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Permeation and inhibition of polycystin-L channel by monovalent organic cations

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Abstract

Polycystin-L (PCL), homologous to polycystin-2 (71% similarity in protein sequence), is the third member of the polycystin family of proteins. Polycystin-1 and -2 are mutated in autosomal dominant polycystic kidney disease, but the physiological role of PCL has not been determined. PCL acts as a Ca-regulated non-selective cation channel permeable to mono- and divalent cations. To further understand the biophysical and pharmacological properties of PCL, we examined a series of organic cations for permeation and inhibition, using single-channel patch clamp and whole-cell two-microelectrode voltage clamp techniques in conjunction with *Xenopus* oocyte expression. We found that PCL is permeable to organic amines, methlyamine (MA, 3.8 Å), dimethylamine (DMA, 4.6 Å) and triethylamine (TriEA, 6 Å), and to tetra-alkylammonium cation (TAA) tetra-methylammonium (TMA, 5.5–6.4 Å). TAA compounds tetra-ethylammonium (TEA, 6.1–8.2 Å) and tetra-propylammonium (TPA, 9.8 Å) were impermeable through PCL and exhibited weak inhibition on PCL (IC_{50} values>13 mM). Larger TAA cations tetra-butylammonium (TBA, 11.6 Å) and tetra-pentylammonium (TPeA, 13.2 Å) were impermeable through PCL as well and showed strong inhibition (IC_{50} values of 2.7 mM and 1.3 μM, respectively). Inhibition by TBA was on decreasing the single-channel current amplitude and exhibited no effect on open probability (NP_o) or mean open time (MOT), suggesting that it blocks the PCL permeation pathway. In contract, TEA, TPA and TPeA reduced NP_o and MOT values but had no effect on the amplitude, suggesting their binding to a different site in PCL, which affects the channel gating. Taken together, our studies revealed that PCL is permeable to organic amines and TAA cation TMA, and that inhibition of PCL by large TAA cations exhibits two different mechanisms, presumably through binding either to the pore pathway to reduce permeant flux or to another site to regulate the channel gating. These data allow to estimate a channel pore size of ~7 Å for PCL.

Keywords: Patch clamp; Voltage clamp; *Xenopus* oocyte; Polycystic kidney disease; Pore size; Organic amine; Tetra-alkylammonium

1. Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited nephropathy, primarily characterized by the formation of fluid-filled cysts in the kidneys. It is one of the most frequent genetic disorders affecting approximately 0.1% of individuals and accounting for up to 10% of end-stage renal failure. ADPKD also exhibits extrarenal manifestations, such as hepatic and pancreatic cystogenesis, intracranial aneurysms and cardiac mitral valve prolapse, and is often associated with hypertension. *PKD1* and *PKD2*, encoding polycystin-1 (PC1) and -2 (PC2), respectively, are the two known genes responsible for about 95% of ADPKD and have been mapped to chromosomes 16p13.3 and 4q21–23, respectively [26]. Polycystin-L (PCL), encoded by *PKDL*, is the third member of the polycystin family [16]. As members of the transient receptor potential (TRP) channels superfamily, PC1, PC2 and PCL have also been called TRPP1, TRPP2 and TRPP3 (TRP-polycystin), respectively [7]. PC1 is a large receptor-like integral membrane protein involved in cell–cell and cell–matrix interactions.

PCL and PC2 are highly homologous (71% similarity) but PCL seems not to be directly linked to ADPKD renal cyst formation. However, the mouse orthologue of PCL is deleted in *krd* (kidney and retinal defects) mice, resulting in defects in kidney and retina [10,16]. PCL may be one of the candidates linked to unmapped human genetic cystic disorders such as dominantly transmitted glomerulocystic kidney disease of post-infantile onset, isolated polycystic liver disease, and Hajdu–

