 2.35) and 2.2 (SD 0.39) for Cl<sup>-</sup>. Conversely, 70-pS K channel activity was inhibited by internal K<sup>+</sup> 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<sub>4</sub><sup>+</sup> and Ca<sup>2+</sup>. 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<sub>4</sub><sup>+</sup> ( $P_{NH_4}/P_K = 0.2$ ). We conclude that the apical 70-pS K channel in TAL cells is a large-conductance Na<sup>+</sup>- and Cl<sup>-</sup>-activated potassium channel functionally resembling a K<sub>Na</sub>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<sup>+</sup>- and Cl<sup>-</sup>-activated K<sub>Na</sub>1.1-like channel.

**Key words:** mouse kidney; thick ascending limb; 70-pS K channel; K<sub>Na</sub>1.1 channel; ROMK channel

47

## Introduction

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

59           It is in general agreement that of the three types of potassium channels, a low-conductance  
60 (30 pS), an intermediate-conductance (70 pS) and a large-conductance (100-200 pS) Ca<sup>2+</sup>-activated  
61 Maxi-K channels, functionally identified in the apical membrane of TAL cells (2) (3) (4) (5) (6), the 30-  
62 pS and 70-pS K channels are the main contributors to the apical membrane potassium conductance  
63 in native cells. During the past years, a great deal of effort has been put into their molecular  
64 identification, a prerequisite for understanding their role in the physiology and pathophysiology of  
65 renal salt reabsorption. There is now compelling evidence that the 30-pS K channel is formed by the  
66 homomeric assembly of inwardly rectifying channel ROMK (Kir1.1; *KCNJ1*) subunits and is critical for  
67 TAL function (7): the native 30-pS K channel shares many biophysical and pharmacological properties  
68 with the recombinant ROMK channel, is functionally detected in ROMK-expressing cells, and is  
69 absent in mice lacking *Kcnj1* and exhibiting the strongly reduced reabsorption of NaCl in the TAL  
70 associated with the Type 2 Bartter's syndrome caused by loss-of-function mutations in *KCNJ1*. In  
71 contrast, the molecular basis of the 70-pS K channel, a major contributor to the apical membrane  
72 potassium conductance in the rat (8), is still not clear. On the one hand, that the ROMK and 70-pS K

73 channels differ by their unitary conductances, kinetics and sensitivities to inhibitors early suggested  
74 two distinct molecular entities. On the other hand, studies in which the complete absence of 70-pS K  
75 channel activity in *Kcnj1*-null mice clearly established the requirement of *Kcnj1* expression for its  
76 functional expression (9) (10). From that emerged the paradigm of ROMK subunits forming part of  
77 70-pS K channel structure, a whole apical membrane potassium conductance underlied by two  
78 channels types involving the ROMK subunit thereby providing a satisfactory explanation for the  
79 impaired NaCl reabsorption in the TAL of *Kcnj1*-null mice. It was then legitimately postulated that the  
80 properties shared by the ROMK and 70-pS K channels are conferred by the ROMK subunit, those  
81 specific to the 70-pS K channel being accounted by the contribution of a yet-to-be-identified  
82 associated subunit. In either case, however, none of the currently known functional properties of the  
83 70-pS K channel, largely established in the rat, is sufficiently distinctive to correlate with either one of  
84 the potassium channels subunits molecularly expressed in TAL cells (11).

85         The aim of this study was therefore to identify new biophysical and regulatory properties of  
86 the 70-pS K channel that may bring clues on its molecular identity. To this end, we considered the  
87 briefly mentioned absence of channel opening in the rat TAL unless its inner face was exposed to  
88 millimolar concentrations of Na<sup>+</sup> (3), a feature not shared by the ROMK channel in the rabbit TAL (5).  
89 We hypothesized that such a requirement of internal Na<sup>+</sup> for 70-pS K channel activity might have  
90 reflected a sensitivity to intracellular Na<sup>+</sup> reminiscent that exhibited by few potassium channels,  
91 namely the small-conductance G protein-coupled inwardly-rectifying (GIRK) Kir3.2 (GIRK2; *KCNJ6*)  
92 and Kir3.4 (GIRK4; *KNCJ5*) channels, and the large-conductance Na<sup>+</sup>- and Cl<sup>-</sup>-activated K<sub>Na</sub>1 channels  
93 K<sub>Na</sub>1.1 (previously listed as Slack or Slo2.2; *KCNT1*) and K<sub>Na</sub>1.2 (previously listed as Slick or Slo2.1;  
94 *KCNT2*) (12) (13). However, only *KCNT1* is expressed in TAL cells where it very likely encodes for a  
95 potassium channel functionally resembling a K<sub>Na</sub>1.1 channel in the basolateral membrane (14) (15)  
96 (16) (17) (18) (19). Here, we examine whether the 70-pS K channel in the apical membrane of mouse  
97 TAL cells also exhibits the ions sensitivity and the conductive properties of a K<sub>Na</sub>1.1 channel with the  
98 use of the patch-clamp technique on split-open tubules.

