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The calcium-binding EF-hand in polycystin-L is not a domain for channel activation and ensuing inactivation

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Abstract Polycystin-L (PCL) shares high homology with polycystin-2, the product of polycystic kidney disease gene-2. It was previously shown that the PCL forms a non-selective cation channel activated by calcium influx. However, it remains unclear whether calcium activates/inactivates PCL by binding to the EF-hand motif located on the cytoplasmic carboxyl-terminus. Here we obtained two PCL splice variants from liver (PCL-LV, lacking the EF-hand) and testis (PCL-TS, lacking 45 amino acids on the carboxyl tail) using PCR-based approaches. When expressed in *Xenopus* oocytes and studied using electrophysiology both splice variants exhibited basal cation channel activity and calcium-induced channel activation. While PCL-TS displayed similar activation to PCL, PCL-LV exhibited a three-fold increased activation. All five PCL C-terminal artificial truncation mutants also exhibited basal and calcium-activated channel activities, in particular the mutant T622X lacking the EF-hand was associated with increased activation. Our data demonstrate that the EF-hand and other parts of the carboxyl tail of PCL are not determinants of channel activation/inactivation although the EF-hand seems to be involved in the modulation of these processes. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Autosomal dominant polycystic kidney disease; Splice variant; Cation channel; Calcium; Electrophysiology; *Xenopus* oocyte

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is a frequent genetic disorder that occurs in about 0.1% of the population worldwide. Cysts in ADPKD develop from dilation of any part of the nephron and the early stages of the disease are characterized by thickening and disorganization of the basement membrane. ADPKD also manifests extrarenal cysts in liver, pancreas, spleen and central nervous system, and results in gastrointestinal and cardiovascular abnormalities [1]. Abnormal fluid secretion, hyperproliferation of tubular epithelial cells, altered ion transport properties, and mistargeting of normally polarized membrane proteins such as Na^+–K^+ ATPase are also associated with ADPKD [2–4]. About 95% of ADPKD are associated with mutations in either the *PKD1* (accession number: U24497) [5] encoding polycystin-1 or *PKD2* (U50928) [6] encoding polycystin-2 (PC2) and possibly one or more genes accounting for the other 5% of ADPKD remain to be identified. New members of the ADPKD family have recently been identified, including *PKDL* (AF073481) [7] encoding polycystin-L[1] (PCL) [8], *PKD2L2* (NM_014386) [9,10] encoding PC2L2, *PKDREJ* (AF116458) [11], and *PKD1L1* [12]. Polycystin homologues have also been identified from sea urchin sperm [13], *Caenorhabditis elegans* [14] and *Drosophila* [15]. Although these new members are unlikely to be associated with ADPKD, some are involved in mating or fertilization [13,14]. It is thus important to understand their functions and regulations at the molecular level in order to elucidate mechanisms underlying their different physiological roles.

PC2, -L, and -2L2 exhibit similar membrane topologies as well as modest sequence homology to the α-subunits of voltage-gated Na^+, K^+ and Ca^{2+} channels and transient receptor potential family channels [16–18]. PCL was recently shown to be a calcium-regulated, calcium-permeable non-selective ion channel [19]. PC2 was later demonstrated to be a cation channel as well [20–22], with functional characteristics similar to but distinct from PCL. A pathogenic C-terminal truncation mutant of PC2, R742X, was also found to have cation channel activity but with altered membrane targeting [23]. Since the cytoplasmic C-terminus of PCL (D562–S805) contains several motifs such as a Ca^{2+}-binding EF-hand, coiled-coil structure and phosphorylation sites, this region might be important for the function and regulation of PCL. Also, several recently identified splice variants of PCL correspond to deletion mutations in this region. Alternative splicing is also seen in other cation channels with a similar structure and sequence to PCL [24–26]. Through functional studies of such splice variants and artificial mutants, roles of some important domains may be elucidated, which will be helpful to the further understanding of the molecular functions of PCL and PC2. In particular, the roles of the EF-hand motif in these two polycystin channels are still unclear. According to previous reports, this motif in voltage-gated cation channels may [27–29] or may not [30] be involved in channel inactivation.

In the present study, we obtained two naturally occurring splice variants of PCL using PCR-based cloning and constructed a number of artificial PCL mutants truncated at different sites in the cytoplasmic C-terminal tail using site-directed mutagenesis. Functional studies using electrophys-

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[1] *PKD2L* and PC2L have also been used in some literature instead of *PKDL* and PCL, respectively.

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iology and *Xenopus* oocyte expression system were performed to elucidate the role of the C-terminus and particularly that of the EF-hand with respect to calcium-induced activation and the ensuing channel inactivation.

2. Materials and methods

2.1. Molecular biology

Human *PKDL* cDNA amplification was performed using kidney, liver, brain and heart Matchmaker libraries, Marathon-Ready™ testis cDNA, and liver poly(A)$^+$ RNA (Clontech, Palo Alto, CA, USA). The forward primer 5′-ATCGTCTAGATCACCATGAATGCTGTGGGAAGTCC and the reverse primer 5′-ATCGAGATCTCTCCGGGAGTGCCTCACACTTA were used to amplify the *PKDL* cDNA (AF073481) [7] fragment spanning the translational start and termination code. The PCR product was ligated to the pTLN2 vector [31], a generous gift of Drs. Jentsch and Fong, for expression in *Xenopus* oocytes. For amplification of a *PKDL* 3′ fragment (T1683–G2435), the following pair of primers was used: 5′-TGACACATATTCAGAGGTCAAG and 5′-CCGGGAGTGCCTCACACTTA. Truncation mutants of the kidney PCL (T622X, V670X, G692X, and R740X, see Fig. 1C) were obtained by introducing a stop codon at specified sites to the human kidney *PKDL* cDNA inserted in the pTLN2 vector using the QuickChange™ kit (Stratagene, La Jolla, CA, USA). All mutations were confirmed by sequencing.