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Cheney syndrome/serpentile fibula syndrome [16]. PCL is present in multiple tissues, including brain, retina, kidney, heart, testis, liver, pancreas and spleen. In adult kidney, PCL is predominantly localized in the apical region of the principal cells of inner medullary collecting ducts [3]. In embryonic kidney, it is found in the apical membrane of tubular epithelial cells. PCL shares similar membrane topology and modest sequence homology with TRP channels and the α-subunits of voltage-gated K, Na and Ca channels. For example, it comprises six transmembrane-spanning domains with both the carboxyl- and amino-termini intracellularly localized. Indeed, recent studies have demonstrated that PCL is a Ca-activated, Ca-permeable, non-selective cation channel [6,14]. It is still unknown how many subunits make up the pore of the PCL channel. PCL channel possesses an EF-hand calcium-binding motif in its C-terminus, suggesting a possible involvement of intracellular calcium in its function. It was reported that EF-hand serves to control (reduce) calcium-induced PCL channel activation [13]. PCL associates with troponin I which inhibits PCL channel activity [12].

Monovalent cations such as organic amines and tetra-alkylammonium (TAA) compounds have been employed to determine the pore diameters of ion channels. Methylamine (MA) is an endogenous compound that is increased during liver and renal failure, Alzheimer's disease, vascular dementia and diabetes. It alters some neurobehavioral functions, probably by acting as a potassium channel blocker [19]. Dimethylamine (DMA) was used to investigate the pore size of PC2 channel and ryanodine receptor [2,24]. Triethylamine (TriEA), tetra-methylammonium (TMA), tetra-ethylammonium (TEA), tetra-propylammonium (TPA), tetra-butylammonium (TBA) and tetra-pentylammonium (TPeA) are known to block ion permeation through K, Na and Cl channels, anthrax toxin channels, ryanodine and NMDA receptors [18,20,28]. They were also used to probe the structure of the internal and external vestibules of K channel pores [8,15,28] and the pore sizes of ryanodine receptor, K channel KcsA, NMDA receptor, PC2, and vanilloid receptor (TRPV6) [1,2,9,22,27]. Using TAA cations as permeant ions or inhibitors, the pore diameter of the PC2 channel was recently estimated to be at least 11 Å [2]. Previous reports revealed that ryanodine receptor has a minimum pore diameter of ~7 Å and that the NMDA channel pore size of ~11 Å. TRPV6, which belongs to the family of TRP channels, has a pore diameter of ~5.4 Å. These TAA compounds are also blockers of Na channels with affinities positively correlated to the length of alkyl side chains [18]. In the present study, we explored biophysical and pharmacological properties of the PCL channel through examining the permeation of and inhibition by monovalent organic amines and TAA cations.

