Modulation of the human polycystin-L channel by voltage and divalent cations

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Abstract Polycystin-L (PCL) is highly homologous in sequence and membrane topology to polycystin-2, the product of the second gene responsible for autosomal dominant polycystic kidney disease (ADPKD). PCL and polycystin-2 were recently shown to be Ca^{2+}-permeable, Ca^{2+}-activated cation channels. Further characterization of polycystins will help in the understanding of cystogenesis and pathogenesis of ADPKD. In the present study, we expressed human PCL in *Xenopus* oocytes and studied its function utilizing patch-clamp and two-electrode voltage clamp techniques. In addition to its permeability to Ca^{2+}, K^+ and Na^+, PCL was highly permeable to NH_4^+ and Cs^+ with a permeability ratio NH_4^+:Cs^+:Na^+ of 2.2:1.02:1. Voltage modulation of channel properties was studied using cell-attached (C-A) and excised inside-out (I-O) patches. In the C-A mode, the open probability (NP_o) of PCL at negative potentials ($NP_o = 0.22$) was higher than at positive potentials ($NP_o = 0.05$). The mean open time averaged 31.6 ms at negative potentials, and 6.2 ms at positive potentials; single-channel activity exhibited bursts with a mean interburst time of 178 ms. Using I-O patches under symmetrical ionic conditions, single-channel inward conductance was significantly larger than outward conductance, indicating a slight inward rectification. External Mg^{2+} inhibited the PCL channel currents. The inhibitory effect was voltage-dependent and substantially reduced by depolarization. The time course of inactivation depended on external calcium concentration but was independent of voltage and peak current. This study shows that although PCL is not a voltage-gated channel, its channel activity and inhibition by Mg^{2+} are modulated by membrane potential. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Electrophysiology; Autosomal dominant polycystic kidney disease; Patch-clamp; Two-electrode voltage clamp; Calcium; Magnesium; *Xenopus* oocyte

1. Introduction

Mutations in polycystin-1 and polycystin-2 account for the majority of individuals affected by the genetic disorder called autosomal dominant polycystic kidney disease (ADPKD). 0.1–0.2% of the population are affected and, at the age of 50–60, all affected individuals exhibit fluid-filled multiple cysts in their kidneys, which are often accompanied by cysts in their liver and pancreas, and less often by gastrointestinal and cardiovascular abnormalities and cerebral aneurysms [1]. Mutations in at least one other unidentified gene account for the remaining (~5%) ADPKD patients. The *PKDL* gene was identified in 1998 and encodes polycystin-L (PCL), which is 50% identical to polycystin-2 [2,3]. A 7 cM deletion including the locus containing the mouse ortholog of *PKDL* is associated with defects in kidney and retina [2]. Several new members of the PKD gene family have recently been identified, including *PKD2L2*, which encodes polycystin-2L2 [4,5], *PKDREJ* [6], and *PKD1L1* [7]. Polycystin homologs have also been identified in other species, including sea urchin [8], *Caenorhabditis elegans* and *Drosophila* [9–11], and these homologs are involved in fertilization and mating. Polycystins are predicted to be integral membrane proteins. Polycystin-2, -L and -2L2 share similar membrane topology and modest sequence homology with the α-subunits of voltage-gated Na^+, K^+ and Ca^{2+} channels and the transient receptor potential channels [12–15]. PCL has been shown to be a calcium-regulated, calcium-permeable non-selective cation channel [16]. More recently, polycystin-2 and one of the naturally occurring pathogenic mutants of polycystin-2, named R742X, were also shown to be cation channels activated by Ca^{2+} [17–21]. However, despite high homology in both sequence and membrane topology, PCL and polycystin-2 exhibited distinct channel properties and membrane targeting. They differed substantially in single-channel conductance (290 vs. 120 pS with 100 mM K^+ and 150 vs. 23 pS with 100 mM Na^+) [16,21].