2.2. Oocyte preparation

Capped synthetic RNAs were in vitro transcribed from linearized templates using the mMESSAGE mMACHINE™ Kit (Ambion, Austin, TX, USA). H_2O (50 nl) alone or containing 25–50 ng mRNA was injected into *Xenopus* oocytes prepared similarly as before [32]. Briefly, oocytes were extracted from stage V–VI *Xenopus laevis* and were defolliculated using a Ca^{2+}-free solution (in mM, 90 NaCl, 2 KCl, 0.82 $MgSO_4$, 10 HEPES, pH 7.5) containing 2 mg/ml collagenase for 2–2.5 h at ~22°C. Injected oocytes were incubated at 18°C in the Barth's solution containing: 90 NaCl, 2 KCl, 0.82 $MgSO_4$, 0.41 $CaCl_2$, 0.33 $Ca(NO_3)_2$ 10 HEPES, pH 7.5, supplemented with antibiotics. Experiments were performed 2–4 days after injection.

2.3. Electrophysiology

Two-microelectrode voltage-clamp experiments were performed as previously [19] using a TEV-200A amplifier (Dagan Corporation, Minneapolis, MN, USA), Digidata 1320A and pClamp8 (Axon Instruments, Foster City, CA, USA). The standard solution for these experiments contained: 100 NaCl, 2 KCl, 1 $MgCl_2$, 10 HEPES, pH 7.5. Patch-clamp experiments were performed as previously [19] using a 3900A amplifier (Dagan), Digidata 1320A and pClamp8. Data analyses were performed using Clampfit8, Fetchan and pStat (Axon) and figures were generated using Sigmaplot5 (Jandel Scientific, San Rafael, CA, USA) and Corel Draw8 (Corel Corporation, Ottawa, ON, Canada). Data were presented in the mean ± S.E.M. (*n*) form, where *n* indicates the number of oocytes or oocyte patches tested.

2.4. ^{45}Ca uptake measurements

$^{45}CaCl_2$ (Amersham Pharmacia Biotech, Baie d'Urfe, QC, Canada) at 30 μM was added to an uptake solution (specified in figure legends) plus 1 mM non-radioactive $CaCl_2$. Ten oocytes of each sample were incubated in 0.5 ml of the uptake solution for 30 min and the incubation was terminated by washing oocytes in the ice-cold uptake solution.

3. Results

3.1. Cloning of PCL and splice variants

Human PCL exhibits tissue-specific alternative splicing according to recent reports [10,33]. To study the molecular functions of splice variants and to determine functional differences among them, we used PCR-based approaches to amplify *PKDL* from human kidney, liver and testis cDNA libraries. DNA sequencing revealed an identical 5′ end of *PKDL* coding region (1–1921) from the three libraries. The kidney cDNA was identical to the one previously used [19] so we named it *PKDL*. The initially published sequence [7] differed from the kidney *PKDL* in two point mutations at the 3′ end of the coding region: C instead of T (in our kidney *PKDL*) at nucleotide 2203 (Gln instead of Pro at amino acid 716) and A instead of C at nucleotide 2147, with no change in amino acid residue, presumably reflecting single nucleotide polymorphisms (Fig. 1A). The liver cDNA (*PKDL-LV*) exhibited deletion within exon 12. This splicing did not totally obey the GT/AG (donor/acceptor) rule as it was generated by an alternative cryptic donor CT. Similar splicing mechanisms have previously been observed in *PKD2* pathogenic mutations [34] and in *PKDL* from transformed lymphoblasts [33]. This splicing resulted in no frame shift and in missing the putative Ca-binding EF-hand motif (29 amino acid residues E637–L665) in PCL-LV (Fig. 1B). This splicing was confirmed by reverse transcription-PCR amplification from a liver poly(A)$^+$ RNA. The testis cDNA (*PKDL-TS*) corresponded to deletion of entire exon 15 and thus encoded a splice protein (PCL-TS) shorter than the kidney PCL by 45 amino acids on the C-terminus (Fig. 1B,C), which is likely to be identical to one previously identified from human transformed lymphoblasts [33]. Note that *PKDL-LV* and a *PKDL* 3′ fragment (T1683–G2435) amplified from brain and heart (data not shown) possess the same nucleotides C2147 and T2203 as the kidney *PKDL* and that *PKDL-TS* has the same A2147 and C2203 found in the initially published sequence [7].

3.2. Basal conductance and calcium-induced channel activation of splice variants

When the two-microelectrode voltage-clamp technique was used to measure whole-cell currents in *Xenopus* oocytes expressing PCL-LV or PCL-TS, respectively, substantial permeability to Na^+, K^+ and Rb^+, but not to large cation NMDG (*N*-methyl-D-glucamine), was observed in the absence of external Ca^{2+} (Fig. 2A). Similar to PCL, PCL-TS was also permeable to the alkaline earth metals, in the order of $Sr^{2+} > Ba^{2+} > Mg^{2+}$ (Fig. 2C). Radiotracer measurements using ^{45}Ca demonstrated that both channels were permeable to Ca^{2+} (Fig. 2B). Current–voltage (*I–V*) curves obtained from oocytes expressing either splice variant in the presence of the external standard solution (100 mM NaCl) or the solution containing 50 mM NaCl+100 mM mannitol were compared and showed no significant difference in outward current (data not shown), demonstrating that Cl^- is not a permeant of these two splice variants. These data indicated that, like PCL, both PCL-LV and PCL-TS channels retained the nonselective permeability to mono- and divalent cations.