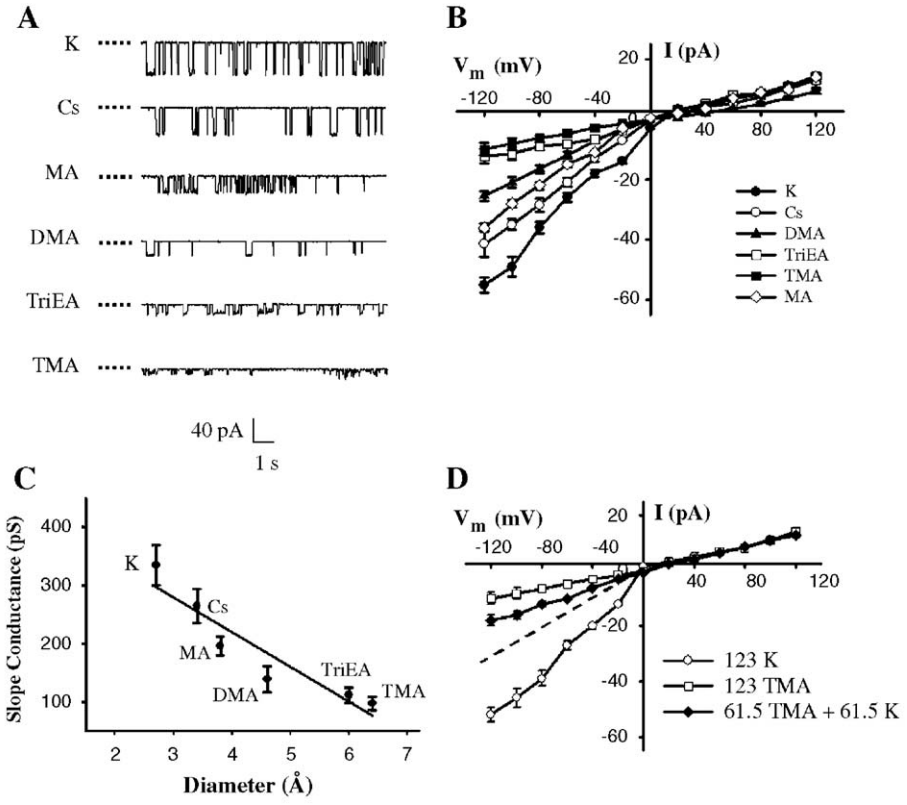

Fig. 1. Permeation of organic amines and TMA. Cell-attached mode of single-channel patch clamp was performed. (A) Downward deflections recorded at −120 mV in the presence of 123 mM pipette K, Cs, MA, DMA, TriEA or TMA. Shown traces were filtered at 200 Hz. The dashed lines indicate the closed state. (B) *I–V* relationships for various cations. (C) Single-channel conductance as a function of cation diameter. (D) *I–V* curves obtained at molar fractions of K and TMA (in mM). Dashed line represents the arithmetic average of the inward currents generated by 123 mM K and TMA.

2. Material and methods

2.1. Oocyte preparation

Capped synthetic human *PCL* mRNA was synthesized by in vitro transcription from a linearized template inserted in the pTLN2 vector, using the mMESSAGE mMACHINE1 Kit (Ambion, Austin, TX, USA). Stage V-VI oocytes were extracted from *Xenopus laevis* and defolliculated by collagenase type I (2.5 mg/mL) (Sigma-Aldrich Canada, Oakville, ON, Canada) in the Barth's solution (in mM: 88 NaCl, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 10 HEPES, and pH 7.5) at room temperature (21–23 °C) for 2 h. Oocytes were injected with 50 nl of water containing 20 or 40 ng of each RNA 3–24 h following defolliculation. An equal volume of RNAase-free water was injected into each control oocyte. Injected oocytes were incubated at 16–18 °C in the Barth's solution supplemented with antibiotics for 3–4 days prior to experiments.

2.2. Two-electrode voltage clamp

Two-electrode voltage clamp was performed as described previously [14]. Briefly, the two electrodes (Capillary pipettes, Warner Instruments, Hamden, CT, USA) impaling *Xenopus* oocytes were filled with 3 M KCl to form a tip resistance of 0.3–2 MΩ. Oocyte whole-cell currents were recorded using a Geneclamp 500B amplifier and a Digidata 1322A AD/DA converter (Axon Instruments, Union City, CA, USA). In experiments using a ramp protocol, currents and voltages were sampled at internals of 200 μs and filtered at 2 kHz using an 8 pole Bessel filter. In experiments using a gap-free protocol, current/voltage signals were sampled at intervals of 200 ms. The modified Choline-Cl-containing solution contained (in mM): 100 Choline-Cl, 2 KCl, 0.2 $MgCl_2$, 10 HEPES, pH 7.5.

2.3. Single-channel patch clamp

Vitelline membranes of oocytes were removed manually following incubation of the oocytes at room temperature in a hypertonic solution containing (in mM): 220 NaCl, 50 sucrose, 1 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.5. Oocytes were then transferred to the Barth's solution and allowed to recover for 10–20 min before patch clamping. Electrodes were filled with a pipette solution containing 123 mM K (in mM: 110 KCl, 13 KOH, 10 HEPES, and pH 7.4) to form a tip resistance of 3–6 MΩ. The Barth's solution was used in the bath. Single channel currents were recorded in cell-attached configuration using PC-ONE Patch Clamp amplifier (Dagan Corp., Minneapolis, MN, USA), DigiData 1322A interface, and Clampex 9 software (Axon Instruments). Recordings started after seal resistance reached at least 3–5 GΩ. Current/voltage signals were sampled every 200 μs and filtered at 2 kHz. All chemicals were purchased from Sigma-Aldrich Canada.