**100 Animals and tissue preparation**

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

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

**123 Single-channel recording and analysis**

124            *Solutions.* Patch-clamp pipettes were filled with a solution containing (in mM) 145 KCl, 1  
125 MgCl<sub>2</sub>, 10 D-Glucose and 10 HEPES, and adjusted to pH 7.4 with KOH. Tubules were bathed in a  
126 medium containing (in mM) 140 NaCl, 5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 D-Glucose and 10 HEPES, and  
127 adjusted to pH 7.4 with NaOH. A high-Na<sup>+</sup> medium typically bathed the inner face of cell-excised  
128 inside-out membrane patches and was modified in order to obtain solutions with various internal  
129 Na<sup>+</sup>, Cl<sup>-</sup>, K<sup>+</sup>, NH<sub>4</sub><sup>+</sup> or Ca<sup>2+</sup> concentrations (see Supplemental Table S1 for composition). When  
130 appropriate, the osmolarity of solutions was measured by an Autocal 13DR freezing point  
131 osmometer (Hermann Roebling Messtechnik, Berlin, Germany). The exchange of internal solution  
132 was performed by gravity and controlled either manually or by a RSC-200 multi-channel rapid  
133 solution changer (BioLogic, Seyssinet-Pariset, France) driven by the Axon Instruments® pClamp™ 10  
134 Clampex software (Molecular Devices S.A.S., Villepinte, France).

135            *Current Recordings.* Currents were amplified by a L/M-EPC 7 (List Electronics, Darmstadt,  
136 Germany) patch-clamp amplifier, filtered at 1 kHz by a LPBF-48DG 8-pole Bessel filter (NPI Electronics  
137 GmbH, Tamm, Germany) and digitized at 2 kHz by an Axon Instruments® Digidata® 1440A A/D  
138 converter (Molecular Devices) for online monitoring and recording by the Clampex software. The  
139 bath reference was 0.5 M KCl in a 4% agar bridge connected to an Ag/AgCl pellet. When appropriate,  
140 clamp voltage  $V_c$  (i.e. =  $-V_{pipette}$ ) values were corrected for liquid junction potential difference as  
141 measured by the Clampex software routine. All experiments were conducted at room temperature  
142 (20-25 °C).

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

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

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

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

154 The channel inward slope conductance in cell-excised inside-out membrane patches exposed  
 155 to asymmetrical transmembrane  $K^+$  concentrations was determined by first fitting the Goldman-  
 156 Hodgkin-Katz (GHK) constant field current equation for  $K^+$  and  $Na^+$  to  $i/V_c$  relationships data over the  
 157 -100 to +40 mV  $V_c$  range using

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

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

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

162 where  $g$  is the conductance at a given  $V_c$ ,  $g_{-\infty}^{in}$  is the inward conductance at infinite negative  $V_c$ ,  $g_{+\infty}^{out}$   
 163 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  
 164 logarithmic sensitivity for an e-fold increase in  $V_c$ .

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

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

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



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

#### 172 **Data presentation and statistics**

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

178

## Results

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

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

204 medium. On the average of seven results obtained according to this sequence, the complete removal  
205 of internal  $\text{Na}^+$  reduced the normalized  $NP_o$  by 97 % (SD 3.5) ( $P = 0.096$  vs 100 % inhibition). From the  
206 five patches where very rare and brief openings could be observed in the absence of  $\text{Na}^+$ , the single-  
207 channel current amplitude was not significantly different from that in the presence of 140 mM  $\text{Na}^+$  ( $P$   
208 = 0.45).