Since PCL-LV contained no Ca^{2+}-binding EF-hand motif in its C-terminus and PCL-TS had a shorter C-terminus compared to PCL, we determined their capability of mediating Ca^{2+}-induced activation. Addition of 5 mM Ca^{2+} to the standard solution evoked large inward currents in oocytes voltage-clamped at −50 mV (Fig. 3A), demonstrating that the EF-hand is not essentially required for Ca^{2+}-induced channel activation. This activation occurred at the whole range of membrane potentials tested, as shown by the *I–V* curve #2 (Fig. 3B). The reversal potential (V_{rev}) obtained from *I–V* curves #2 (at activation peak) showed no substantial difference for the three isoforms and averaged −26 ± 3 mV ($n = 15$), which seems to infer a major contribution from the Cl^- current. However, when 70 mM glutamate replaced the equimolar

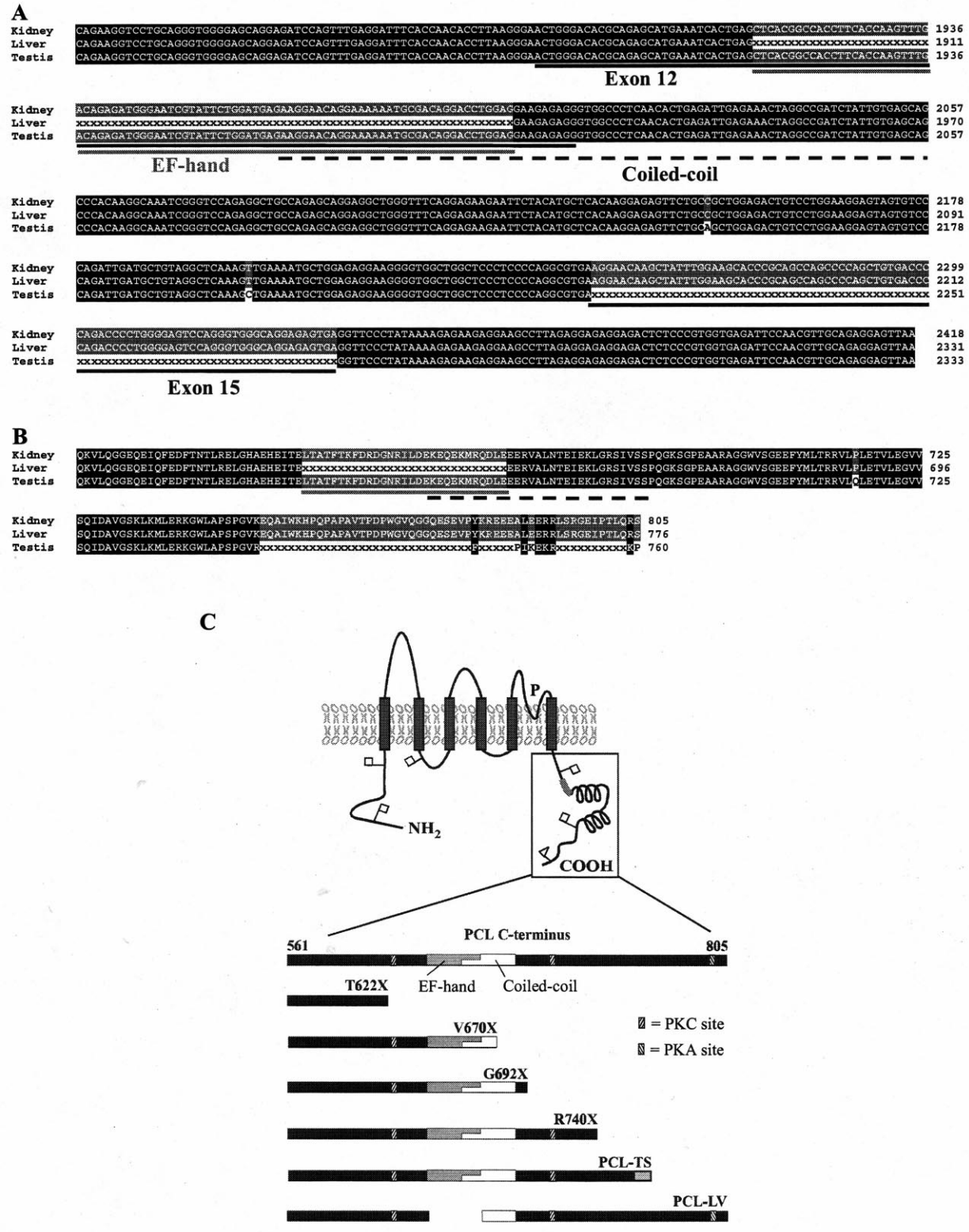

Fig. 1. Sequence alignment and schematic illustration for human PCL splice variants and truncation mutants. A,B: Black areas indicate identical sequence among the three splice variants while gray areas indicate sequence mismatches. A: cDNA sequence alignment of the 3′ ends of human *PKDL* isolated from kidney, liver and testis. The region encoding the EF-hand motif, the coiled-coil structure and those corresponding to exons 12 and 15 are underlined by gray, dotted and black bars, respectively. B: Amino acid sequence alignment of the C-terminus (Q606–) of PCL (kidney), PCL-LV (liver), and PCL-TS (testis). C: Top, putative membrane topology predicted for PCL based on amino acid hydrophobicity. Square and triangle flags indicate PKC and PKA phosphorylation sites, respectively. 'P' indicates the putative pore region. Bottom, the C-terminal fragments (N561–) of the four PCL truncation mutants and two splice variants are illustrated. The box at the end of the bar for PCL-TS indicates the nine amino acid residues that are different from those in PCL (see B).