2.4. Statistics and data analysis

Data obtained from the two-microelectrode voltage-clamp and patch-clamp experiments were analyzed using Clampfit 9. Single-channel conductance values were obtained from Gaussian fits to 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, recordings with single openings were used and filtered at 500 Hz (Gaussian). Analyzed data were plotted using Sigmaplot 9 (Jandel Scientific Software, San Rafael, CA, USA) and expressed in the form of mean ± SE (N), where SE 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 Clampfit 9 or Sigmaplot 9. Dose–

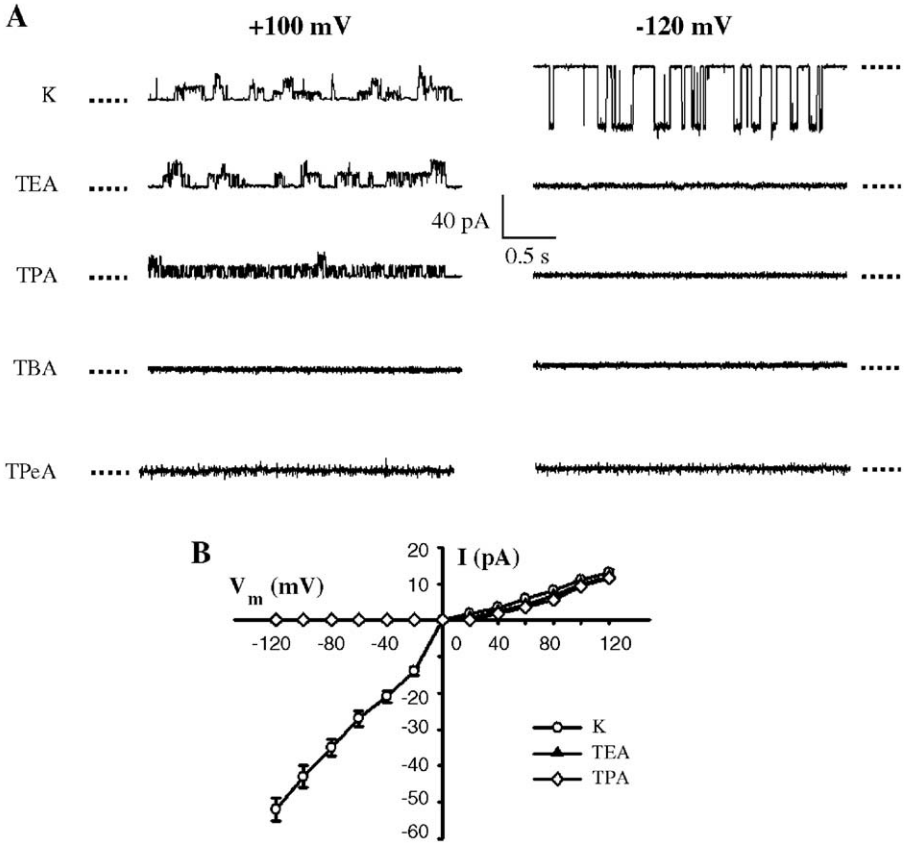

Fig. 2. Permeation of and inhibition by TAA cations TEA, TPA, TBA and TPeA. (A) Representative traces recorded in the presence of 123 mM K, TEA, TPA, TBA or TPeA in the pipette at +100 or −120 mV. (B) Averaged *I–V* relationships for K, TEA and TPA (N=12) obtained under the same conditions as in panel A.

response inhibition data were fitted with the Logistic equation: $I/I_{max}=1/\{1+([S]/IC_{50})^p\}$, where [S] represents the concentration of an inhibitor and p is the power (or Hill coefficient). Comparison between two sets of data was analyzed by t-test or two-way ANOVA, and a probability value (P) of less than 0.05 (or 0.01) was considered significant (or very significant).

3. Results

To gain more insights into biophysical and pharmacological properties of PCL we tested a series of organic amines and TAA cations with varying ionic diameters for their permeability through and inhibition of PCL channel expressed in *Xenopus* oocytes. PCL is a large conductance cation channel of 366 pS in the presence of 123 mM K [14]. Using the cell-attached mode of single-channel patch clamp, we found that MA (3.8 Å), DMA (4.6 Å), TriEA (6 Å) and TMA (5.5–6.4 Å) were permeable through PCL channel with current amplitudes dependent on cation size (Fig. 1A and B). In fact, single-channel conductance inversely correlated with cation size (Fig. 1C). As expected, the outward single-channel current amplitudes at positive voltages ($+V_m$, +20 to +120 mV), presumably elicited by endogenous permeant cations inside oocytes, mainly K, were not significantly different, in the presence of extracellular 123 mM K, Cs, MA, DMA, TriEA or TMA (Fig. 1B).