Both PCL and polycystin-2 channels possess an EF-hand calcium-binding motif in their intracellular carboxy-terminus, suggesting a possible involvement of intracellular calcium in their activity. However, our recent studies have demonstrated that neither the EF-hand nor the rest of the C-terminus in human PCL was essential for the basal channel activity and calcium-induced channel activation [22]. It is thus possible that calcium binds to a non-EF-hand site in PCL to trigger the activation or that an intermediate calcium-binding protein exists which associates with PCL for mediating the activation. Although PCL and polycystin-2 share similar membrane topology and modest sequence homology with the α-subunits of voltage-gated channels, they are not voltage-gated channels. However, there are three positively changed amino acid residues in the S4 transmembrane domain of PCL and polycystin-2, in contrast to five to nine such residues forming a voltage

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sensor in the α-subunits of voltage-gated channels [2], which seems to suggest some voltage dependence of polycystin channels. In the present study, we expressed human PCL in *Xenopus* oocytes and employed the two-microelectrode voltage-clamp and patch-clamp techniques to study the modulation of the channel activity of PCL by voltage and divalent cations.

2. Materials and methods

2.1. Oocyte preparation

Capped synthetic human PKDL RNA was synthesized by in vitro transcription from a linearized template inserted in the pTLN2 vector [23], using the mMESSAGE mMACHINE™ Kit (Ambion, Austin, TX, USA). 50 nl H_2O containing 40 ng RNA was injected into *Xenopus laevis* oocytes prepared as before [22]. Equal volumes of H_2O were injected into control oocytes. Experiments were performed 2–4 days after injection.

2.2. Electrophysiology

Two-microelectrode voltage-clamp experiments were performed as described previously [24]. Capillary pipettes (Warner Instruments, Hamden, CT, USA) were used to form current and voltage electrodes with tip resistance of 0.2–1.0 and 0.5–2.0 MΩ, respectively. In experiments using a ramp, jump or gap-free protocol [16], current/voltage signals were sampled at intervals of 0.2, 0.2 or 200 ms, respectively. The standard solution contained (in mM): 100 NaCl, 2 KCl, 1 $MgCl_2$, 10 HEPES, pH 7.5. When 100 mM Na^+ was replaced with equimolar amounts of other cations, the resulting solutions were named accordingly, e.g. choline-substituted solution. Relative permeability coefficients for monovalent cations were calculated using the equation derived from the Goldman–Hodgkin–Katz equation [25]: $P_X/P_{Na} = \exp(\Delta V_r/58.5)$, where ΔV_r (mV) is the change in reversal potential. Patch-clamp experiments were performed using the commercial amplifier 3900A (Dagan, Minneapolis, MN, USA), Digidata 1320A and pClamp 8 (Axon Instruments, Foster city, CA, USA). Electrodes were made of capillary pipettes (Warner Instruments) with tip resistance of 3–10 MΩ. Recording started after seal resistance reached at least 2 GΩ. Single-channel currents and voltages were sampled every 0.2 ms and filtered at 2 kHz using an 8-pole Bessel filter.

2.3. Statistics and data analysis

Data obtained from the two-microelectrode voltage-clamp and patch-clamp experiments were analyzed using Clampfit 8, Fetchan, and/or pStat (Axon Instruments). Single-channel conductance values were obtained from Gaussian fits (in pStat) to Fetchan-generated All-Point Histograms. The open probability times the number of channels in the patch (NP_o, designated 'open probability' hereafter) and channel mean open time (MOT) values were obtained from currents generated either by voltage pulses of 10 s per pulse or by gap-free recordings of 10 s long. For the MOT analysis, only recordings with single openings were used. These currents were analyzed using Fetchan Events List and pStat. MOT was evaluated as the time constant through mono-exponential fits (in pStat) to dwell-time distribution curves generated from Fetchan Events List. Analyzed data were plotted using Sigmaplot 5 (Jandel Scientific Software, San Rafael, CA, USA) or expressed in the form of mean ± S.E.M. (n), where S.E.M. represents the standard error of the mean and n indicates the number of oocytes or oocyte patches tested. Curve fitting and data filtering were performed using Sigmaplot 5.