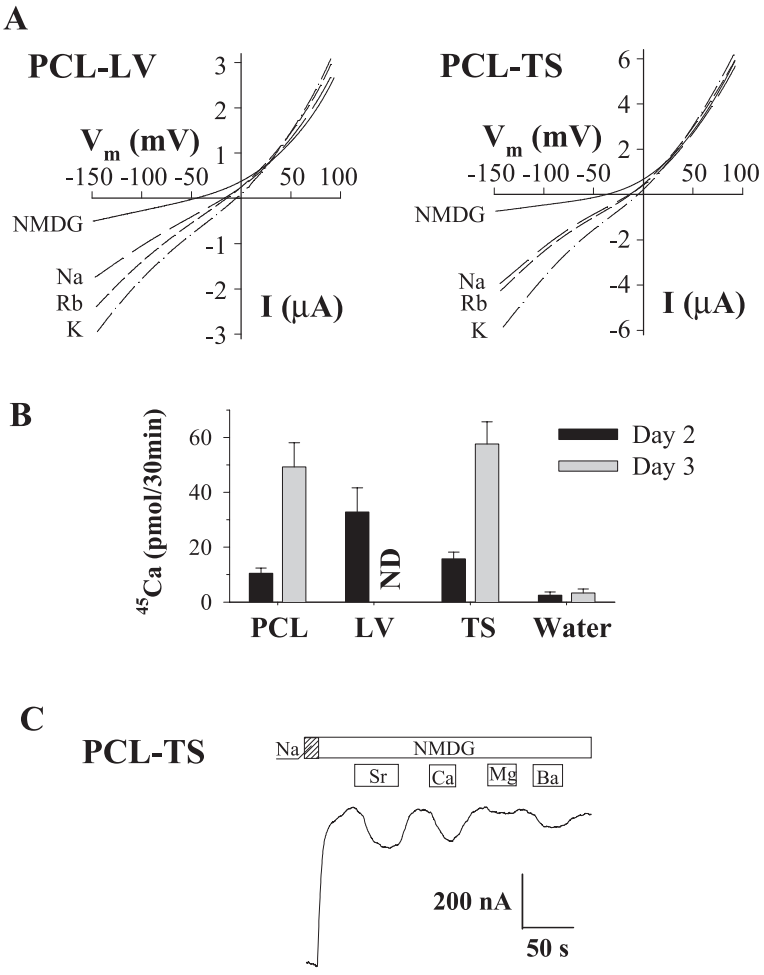

Fig. 2. Non-selective basal permeability to mono- and divalent cations. A: Left, *I–V* curves representative of five oocytes expressing PCL-LV in the presence of the extracellular standard solution ('Na') or solutions where Na^+ was replaced by equimolar NMDG, Rb^+ or K^+. Right, *I–V* curves representative of five oocytes expressing PCL-TS. Experiments were performed using the two-microelectrode voltage-clamp technique using a ramp protocol [19]. B: Calcium entry to oocytes expressing PCL or the splice variants was assessed by radiolabeled ^{45}Ca uptake using the NMDG-substituted solution ('NMDG'). Presented data were averaged from three experiments. Uptake was performed at days 2 and 3, respectively. Oocytes injected with PCL-LV exhibited earlier channel expression than those injected with PCL and PCL-TS (TS) and usually most of them died or were unhealthy at day 3. 'ND' indicates 'not determined'. Oocytes injected with water were used as control. C: Inward currents at −50 mV due to divalent cations (5 mM) contained in 'NMDG'. Experiments were performed 1–3 h following the injection of 28 nL of 50 mM EGTA into oocytes expressing PCL-TS. Assuming an effective volume of 0.7 μl for an oocyte, the final EGTA concentration was estimated to be 2 mM.

amount of Cl^- in the standard solution, this V_{rev} averaged -20 ± 4 mV ($n = 10$) while the predicted V_{rev} value for Cl^- under this condition was about −5 mV, indicating that the majority of the current at activation peak was not due to Cl^-. To determine whether calcium alone could also lead to activation, Na^+ was replaced by the impermeant NMDG. Addition of 5 mM Ca^{2+} to the NMDG-containing solution also elicited large channel activation (Fig. 3C). In oocytes expressing PCL-TS pre-injected with EGTA to an estimated final concentration of 2 mM, no Ca^{2+}-induced activation but only a relatively small inward current (as compared with the Na^+ current) was observed, likely due to calcium entry (Fig. 2C). Thus, these basic functional features (basal permeability to cations in the absence of calcium and activation by calcium) of the two splice channels were similar to those of PCL reported previously [19].

We next wanted to determine whether they differed in the extent of Ca^{2+}-induced activation, assessed by the ratio of the current in the presence of Ca^{2+} (I_{Na+Ca}) to the one in the absence of Ca^{2+} (I_{Na}). The measured ratio (I_{Na+Ca}/I_{Na}) for PCL-LV was about three-fold higher than PCL (Fig. 4). In contrast, the ratio for PCL-TS did not significantly differ from PCL. Thus, in contrast to the speculation that the EF-hand in the intracellular C-terminus may be the Ca^{2+}-binding site for the observed channel activation, the absence of the EF-hand in PCL-LV substantially enhanced its activation ability by calcium. Note that the ratio represents an intrinsic characteristic of channel function and is independent of the expression levels of polycystins in oocyte plasma membrane.

In an attempt to evaluate whether the two splice variants displayed activation and inactivation kinetics distinct from the kidney PCL, we measured the half-rising time and half-decay time as two parameters characterizing the Ca^{2+}-induced activation and inactivation time courses, respectively. Similar

half-rising and half-decay times were observed for the three channels (Fig. 5). No voltage dependence of the two time constants was observed when they were evaluated over the membrane potential range between −40 and −140 mV, suggesting that activation and inactivation do not involve binding (or dissociation) of a charged ligand such as Ca^{2+} to (or from) a PCL transmembrane site. The activation process was faster than inactivation: the half-rising time was 3.8 ± 0.5- (n = 25), 3.5 ± 0.5- (n = 18) and 3.2 ± 0.5- (n = 8) fold smaller than the half-decay time for PCL, PCL-LV and PCL-TS, respectively. Thus, the EF-hand motif and the carboxyl tail (E752–S805) of PCL are not responsible for the time course of activation and the ensuing inactivation processes.