Single channel conductance can depend on the mole fraction of two permeant ions in a peculiar way, such that the conductance is smaller than the one calculated based on mole fraction (anomalous mole-fraction behavior). To test whether PCL channel exhibits anomalous mole-fraction dependence, we measured the inward currents through PCL channel in the presence of K and TMA (61.5 mM each). We found that the resulting amplitudes were smaller than the arithmetic average of those obtained in the presence of 123 mM K and TMA alone (Fig. 1D). This result indicates that the two permeant cations exhibit interaction between them when passing through PCL channel.

We next tested the permeation of larger TAA cations through the PCL channel. In the presence of 123 mM TEA (6.1–8.2 Å) or TPA (9.8 Å) in the pipette, channel openings

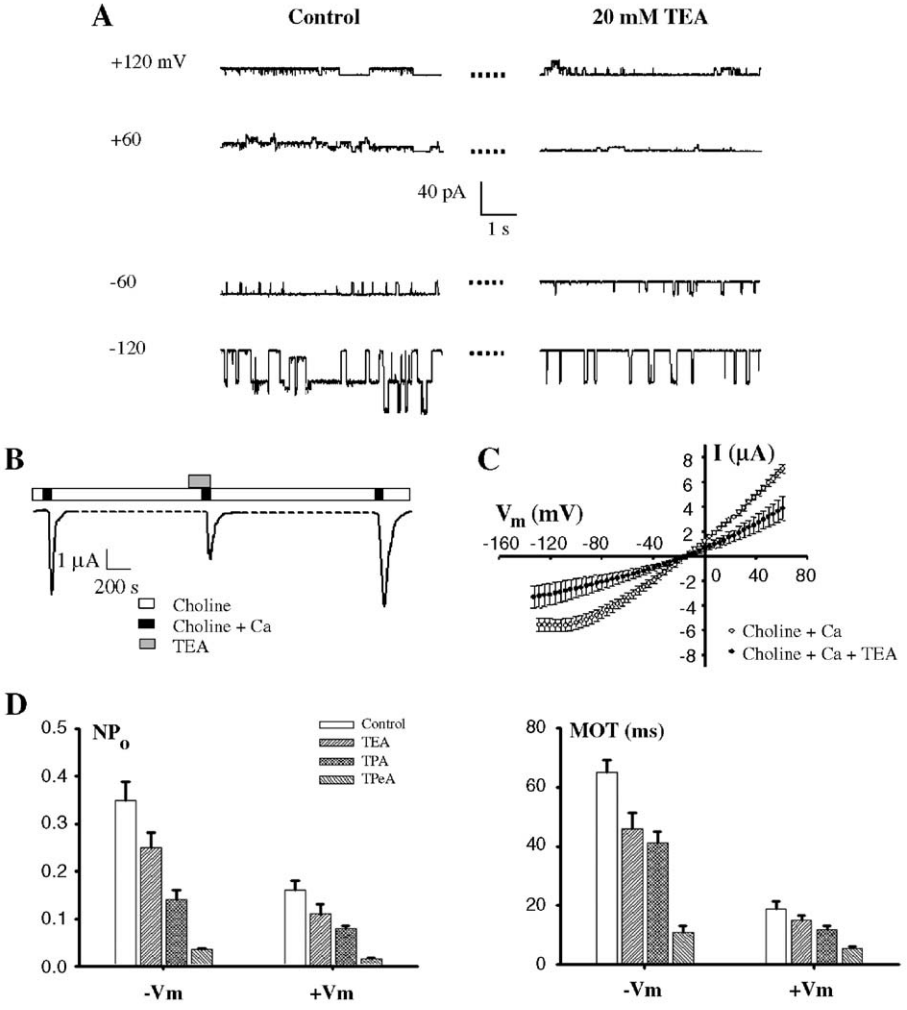

Fig. 3. Effects of TEA on PCL. (A) Representative single-channel recordings obtained with or without TEA (20 mM). (B) Representative whole-cell recording in a single oocyte voltage clamped at −50 mV. "Choline" indicates the solution containing (in mM): 100 Choline-Cl, 2 KCl, 0.2 $MgCl_2$, 10 HEPES, pH 7.5, and "Ca" = 5 mM $CaCl_2$. The dashed lines represent 10-min periods during which the oocyte was not voltage clamped. (C) Averaged whole-cell I–V curves before and after addition of 20 mM TEA (N=10). (D) Effects of 20 mM TEA (N=10), 20 mM TPA (N=11), and 1 μM TPeA (N=14) on NP_o and MOT at $-V_m$ and $+V_m$.

were observed at positive voltages but not at negative voltages (−180 to −20 mV) (Fig. 2A and B). This indicates that TEA and TPA were impermeable through PCL. There was no difference in the single-channel amplitudes of the PCL channel $+V_m$, whether the pipette contained 123 mM K, TEA or TPA (Fig. 2B, $N=12$). In contrast, in the presence of 123 mM TBA (11.6 Å) or TPeA (13.2 Å) in the pipette, at voltages between −180 and +180 mV, no channel openings were observed, indicating that these two cations are impermeable through PCL as well and inhibit outward currents associated with effluxes of oocytes endogenous permeant cations through PCL.