3. Results

It was previously demonstrated that PCL was permeable to monovalent cations, including Na^+, K^+, Rb^+ and Li^+, but was impermeable to protons and large cations such as *N*-methyl-D-glucamine (NMDG) and choline [16]. Large inward and outward whole-cell currents were observed in oocytes expressing human PCL channels in the presence of extracellular Cs^+ or NH_4^+ (100 mM). While the currents generated using Cs^+ as the charge carrier were comparable to the currents generated by Na^+, NH_4^+ generated much larger currents (Fig. 1). The permeability ratio NH_4:Cs:Na was determined to be 2.2:1.02:1 ($n = 7$). The physiological implication of this high permeability to NH_4^+ remains unknown. In addition to the basal channel activity (in the absence of Ca^{2+}), PCL channel is activated in the presence of external Ca^{2+} (usually ≥ 1 mM) but is not activated by Ba^{2+} (up to 10 mM).

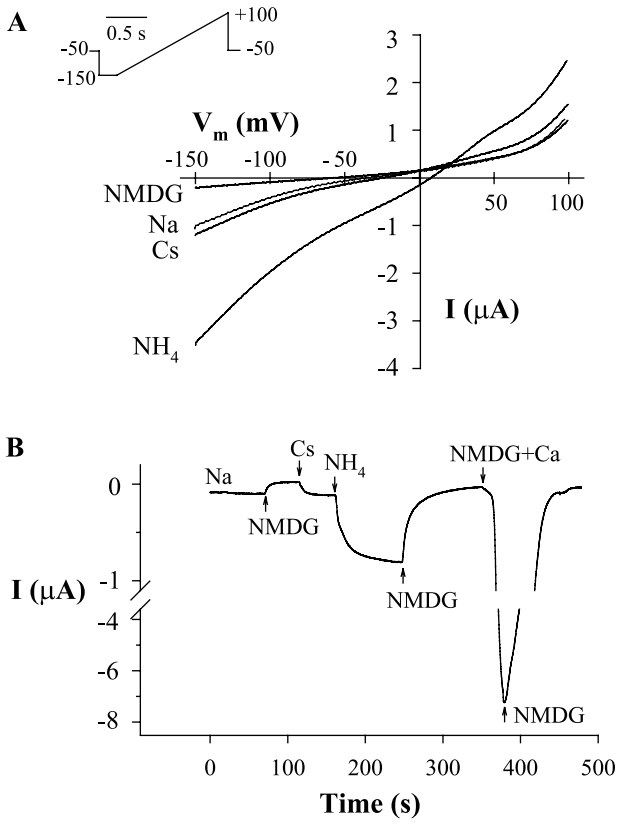

Fig. 1. Permeability of human PCL to Cs^+ and NH_4^+. A: Representative ion-selectivity I–V curves obtained in the presence of the standard solution (Na), Cs-, NH_4- or NMDG-substituted solution. Current was recorded using a ramp protocol from −150 to +100 mV as illustrated in upper-left corner of the panel. Permeability ratio NH_4^+:Cs^+:Na^+ averaged from seven oocytes was 2.20 ± 0.17: 1.02 ± 0.03:1. B: Current recorded from an oocyte expressing PCL voltage clamped at −50 mV in the presence of different monovalent cations (100 mM) ± Ca^{2+} (5 mM) as indicated. Solution changes started at time indicated by arrows.