3.3. Single-channel currents of splice variants

Patch-clamp experiments revealed that PCL-LV and PCL-TS, like PCL, exhibited large conductance to K^+. In a cell-attached configuration with the pipette solution containing (in mM) 110 KCl, 13 KOH, 5 HEPES, 1 EGTA, and pH 7.4, large downward deflating currents were observed at negative membrane potentials in oocytes expressing PCL-LV or PCL-TS (Fig. 6). When the slope of the I–V curves was used to evaluate the single-channel conductance, no significant difference was observed between the two splice variants. The inward single-channel conductance was 369 ± 11 (n = 12), 368 ± 2 (n = 7), 359 ± 10 pS (n = 5) for PCL, PCL-LV and PCL-TS, respectively. At positive membrane potentials, both PCL and splice variants exhibited smaller outward unitary currents as compared to inward currents, partly due to the presence of lower monovalent cation concentrations (~90 mM K^+ and ~20 mM Na^+) [35] and the presence of millimolar inhibitory Mg^{2+} [36], the most abundant physiological divalent cation within the cell. Water-injected and non-injected control oocytes exhibited inward single-channel currents in 11 out of 45 patches under the same cell-attached conditions, with a mean unitary conductance of 60 ± 10 pS (n = 11). No values at positive potentials were calculated because less frequent outward openings (with small current amplitudes) were observed and hardly resolvable from background noise.

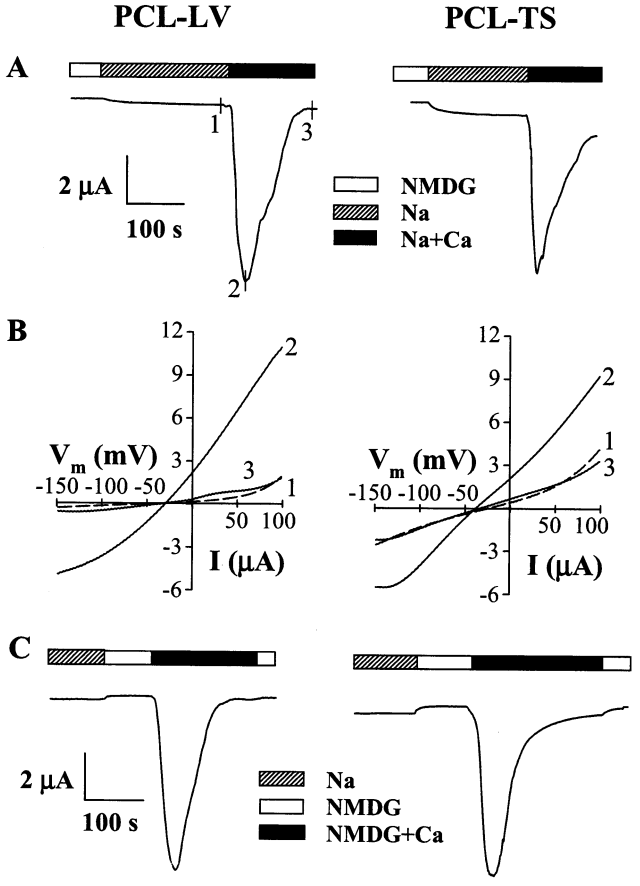

Fig. 3. Ca^{2+}-activated channel currents in oocytes expressing PCL-LV or PCL-TS. A: Representative whole-cell current recordings under voltage-clamp (−50 mV). 'Na+Ca' = 'Na' plus 5 mM $CaCl_2$. '1', '2' and '3' indicate time points where the voltage ramp protocol may be applied. B: I–V curves obtained under the basal ('1'), Ca^{2+}-activated ('2') and inactivated ('3') conditions. They were recorded at the time corresponding to points 1, 2 and 3 indicated in A. C: Ca^{2+} activation of PCL-LV and PCL-TS in 'NMDG+Ca' ('NMDG' plus 5 mM $CaCl_2$) at −50 mV.