We next examined inhibition of K currents by TEA, TPA, TBA and TPeA, using both single-channel patch clamp and whole-cell voltage clamp. Addition of TEA at 20 mM to the pipette containing 123 mM K reduced the NP_o and MOT values of inward and outward single-channel currents (Fig. 3A). In average, NP_o decreased from 0.35±0.04 ($N=40$) and 0.16±0.02 ($N=35$), in the absence of TEA, to 0.25±0.03 ($N=15$, $P<0.05$) and 0.11±0.02 ($N=12$, $P<0.05$), in the presence of pipette TEA (20 mM), at $-V_m$, and $+V_m$, respectively (Fig. 3D). Under the same condition, MOT decreased from 65±4 ($N=40$) and 19±2 ms ($N=35$) to 46±5 ($N=15$) and 15±2 ms ($N=12$) at $-V_m$ and $+V_m$, respectively. In contrast, TEA did not exhibit significant effect on the outward single-channel current amplitude. Inhibitory effect of TEA was also observed with whole-cell recordings (Fig. 3B and C). Inward currents at −50 mV in the presence of 100 mM Choline and 5 mM Ca decreased 44±7% ($N=8$) by 20 mM TEA, with an IC_{50} value of 23±3 mM ($N=31$).

TPA exhibited similar characteristics as TEA (Fig. 4A–C). TPA at 20 mM inhibited the single-channel activities of the PCL channel (Fig. 4A). In average, NP_o decreased to 0.14±0.02 ($N=12$, $P<0.01$) and 0.08±0.01 ($N=11$, $P<0.01$) at $-V_m$ and $+V_m$, respectively (Fig. 3D). MOT decreased to 41±4 ms ($N=12$) and 12±1 ms ($N=11$), respectively. Like TEA, TPA had no effect on the current amplitude. Inhibitory effect of TPA was also supported by whole-cell data (Fig. 4B and C). Inward currents at −50 mV in the presence of 100 mM Choline and 5 mM Ca decreased 68±7% ($N=9$) by 20 mM TPA (Fig. 4B), with an IC_{50} value of 13±2 mM ($N=29$).

In contrast to TEA and TPA, TBA exhibited inhibition of the PCL single-channel amplitude at the whole voltage range tested and had no effects on NP_o and MOT (Fig. 5A). The concentration dependence of the inhibition was represented by averaged current–voltage (I–V) curves obtained in the presence 0.1, 1, 10, 33 mM TBA in the pipette (Fig. 5B). For example, at −120 mV, the presence of 0.1, 1, 10 and 33 mM TBA in the pipette containing 123 mM K significantly reduced channel inward-current amplitude to 95±11% ($P>0.05$, $N=7$), 75±9% ($P<0.05$, $N=10$), 46±3% ($P<0.01$, $N=15$) and 25±4% ($P<0.01$, $N=8$), respectively. The IC_{50} values decreased with hyperpolarization, from 16.2 mM at −40 mV to 6.8 mM at −120 mV, suggesting voltage-dependent inhibition by TBA (Fig. 5C). TBA inhibition was also demonstrated at the whole-cell level (Fig. 5D and E). Inward currents at −50 mV activated by 5 mM Ca were reduced to 18% by 10 mM TBA (Fig. 5D).