3.1. Voltage-dependent single-channel properties

In the presence of 123 K^+ in the pipette under a cell-attached (C-A) mode, PCL channels had a larger inward conductance (366 ± 10 pS, $n = 12$) than outward conductance (150 ± 20 pS, $n = 5$) (Fig. 2A), due in part to an asymmetrical presence of permeant ions across the membrane. No large unitary conductance was observed in H_2O-injected control oocytes ($n = 37$). The open probability was voltage-dependent, with NP_o values equal to 0.22 ± 0.03 at membrane potentials (V_m) between −40 and −120 mV and reduced to 0.05 ± 0.02 at V_m between +40 and +100 mV (Fig. 2B). The channel MOT also exhibited voltage dependence, averaging 31.6 ± 3.5 ms ($n = 39$) and 6.2 ± 1.5 ms ($n = 17$) at hyperpolarization and depolarization, respectively (Fig. 2C). In addition to much smaller MOT values at positive voltages, PCL channel

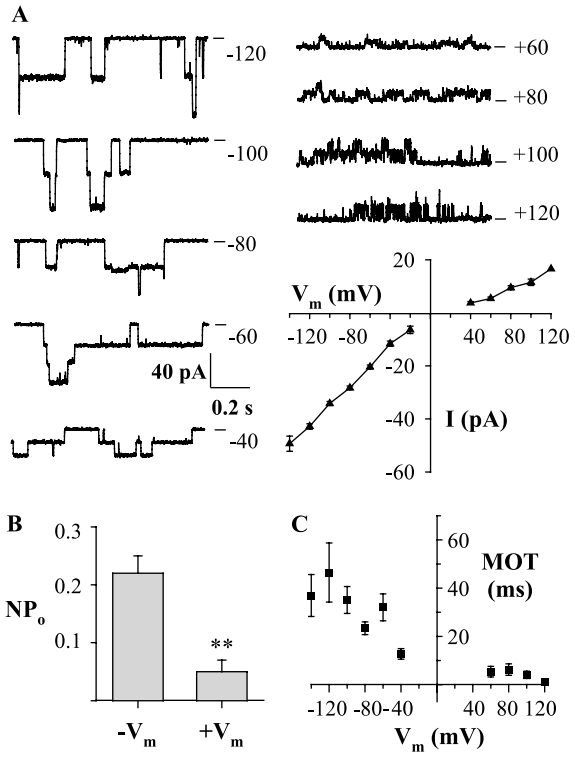

Fig. 2. Single-channel properties of PCL on C-A configuration. A: Representative recordings at various membrane potentials between −120 and +120 mV, as indicated. The pipette (external) solution contained 123 mM K^+ (see Section 2). The closed level in each recording is indicated by a horizontal bar. Traces corresponding to negative and positive voltages were Gaussian filtered at 200 and 500 Hz, respectively, in the Sigmaplot 5 program. The average *I–V* curve was obtained from 12 and five oocyte patches for negative and positive potentials, respectively. B: NP_o was averaged ($n=7$) over negative ($-V_m$: −40 to −120 mV) and positive potentials ($+V_m$: 40–100 mV), respectively. C, MOT was obtained from 11 oocyte patches and average values are plotted versus the membrane potential.

exhibited bursts at depolarized potentials (Fig. 3A). The mean interburst duration was determined to be 178 ± 36 ms ($n=7$) at +100 mV, in contrast to the mean intraburst close time of 1.2 ± 0.2 ms. The mean burst duration was 78 ± 28 ms and there were on average 7.3 ± 2.4 closings within each burst.

PCL channels expressed in *Xenopus* oocytes were also studied using inside-out (I-O) excised patches under symmetrical ionic conditions: both intracellular and pipette (extracellular) solutions contained (in mM) 110 KCl, 13 KOH, 5 HEPES, and 1 EGTA, pH 7.4. However, single-channel opening, unitary conductance and MOT still displayed asymmetrical properties (Fig. 3B–D). The inward unitary channel currents were 40.9 ± 2.3 and 20.4 ± 1.3 pA ($n=7$) at −120 and −60 mV, respectively, which were significantly larger than 29.8 ± 4.2 and 16.2 ± 2.9 pA ($n=5$) observed at +120 and +60 mV, respectively (Fig. 3C).