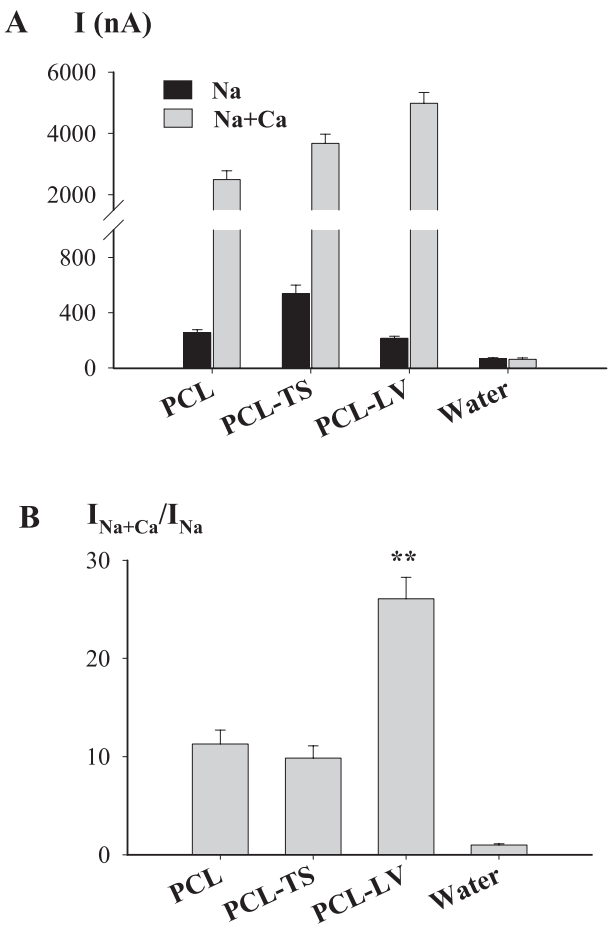

Fig. 4. Ca^{2+} activation ability of PCL and splice variant channels. A: Averaged currents measured in 'Na' or 'Na+Ca' at −50 mV for PCL (n = 32), PCL-TS (n = 46), PCL-LV (n = 46), and control (water) (n = 23). B: The Ca^{2+} activation ability was assessed by the ratio of the whole-cell channel current in the presence of 5 mM Ca^{2+} to the one obtained without Ca^{2+}. '**' on the PCL-LV bar means that the ratio is very significantly larger ($P < 0.01$) than other ratios.

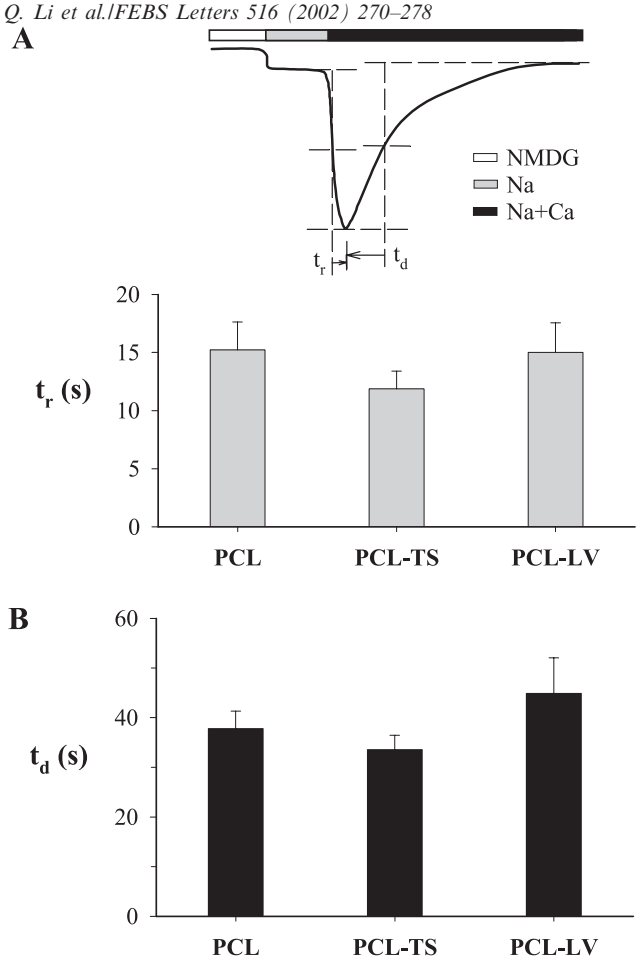

Fig. 5. Ca^{2+}-induced activation and inactivation time courses. The half-rising time (t_r) and half-decay time (t_d) obtained from each inward current time course following calcium-induced activation. A: The upper panel shows how the two parameters were defined and measured. Activation time course was characterized by t_r (lower panel). B: Inactivation time course was characterized by t_d. Shown t_d and t_r were averaged over various voltages ranging from −40 to −140 mV.

3.4. Functional properties of PCL C-terminal truncation mutants

To determine how other parts of the C-terminal region affect the PCL function, we truncated PCL at various locations using site-directed mutagenesis. As illustrated (Fig. 1C), the mutant T622X lacked most of the C-terminus, including the EF-hand, while V670X, G692X and R740X all retained the EF-hand. It turned out that all these mutants, like the wild-type PCL, retained basal non-selective permeability to monovalent (e.g. Na^+, Fig. 7A) and divalent cations (data not shown), indicating that the C-terminus is not critical for defining basal channel activity. When evaluating the characteristic ratio I_{Na+Ca}/I_{Na}, we found that the ratio was more than two-fold larger for the mutant T622X (23.7 ± 4.1, $n = 32$) than PCL (11.0 ± 1.0, $n = 46$), while those for V670X, G692X and R740X were only modestly (though significantly) different from PCL (13.9 ± 2.1, 7.1 ± 1.1, and 7.3 ± 1.1, $n = 20$, 17, and 21, respectively) (Fig. 7B). These data support the observations obtained from our studies of the two splice variants that removal of the EF-hand enhances the polycystin's Ca^{2+} activation ability and that the C-terminus is not essential for basal activity and Ca^{2+}-induced channel activation.

4. Discussion

Alternative splicing is a common phenomenon in many human genes and constitutes an efficient way to create diversity using a minimum number of independent genes. It also provides a mechanism to respond to tissue-specific needs and developmental regulations [24]. Splice variant channels have often been shown to exhibit similar but distinct channel properties compared to their wild-type proteins [25,26]. However, functional difference between the wild-type and splice variants is generally not well investigated. In the present study, we have addressed the problem through functional studies of two human PCL splice variants, PCL-LV and PCL-TS, identified from liver and testis, respectively. PCL-LV lacked the EF-hand motif in its carboxyl segment and PCL-TS was a protein shorter than PCL by 45 amino acids on the carboxyl tail. We reported here that both splice variants retained a basal conductance to cations in the absence of calcium, as well as channel activation by calcium.