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

213 Since the  $\text{Na}^+$ -dependent  $K_{\text{Na}1.1}$  channel is also activated by internal  $\text{Cl}^-$  (22) (24), the effect of  
214 altering the internal  $\text{Cl}^-$  concentration on the activity of the 70-pS K channel was then investigated.  
215 Figure 4 shows that its activity in a membrane patch bathed throughout with 140 mM  $\text{Na}^+$  was  
216 strongly reduced when the internal  $\text{Cl}^-$  concentration was lowered from 145 mM down to 5 mM but  
217 rapidly recovered upon the reintroduction of 145 mM  $\text{Cl}^-$ . On the average of nine separate  
218 measurements, the lowering of internal  $\text{Cl}^-$  concentration reduced the normalized  $NP_o$  by 79 % (SD  
219 10.3) ( $P = 0.0002$  vs 100 % inhibition), and did not affect the single-channel current amplitude ( $P =$   
220 0.4). Channel activity increased with the internal  $\text{Cl}^-$  concentration, cumulated data from five  
221 membrane patches yielding a relative  $EC_{50}$  of 29.3 mM  $\text{Cl}^-$  (SD 2.35), a  $n_H$  of 2.2 (SD 0.39) and a  
222 predicted normalized  $NP_o$  of 0.14 (SD 0.037) in the complete absence of  $\text{Cl}^-$  (Figure 5).

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

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

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

253 Given the inhibition of the 70-pS K channel by internal  $\text{K}^+$ , the effect of  $\text{NH}_4^+$ , a good  
254 surrogate for  $\text{K}^+$  through ionic channels and transporters, was assessed in the presence of 140 mM  
255  $\text{Na}^+$ . Figure 9 shows that the channel was also negatively regulated by internal  $\text{NH}_4^+$ , the normalized

256  $NP_o$  being reduced by 25 % (SD 12.8) ( $P = 0.03$  vs no inhibition) when 10 mM  $\text{NH}_4^+$  were added to the  
257 bathing medium ( $n = 4$ ), and by 98 % (SD 3.8) ( $P = 0.35$  vs 100 % inhibition) with 145 mM  $\text{NH}_4^+$  ( $n = 4$ ).

258 The 70-pS K channel conductance of  $\sim 85$  pS we determined in cell-attached membrane  
259 patches at  $V_c = 0$  mV ( $g_o$  in Table 1) was in reasonable agreement with the 60-75 pS range of  
260 conductance reported by others at this voltage and hitherto defining the channel as an intermediate-  
261 conductance channel (2) (3) (9), but did not conform to the large inward conductance of  $\sim 180$  pS  
262 characterizing the cloned  $\text{K}_{\text{Na}1.1}$  channel (22). However, the steepness of the  $i/V_c$  relationship at  
263 negative membrane potential differences shown in Figure 1b, as well as of previously published ones  
264 (2) (3) (9), strongly suggested a 70-pS K channel of larger inward conductance. Therefore, its  
265 conductive properties were reinvestigated in cell-excised inside-out membrane patches under  
266 precisely controlled ionic conditions (Figure 10). In the presence of 145 mM  $\text{K}^+$  in the pipette, and of  
267 9 mM  $\text{K}^+$  and 140 mM  $\text{Na}^+$  in the bath, the GHK equation (equation 2) fitted well the obtained  $i/V_c$   
268 data with a  $P_K$  of  $0.41 \cdot 10^{-12} \text{ cm}^3 \text{ s}^{-1}$  (SD  $0.012 \cdot 10^{-12}$ ) ( $n = 5$ ) close to the values obtained for the channel  
269 in the rat TAL and for native  $\text{K}_{\text{Na}1}$  channels (3) (26). The fit of the Boltzmann equation (equation 3) to  
270 the first derivative of GHK data then yielded an inward conductance at an infinite negative clamp  
271 potential difference of 172 pS (SD 5.2) ( $n = 5$ ) (Figure 10 inset), indicating a 70-pS K channel of actual  
272 large conductance.