MOT was also substantially greater at negative voltages than at positive voltages (Fig. 3D). Similar to data obtained using the C-A mode, single-channel bursts were present at depolarized potentials. Our data showed that the PCL channel in a symmetrical solution environment mediated asymmetrical inward and outward unitary currents, i.e. PCL channel current was, to a certain degree, inwardly rectified. This suggests that the membrane organization of the PCL protein is asymmetrical with respect to the two sides of the membrane.

3.2. Inhibition of PCL by external Mg^{2+}

Unlike the divalent cations Ca^{2+}, Ba^{2+} and Sr^{2+} which displayed dual effects as being both permeant and inhibitor of PCL, Mg^{2+} inhibited the PCL activity but was not a permeant of PCL. Other tested divalent cations, including Pb^{2+}, Zn^{2+}, Cd^{2+}, and Co^{2+} at up to 100 μM, had no observable effect on PCL channel activity. Inhibition by external Mg^{2+} was enhanced at hyperpolarization and insignificant at depolarization (Fig. 4A), indicating that Mg^{2+} inhibition was voltage-dependent. This suggests that inhibitory binding of Mg^{2+} to the PCL channel protein may traverse a membrane electrical field. Negative voltages may have facilitated Mg^{2+} binding while positive voltages hindered the binding, in agreement with the access of a positively charged ion (Mg^{2+}) to the PCL channel from outside the membrane. The half-maximal inhibition constant (IC_{50}) at −50 and −120 mV was determined to be 0.18 ± 0.05 and 0.09 ± 0.04 mM ($n=6$), respectively. Assuming that IC_{50} $(V_m) = IC_{50}$ $(0) \times \exp(\delta z F V_m / RT)$, where $z=2$ (for Mg^{2+}) and F, R and T have their usual thermodynamic meaning, we estimated δ, the fractional electrical distance, to be 0.12.

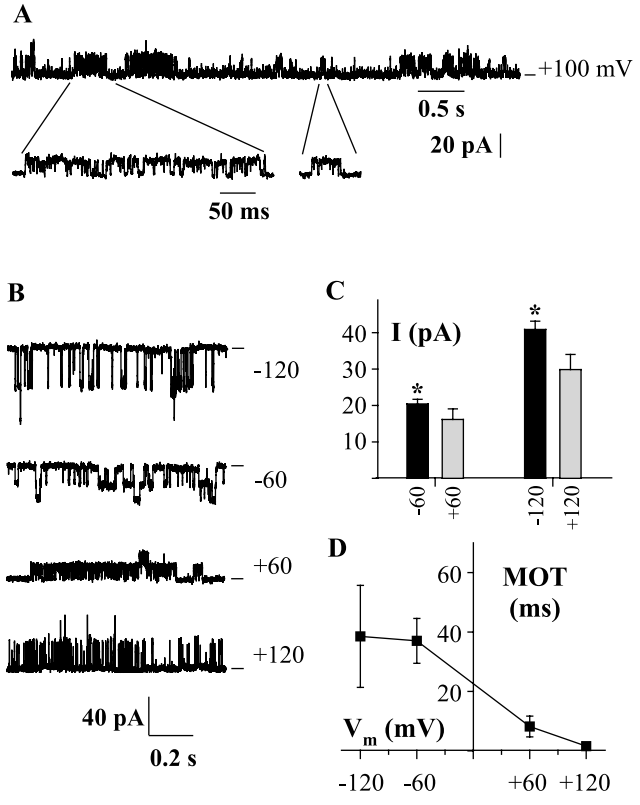

Fig. 3. Channel bursts (C-A) and channel properties (I-O). A: Representative trace obtained on the C-A mode at 123 mM pipette K^+. Oocyte was clamped at +100 mV. Expanded traces are shown in the second line. B: Representative current traces recorded using the I-O configuration with a symmetrical solution. 123 mM K^+ was present on both sides of the membrane. C: Averaged single-channel conductance at −120, −60, +60, and +120 mV, obtained under the same condition as in B. The asterisk indicates that the difference is significant between single-channel conductance at opposite voltages. D: Average MOT obtained from six oocyte patches.