4.1. Endogenous channels in oocytes

Xenopus oocytes possess endogenous Cl^- channels that, like PCL, can also be activated by calcium entry [37,38]. Our previous studies showed that PCL channel activation persisted in the absence of Cl^- (figure 4e in [19]). In the present study, the average reversal potential of −20 mV obtained at activation peak under the approximately symmetrical Cl^- condition was substantially different from the predicted value of −5 mV, demonstrating that the majority of the observed current was not due to Cl^- through endogenous channels. Interestingly, C-terminal fragments of polycystin-1 were reported to up-regulate an endogenous cation channel in *Xenopus* oocytes [39]. This channel was non-selectively permeable to cations with a unitary conductance of 20 pS in the presence of 100 mM Na^+, much smaller than the one for PCL (150 pS, see [19]), and was observed in control oocytes at a much lower probability. Furthermore, PCL expressed in human embryonic kidney cells was recently reported to display a similar basal unitary conductance (in the presence of Na^+ and absence of Ca^{2+}) to the one obtained using oocytes [19,40], indicating that *Xenopus* oocytes constitute an appropriate expression system for functional studies of PCL.

4.2. Roles of the carboxyl-terminus in PCL basic functions

C-terminal deletions in the α-subunit of calcium channels were reported to retain the channel function but result in increased currents without significantly affecting expression levels [41]. Both of our splice variant channels displayed the basal conductance to a number of mono- and divalent cations in the absence of Ca^{2+}, including Na^+, K^+, Rb^+, Sr^{2+}, and Ba^{2+}. Like PCL, they were impermeable to large cations such as NMDG and choline and were inhibited by Mg^{2+}. With respect to calcium-induced channel activation/inactivation, the two splice channels exhibited characteristically similar responses to calcium entry following an extracellular calcium application. Since PCL-LV does not possess a known calcium-binding motif, these results indicate that the EF-hand in PCL is not essential for the channel activation and that the action of Ca^{2+} on the channel may be mediated through an alternate mechanism. The results obtained from studies of artificial carboxyl-terminal truncation mutants were consistent with those from the two splice variants. All four mutants

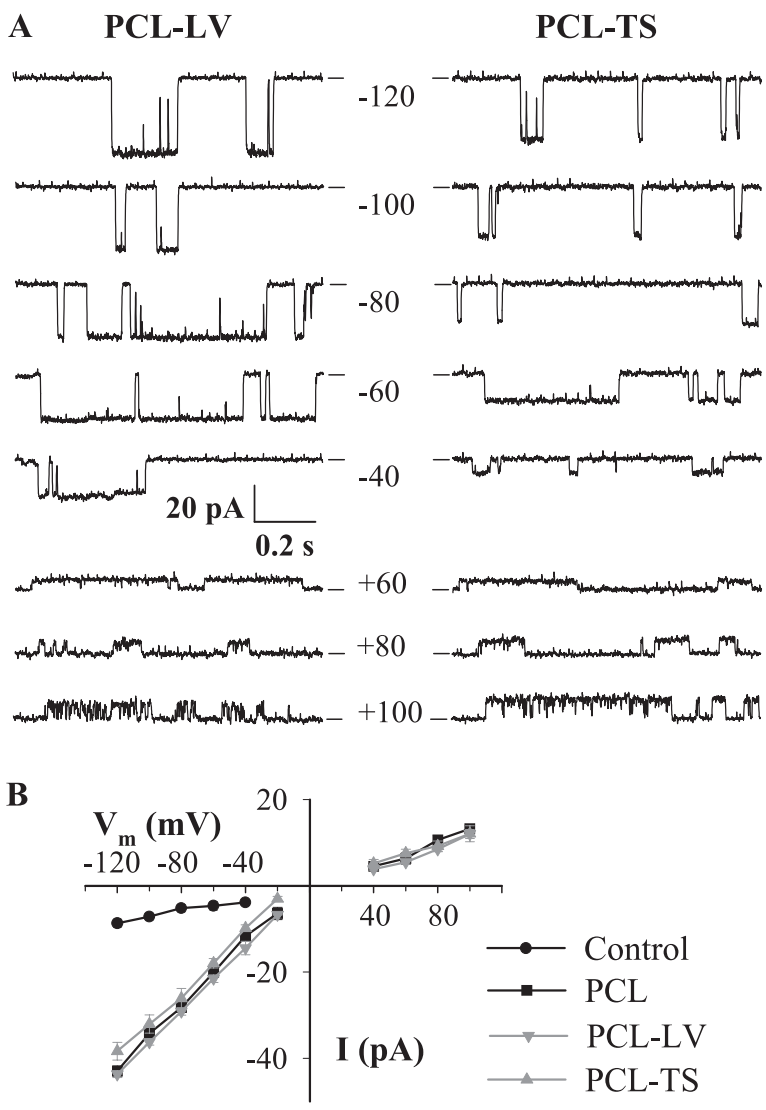

Fig. 6. Single-channel currents of PCL-LV and PCL-TS under the cell-attached configuration. A: Representative recordings in oocytes expressing one or another splice variant. The oocyte chamber contained the Barth's solution. Membrane potential was applied from the pipette and was indicated on each recording. Inward (downward) currents were carried by cations of the pipette solution while outward currents were carried by oocyte endogenous cations. B: *I–V* relationships for PCL, the two splice variants, and oocyte endogenous channels.

(T622X, V670X, G692X and R740X) retained the basal conductance to Na^+ and the capability of calcium-induced channel activation and ensuing inactivation. Our data together demonstrated that the C-terminus is not crucial to these basic functions of PCL. In particular, the EF-hand is not required for calcium-induced channel activation/inactivation.

Despite the apparent similarity between PCL and the splice variant channels in terms of basal conductance and calcium-triggered activation, further examination revealed that their Ca^{2+}-induced activation capability, assessed by the activation ratio I_{Na+Ca}/I_{Na}, a parameter independent of channel expression level, differed substantially from each other. The four artificial truncation mutants also exhibited different activation ratios. PCL-LV and T622X, both lacking the EF-hand motif, were associated with two- to three-fold higher activation ratios than those possessing the motif, indicating that the motif may have a modulation effect on the channel activation. It is possible that calcium ion binds to the motif in order to regulate PCL function and prevent it from over-activation. It is still unclear what is the physiological affinity of the motif for calcium-binding and why liver would not need such a regulation of channel activation.