273 By taking advantage of the remaining activity when the channel was exposed to 145 mM  
274 internal  $\text{K}^+$  or  $\text{NH}_4^+$  in the conditions given in Figures 7 and 9, we were able to establish the first  $i/V_c$   
275 curves under a high internal  $\text{K}^+$  or  $\text{NH}_4^+$  concentration (Figure 10). Under symmetrical 145 mM  $\text{K}^+$   
276 condition, the currents through the channel reversed at -0.1 mV (SD 1.2) and the  $i/V_c$  relationship for  
277 inward currents was linear with a slope ( $g_{in}$ ) of 173 pS (SD 4.5) ( $n = 4$ ) that further established the  
278 large channel inward conductance. In addition, the outward currents were smaller than expected  
279 from Ohm's law with an outward chord conductance ( $g_{chord-out}$ ), as defined by the slope of the curve  
280 between the reversal potential and  $V_c + 40$  mV, of 101 pS (SD 3.4) ( $n = 4$ ) that yielded a rectification  
281 coefficient ( $g_{in} / g_{chord-out}$ ) of 1.7. With 145 mM  $\text{NH}_4^+$  added to the high- $\text{Na}^+$  medium, the channel

282 inward conductance was 164 pS (SD 8.1) (n = 4) and the fit of the GHK equation (equation 4) to the  
283 mean inward current data yielded a  $P_{NH_4}/P_K$  of around 0.2.

284         Lastly, the 70-pS K channel exhibited the multiple conductance substates described for the  
285  $K_{Na1.1}$  channel (22). An example is given in Supplemental Figure S4 where partial channel openings  
286 and closures during a burst of activity are visible. The corresponding all-points current amplitude  
287 histogram showed that the data points between the fully open and closed states exceeded the values  
288 predicted from a double-Gaussian curve by the contribution of several substates.

289

## Discussion

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

301 Our results show that the 70-pS K channel recapitulates the salient functional properties of  
302 the basolateral  $K_{Na}1.1$ -like and cloned  $K_{Na}1.1$  channels. First, its activity increases with the internal  
303  $Na^+$  concentration, with a relative  $EC_{50}$  of  $\sim 40$  mM  $Na^+$  and a  $n_H$  of  $\sim 2.5$  that are within the ranges of  
304  $EC_{50}$  (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)  
305 (31) and in good agreement with the  $EC_{50}$  of 30 mM  $Na^+$  and the  $n_H$  of  $\sim 4$  we previously determined  
306 for the basolateral channel (17). Second, the channel is also activated by internal  $Cl^-$ , with a relative  
307  $EC_{50}$  of  $\sim 30$  mM  $Cl^-$  in the presence of an elevated internal  $Na^+$  concentration that falls well within  
308 the ranges of  $EC_{50}$  (8-35 mM  $Cl^-$ ) reported for the basolateral and the cloned channels under similar  
309 conditions (17) (24) (30), and a  $n_H$  of  $\sim 2$  close to the  $n_H$  of  $\sim 1$  reported for the basolateral channel  
310 and that can be deduced from data on the cloned  $K_{Na}1.1$  channel (17) (30). Furthermore, as observed  
311 for the basolateral  $K_{Na}1.1$ -like and cloned  $K_{Na}1.1$  channels which are inactive in the absence of  
312 internal  $Na^+$  but exhibit a significant activity in a  $Cl^-$ -free medium (17) (22) (30), the presence of  
313 internal  $Na^+$ , but not that of  $Cl^-$ , is an absolute prerequisite for 70-pS K channel activity. Third, its  
314 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  $\sim 100$  mM  $K^+$   
325 and the  $n_H$  of  $\sim 5$  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  $K_{Na}1.1-\alpha$  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  $K_{Na}1.1-\alpha$  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



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

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

382           To be functionally relevant, the sensitivity of the apical 70-pS K channel to internal ions  
383 should occur in the ionic conditions that exist in TAL cells. However, channel sensitivity to internal  
384 ions in cell-free membrane patches predicts a very low activity at the respective resting intracellular  
385  $Na^+$ ,  $K^+$  and  $Cl^-$  concentrations of 15, 155 and 35 mM prevailing in TAL cells (43) (44) (45).  
386 Furthermore, given the known complex interplay between internal  $Na^+$ ,  $Cl^-$  and  $K^+$  in regulating native  
387  $K_{Na}1$  channels in excitable cells or the cloned  $K_{Na}1.1$  channel, it is very likely that the sensitivity of the  
388 70-pS K channel to internal ions might have been underestimated. Indeed, the cloned  $K_{Na}1.1$  channel is  
389 cooperatively activated by intracellular  $Na^+$  and  $Cl^-$  ions so that an increase in the internal  
390 concentration of either of them increases the sensitivity to the other (30). Accordingly, the lowering  
391 of internal  $Cl^-$  concentration from 145 mM to 40 mM causes a ~ 2.5-fold decrease in the sensitivity to  
392 internal  $Na^+$  of the basolateral  $K_{Na}1.1$ -like channel (17). Conversely, an increase in internal  $K^+$