3.3. Channel activation and inactivation

It was previously shown that PCL exhibited calcium influx-induced channel activation and ensuing inactivation [16]. It is still unclear whether membrane potential and intracellular calcium concentration ($[Ca^{2+}]_i$) affect the time course of PCL activation and inactivation. To avoid triggering channel activation before external calcium is uniformly applied to the whole oocyte surface, the oocyte was voltage-clamped at depolarized potentials (0 or +10 mV) or not voltage-clamped until the solution was completely changed. Following voltage clamp at negative potentials (−40 to −140 mV), PCL channels exhibited slow activation and followed by slower inactivation while the oocyte was continuously perfused with a calcium-containing solution [16]. The time course of activation and inactivation was quantitatively characterized by the half-rise (t_r) and half-decay (t_d) time (Fig. 5A). These two parameters were determined at various clamp voltages using oocytes of different expression levels. t_r and t_d were voltage-independent (Fig. 5B) and determined to be 16.0 ± 1.6 s ($n = 31$) and 34.1 ± 2.3 s ($n = 38$), respectively, averaged over the tested voltage range. This suggests that calcium influx-induced channel activation and the ensuing inactivation are not dependent on membrane potential.

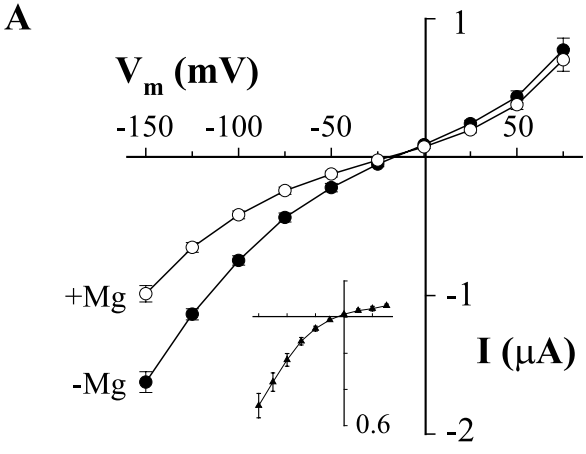

Fig. 4. Voltage dependence of inhibition by Mg^{2+}. A: *I–V* curves were obtained using a pulse protocol before and after addition of 5 mM Mg^{2+} to the solution containing (in mM) 100 NaCl, 2 KCl, 1 $CaCl_2$, 10 HEPES, pH 7.5. The inset represents the Mg^{2+}-inhibited current obtained as the difference between the currents before and after Mg^{2+} application. B: Concentration dependence of PCL-mediated membrane current measured at −50 mV as a function of external Mg^{2+} concentration ($[Mg^{2+}]$). Currents in the absence of Mg^{2+} were normalized to 1 and averaged from six oocytes. A Michaelis–Menten fit to the curve gave an IC_{50} of 0.18 ± 0.05 mM.