Whether the EF-hand motif links to channel inactivation in the super family of cation channels possessing six transmembrane domains is still controversial. For example, Zhou and colleagues reported that the motif is not responsible for the calcium-induced feedback inactivation in the α1C-subunit [30] that shares a common membrane topology and modest sequence similarity with PCL. However, other reports have demonstrated that the motif is indeed involved in the channel inactivation process [27–29]. In the case of PCL, although we did not observe a direct involvement of the consensus Ca^{2+}-binding EF-hand in channel inactivation, the augmented activation ratio might be the result of reduced inactivation ability. Thus, although the motif is not essential for channel activation/inactivation, it clearly exhibited modulation roles in the processes.

4.3. Comparison with PC2

Despite the high similarity between PCL and PC2 in amino

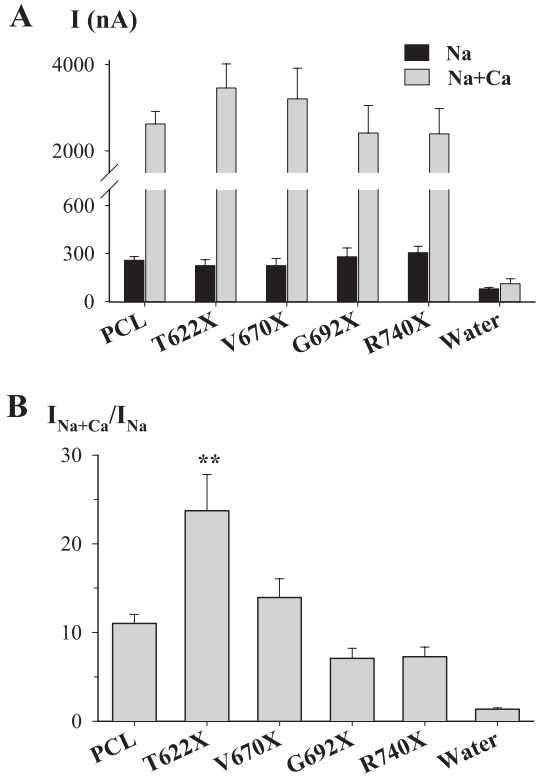

Fig. 7. Ca^{2+} activation ability of the PCL C-terminal truncation mutants. A: Averaged currents measured in 'Na' and 'Na+Ca' in oocytes expressing PCL ($n=46$), T622X ($n=32$), V670X ($n=20$), G692X ($n=17$), R740X ($n=21$), or water control oocytes ($n=47$). Membrane was voltage-clamped at −50 mV. B: Ca^{2+} activation ratios were evaluated as I_{Na+Ca} divided by I_{Na}. The ratio for T622X was very significantly larger than that for PLC channel, as indicated by '**' ($P<0.01$).

acid sequence and membrane topology, they substantially differed in single-channel conductance (290 vs. 120 pS with 100 mM K^+ and 150 vs. 23 pS with 100 mM Na^+). Since there is about 20-fold more extracellular Na^+ than K^+, the entry of Na^+ through PCL should be predominant at physiological (negative) potentials (e.g. −50 mV) despite of the two-fold higher conductance for K^+. The membrane targeting information seems to be contained in different segments of PCL and PC2. Our present study and that led by Dr. Somlo [42] suggest that membrane targeting of PCL is not determined by its C-terminus. In contrast, the PC2 C-terminus appeared to be responsible for this process. A PC2 pathogenic truncation mutant lacking the major part of the C-terminus, named R742X, expressed predominantly in oocyte plasma membrane while the wild-type was mostly localized in intracellular organelles [23]. Expression of PC2 in CHO cells resulted in its predominant localization in intracellular organelles as well while its co-expression with polycystin-1 resulted in its targeting to the plasma membrane [22]. This later study demonstrated that the PC2 C-terminus is important for both membrane targeting and formation of a functional complex with polycystin-1 in the plasma membrane. However, the membrane localization of PC2 has rather been controversial [20,43,44]. Although there has been no reported controversy regarding PCL subcellular localization, much work has yet to be done in order to elucidate the PCL domains involved in calcium-induced activation and membrane targeting.

Although there is no other Ca^{2+}-binding motif in PCL, it is still possible that calcium binds to another site in PCL to trigger the activation. It was recently reported that a PCL splice variant cloned from human brain lacking part of the N-terminus targeted to intracellular compartments of CHO-K1 cells and resulted in loss of channel function when expressed in oocytes [42]. Deletion of the entire N-terminus of PCL resulted in the complete loss of channel activity in oocytes (Li et al., unpublished data). On the other hand, it is possible that the observed channel activation is not due to a direct association between Ca^{2+} and PCL at all. This would mean that there exists an intermediate Ca^{2+}-binding protein subunit which associates with PCL to mediate channel activation. Upon association, the PCL–subunit complex would undergo conformational changes triggered by calcium-binding to the subunit, which then results in activation.

While removal of the putative Ca^{2+}-binding EF-hand motif in the carboxyl tail of PCL resulted in the observed increase in activation capability, it remains to be elucidated which residues in the motif are responsible for the increase. An alternatively spliced isoform of a voltage-gated channel, missing the EF-hand motif, has previously been identified in rat hippocampal neurons [24]. This splice variant was shown to be developmentally regulated in a way different from the wild-type. Thus, it will be of great importance to clarify how PCL-LV and PCL protein (if present) in liver might be developmentally regulated, and why liver needs such altered channel functions.

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