393 decreases the sensitivity to internal  $\text{Na}^+$  of native  $\text{K}_{\text{Na}1}$  channels in excitable cells and vice versa (26)  
394 (32). Therefore, one would expect a channel activity in situ even lower than that directly  
395 extrapolated from our measurements. Yet, the 70-pS K channel exhibits the significant activity in cell-  
396 attached membrane patches ( $NP_o$  of  $\sim 0.9$ , see Table 1) previously observed by others for the channel  
397 in mice and rats (2) (3) (4) (8) (9) (43). Such a paradox has been frequently reported regarding the  
398 sensitivity of  $\text{K}_{\text{Na}1}$  channels in excitable cells to internal  $\text{Na}^+$  and early proposed to reflect its  
399 modulation by an endogenous cytosolic factor(s) lost upon membrane patch excision (23). Very little  
400 is currently known on the modulation of the sensitivity of native and cloned  $\text{K}_{\text{Na}1}$  channels to internal  
401 ions. Indeed, only one study reports a shift in the  $EC_{50}$  for  $\text{Na}^+$  from 50 mM to 17 mM upon the direct  
402 binding of the oxidized form of nicotinamide dinucleotide ( $\text{NAD}^+$ ) to  $\text{K}_{\text{Na}1}$  channels in rat dorsal root  
403 ganglion neurons (46). Whether  $\text{NAD}^+$  increases the sensitivity of the 70-pS K channel to internal  $\text{Na}^+$   
404 also in TAL cells, thereby making plausible its regulation by low cytosolic  $\text{Na}^+$  concentrations in situ,  
405 remains to be established. More generally, the identification of factors modulating the sensitivity of  
406 the apical and basolateral  $\text{K}_{\text{Na}1.1}$ -like channels not only to internal  $\text{Na}^+$  but also to  $\text{Cl}^-$ ,  $\text{K}^+$  and  $\text{NH}_4^+$ ,  
407 whether they be a diffusible cytosolic factor or some other undefined process, should more precisely  
408 define the conditions under which physiologically relevant ion concentrations control channels  
409 activity in their native environment.

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

419 and the basolateral Na<sup>+</sup>/K<sup>+</sup> ATPase. In order to maintain steady-state intracellular K<sup>+</sup> concentration,  
420 and hence the potential difference across apical and basolateral membranes, these K<sup>+</sup> influxes must  
421 be coordinately counterbalanced by appropriate K<sup>+</sup> effluxes (*i.e.* K<sup>+</sup> recycling) through apical and  
422 basolateral potassium channels. We previously proposed that the sensitivity of the basolateral K<sub>Na</sub>1.1  
423 channel to Na<sup>+</sup> and Cl<sup>-</sup> may provide a signaling linkage between the entry and the basolateral exit of  
424 K<sup>+</sup> (17). The identification of internal Na<sup>+</sup> and Cl<sup>-</sup> as new regulators of the apical 70-pS K channel  
425 suggests that a similar coupling between the entry of K<sup>+</sup> and its apical recycling through the channel  
426 may exist. On the other hand, the inhibitory effect of internal K<sup>+</sup> on the activity of the apical and  
427 basolateral channels in the context of NaCl reabsorption is puzzling since the associated increase in  
428 intracellular K<sup>+</sup> concentration would decrease channels activity and hence antagonize the apical and  
429 basolateral exits of K<sup>+</sup>. Thus, as in cardiac cells, K<sub>Na</sub>1.1 channels activation in TAL cells might also take  
430 place in specific pathological conditions, such as digitalis toxicity where the increase in internal Na<sup>+</sup>  
431 concentration is accompanied by a decrease in K<sup>+</sup> concentration in rat TAL cells (49), and protect  
432 from damage by maintaining hyperpolarized the cell membranes. Finally, regarding the apical 70-pS  
433 K channel, also intriguing is its inhibition by internal NH<sub>4</sub><sup>+</sup>. The TAL is an important site of NH<sub>4</sub><sup>+</sup>  
434 reabsorption and numerous studies established that apical NH<sub>4</sub><sup>+</sup> entry into TAL cells is mediated  
435 predominantly by NKCC2 functioning as a Na<sup>+</sup>-NH<sub>4</sub><sup>+</sup>-2Cl<sup>-</sup> cotransporter (50). Our observations that the  
436 channel is substantially permeable to NH<sub>4</sub><sup>+</sup> and able to conduct current in the presence of internal  
437 NH<sub>4</sub><sup>+</sup> would make it in a position to also contribute to the luminal uptake of NH<sub>4</sub><sup>+</sup>. However, the  
438 extent to which its inhibition by internal NH<sub>4</sub><sup>+</sup>, which may partly account for its reduced activity, and  
439 lower apical membrane potassium conductance and voltage in intact rat TAL tubules lumenally  
440 exposed to 10 mM NH<sub>4</sub><sup>+</sup> (51), may limit its contribution to the net NH<sub>4</sub><sup>+</sup> absorption by the TAL needs  
441 further investigation.