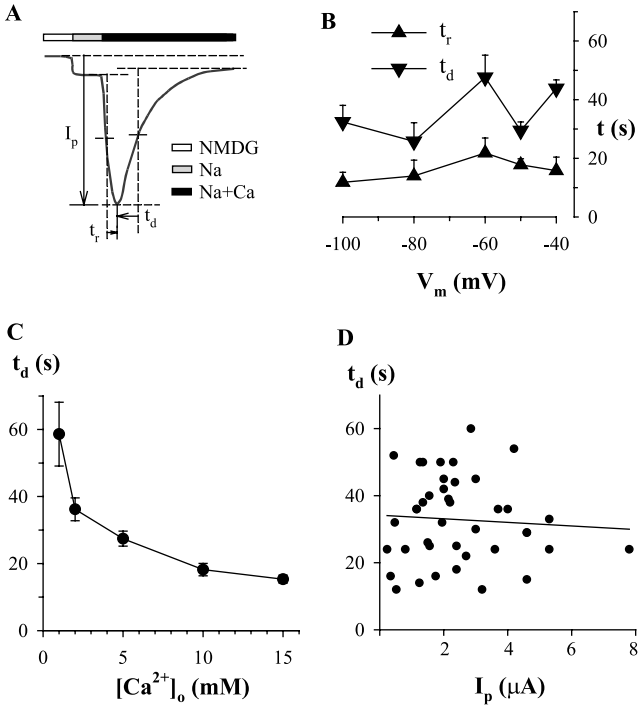

Fig. 5. Dependence of the half-rise and half-decay time on voltage, $[Ca^{2+}]_o$ and peak current. A: Schematic illustration of how the half-rise (t_r) and half-decay (t_d) time were defined and measured. 'NMDG' and 'Na' indicate the NMDG-substituted and the standard solution, respectively, and 'Na+Ca' indicates the standard solution plus 5 mM $CaCl_2$. B: The two parameters were plotted against the membrane potential at which oocytes were clamped. C: Dependence of the half-decay time on external calcium concentration. Each data point represents an average obtained from 10 (1 mM), 21 (2 mM), 22 (5 mM), 26 (10 mM), and 23 (15 mM) oocytes (four batches). D: t_d versus the peak current (I_p) obtained from oocytes voltage-clamped between −40 and −100 mV. A constant 5 mM $[Ca^{2+}]_o$ was used and data presented in this panel were not included in panel C. The straight line represents a linear regression that yielded a correlation coefficient of 0.005.

To determine whether the inactivation process depends on calcium, we measured the half-decay time as a function of external calcium concentration ($[Ca^{2+}]_o$). We found that t_d decreased when $[Ca^{2+}]_o$ increased (Fig. 5C). Because calcium is highly permeable through the plasma membrane of oocytes expressing PCL channels, $[Ca^{2+}]_o$ positively correlates with calcium influx and $[Ca^{2+}]_i$. Our result indicates that the channel inactivation may depend on one or more of the three factors: $[Ca^{2+}]_o$, $[Ca^{2+}]_i$ and calcium flux. The local intracellular calcium concentration can be roughly represented by the inward peak current. By plotting the half-decay time against the peak current generated using a constant $[Ca^{2+}]_o$ (5 mM), we found no significant correlation between the two parameters, with a correlation coefficient $r = 0.005$ (Fig. 5D), suggesting that the inactivation process was not affected by $[Ca^{2+}]_i$ and calcium flux through individual channels. Thus, our data support that inactivation is affected by external calcium concentration.

4. Discussion

In the present study, we have used the patch-clamp and two-electrode voltage clamp techniques to examine the voltage dependence and modulation by Mg^{2+} and Ca^{2+} of the human PCL channel. Together with our previous study [16], we have shown that PCL acts as a non-selective cation channel, permeable to several mono- and divalent cations, including NH_4^+, K^+, Na^+, Rb^+, Cs^+, Ca^{2+}, Sr^{2+} and Ba^{2+}, but impermeable to large cations such as choline, NMDG and tetraethylammonium. This channel was insensitive to Pb^{2+}, Zn^{2+}, Cd^{2+} and Co^{2+}, but it was inhibited by H^+, Mg^{2+}, La^{3+} and Gd^{3+}. PCL's permeability to ammonium (NH_4^+) was much higher than that to other monovalent permeants but the physiological importance of this high permeability is unclear. In kidney, PCL is mostly localized in the principal cells of inner medullary collecting duct (IMCD) [26]. Since the intercalated cells play a major role in acid–base homeostasis in IMCD, PCL is unlikely to be essential to this regulation although it may be involved in the NH_4^+ excretion to urine. Unlike uptake of NH_4^+ through the bumetanide-sensitive Na^+-NH_4^+-$2Cl^-$ co-transport system in thick ascending limb, PCL-mediated NH_4^+ transport was not sensitive to bumetanide up to 80 μM (data not shown).

Although PCL is not a voltage-gated channel, our data have shown that several parameters of the channel were modulated by the membrane potential. Single-channel open probability, conductance, and MOT had higher values at negative potentials than at positive potentials. Channel bursts were observed at positive voltages but not at negative voltages. This was true either in an ionically asymmetrical C-A configuration or in a symmetrical I-O configuration. This weak voltage dependence of the PCL channel may be linked to the fact that there are only three positively charged residues (two Arg and one Lys) in the S4 segment of PCL, in contrast to five to nine such residues (forming the voltage sensor) in the α-subunits of voltage-gated channels. Site-directed mutagenesis to this PCL S4 segment will help to establish a possible relationship between these cationic residues and voltage dependence.

PCL inhibition by external Mg^{2+} was voltage-dependent; inhibition by Mg^{2+} was potent at negative potentials but not at positive potentials. This suggests that hyperpolarization (cytoplasm negative) favors the association of external Mg^{2+} to the site and decreases the dissociation of Mg^{2+} from the site, which results in increased inhibition affinity. Since PCL is permeable to most monovalent metal ions and the divalent cations Ca^{2+}, Sr^{2+}, and Ba^{2+}, voltage-dependent inhibition may be due to (1) the competition of binding between Mg^{2+} and a permeant cation and/or (2) blockade of Mg^{2+} at a binding site inside a narrow (voltage-dependent) intramembrane pathway. Intracellular calcium was reported to induce both activation and inactivation of voltage-gated channels that share similar membrane topology and modest sequence homology with PCL [27,28]. The divalent cation calcium exhibits multiple effects on PCL: it is itself a permeant and activates the PCL channel [16]. It also inhibits the passage of other permeants through the channel. Following calcium influx the human PCL channel activated and then inactivated with much slower time courses than voltage-gated channels (see Fig. 5A or [22]). The half-rise and half-decay time were voltage-independent, suggesting that membrane potential is not involved in activation and inactivation processes. Because these processes are somehow related to channel open probability, this would raise apparent controversy with the voltage-dependent NP_o. However, unlike t_r and t_d which were determined only at negative V_m, NP_o was voltage-dependent when comparing values obtained at negative V_m (−40 to −120 mV) with those obtained at positive V_m (+40 to +100 mV). In fact, no obvious voltage dependence of NP_o was observed within the negative (or positive) V_m range. Of note, NP_o was obtained in the absence of calcium while the PCL channel activation/inactivation requires the presence of calcium (influx). The half-decay time decreased with increased $[Ca^{2+}]_o$ which positively correlates with the amplitude of calcium influx (or the peak current). However, t_d did not depend on the peak current (Fig. 5D). These results together indicate that $[Ca^{2+}]_o$, but not calcium influx or $[Ca^{2+}]_i$, modulates the inactivation process. The mechanism of PCL channel activation triggered by calcium remains to be elucidated. It is possible that an intracellular fragment of the PCL protein undergoes conformational changes which are triggered by intracellular calcium binding either with the PCL protein itself or with an intermediate calcium-binding protein.

In summary, we have shown that PCL channel properties are dependent on voltage despite the fact that it is not a voltage-gated channel. Inhibition by external Mg^{2+} was also voltage-dependent. However, membrane potential did not appear to play a significant role in Ca^{2+}-induced channel activation and inactivation. Because the mechanism of calcium-induced activation remains largely undetermined, further studies will be required to determine whether this is due to calcium binding directly with PCL or with a yet to be identified intermediate calcium-binding protein that triggers the channel activation upon binding with calcium.

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