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

445 expression of the  $K_{Na}1.1-\alpha$  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,  $K_{Na}1.1-\alpha$  subunit protein synthesis or channel trafficking.

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

455

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

457 <https://figshare.com/s/8f234b08cbf16ec42f02>

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459

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466

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468

469 **Author contributions:** M.P. conceived and designed research; E. de C., N.F., L.C. and M.P. performed

470 experiments and analyzed the data; E. de C., N.F., L.C. and M.P. interpreted the results of

471 experiments; M.P. prepared figures; M.P. drafted the manuscript; E. de C., N.F., L.C., S.L. and M.P.

472 edited and revised the manuscript; E. de C., N.F., L.C., S.L. and M.P. approved the final version of the

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## 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<sub>1</sub>  
626 and O<sub>2</sub> 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<sub>o</sub> at resting membrane potential difference (V<sub>c</sub> = 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<sub>o</sub> at V<sub>c</sub> =  
630 0 mV. (b) ROMK and 70-pS K channels i/V<sub>c</sub> 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<sup>+</sup> 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<sup>+</sup> concentrations (in mM). Bath K<sup>+</sup> and Cl<sup>-</sup> concentrations were kept at 9 and 145 mM,  
636 respectively, and V<sub>c</sub> 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<sub>o</sub> 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<sub>o</sub> during the  
641 initial exposure to 140 mM internal Na<sup>+</sup>. \* P < 0.0001 vs NP<sub>o</sub> = 1.

642

643 **Figure 3. Dependence of 70-pS K channel activity on internal Na<sup>+</sup> concentration.** (a) Left : recordings  
644 of channel activity in a cell-excised inside-out membrane patch at the indicated internal Na<sup>+</sup>  
645 concentrations (in mM) in the conditions given in Figure 2, except V<sub>c</sub> set at 0 mV. C- is the current  
646 level corresponding to the closure of all channels and O<sub>1</sub> to O<sub>6</sub> 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  $\text{Na}^+$  concentrations (in mM). (b) Dose-response relationship of the effect of  
649 internal  $\text{Na}^+$  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  $\text{Na}^+$ , and are shown as individual  
651 values ( $\square$ ,  $n = 4$  for each condition) and as mean ( $\blacksquare$ ) 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  $\text{Cl}^-$  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  $\text{Cl}^-$  concentrations (in mM). Bath  $\text{Na}^+$  and  $\text{K}^+$  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 ( $\square$  and dashed lines) and as mean ( $\blacksquare$  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  $\text{Cl}^-$ . \*  $P < 0.0001$  vs  $NP_o = 1$ .

664  
665 **Figure 5. Dependence of 70-pS K channel activity on internal  $\text{Cl}^-$  concentration.** (a) Left : recordings  
666 of channel activity in a cell-excised inside-out membrane patch at the indicated internal  $\text{Cl}^-$   
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  $\text{Cl}^-$   
670 concentrations (in mM). (b) Dose-response relationship of the effect of internal  $\text{Cl}^-$  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  $\text{Cl}^-$ , and are shown as individual values ( $\square$ ) and as mean ( $\blacksquare$ ) 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 ( $\square$  and dashed lines) and as mean ( $\blacksquare$  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

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

706

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

714

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

- 725 linear regression fit to inward currents data (adjusted  $R^2 = 0.997$ ). With 145 mM  $\text{NH}_4^+$  in the bath (o),
- 726 the dotted line is a fit of the GHK equation to the mean data (adjusted  $R^2 = 0.998$ ).