TPeA is the largest cation tested in the current study. TPeA did not permeate PCL (Fig. 2A) but acted as a high-affinity

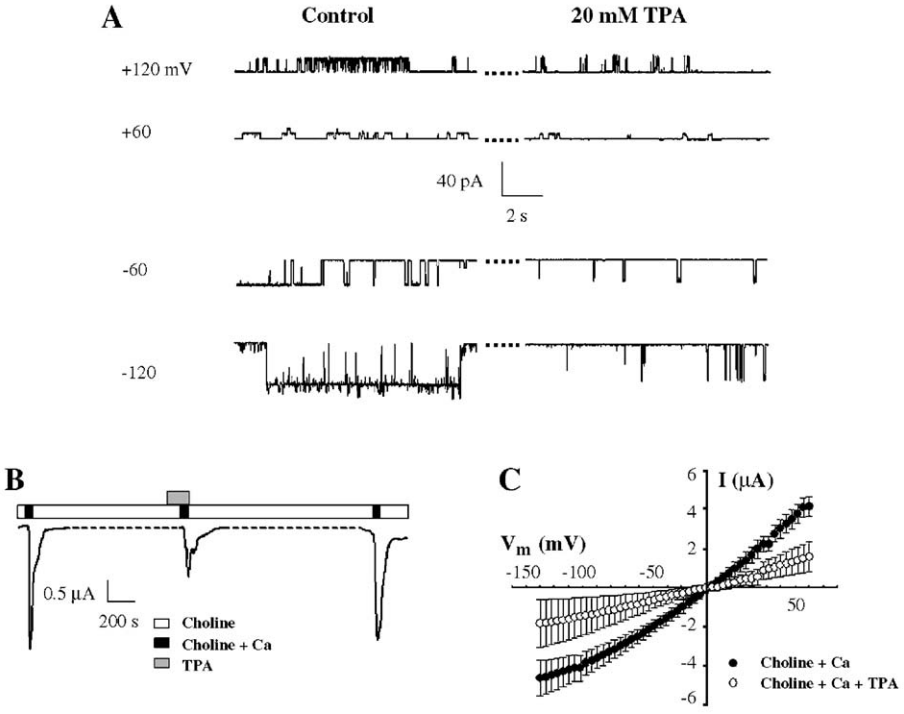

Fig. 4. Effects of TEA on PCL. (A) Representative single-channel recordings obtained with or without TPA (20 mM). (B) Representative whole-cell recording in a single oocyte voltage clamped at −50 mV. (C) Averaged whole-cell I–V curves before and after addition of 20 mM TPA ($N=11$).

inhibitor. Using patch clamp, with 1 μM TPeA in the pipette solution PCL channel activity was almost abolished at both $+V_m$ and $-V_m$. TPeA reduced NP_o and MOT, but not the single-channel conductance (Figs. 3D and 6A). The inhibition by TPeA was concentration-dependent, and TPeA at 10 μM completely abolished PCL channel opening (Fig. 6B). The IC_{50} values for TPeA on NP_o were 1.2±0.1 μM at $+V_m$ and 0.60±0.02 μM at $-V_m$ (Fig. 6B, left). The IC_{50} values for TPeA on MOT were 0.54±0.16 μM at $+V_m$ and 0.72±0.17 μM at $-V_m$ (Fig. 6B, right). TPeA also exhibited potent inhibition of PCL whole-cell currents (Fig. 7A and B), with an IC_{50} value of 1.3±0.1 μM (N=15) at −50 mV, much smaller than that for TBA (2.7±0.2 mM, N=10), TPA (13±1.2 mM, N=11) and TEA (23±3 mM, N=13) (Fig. 7C). Thus, TPeA is the most potent inhibitor tested when compared with the three other inhibitors and the inhibition potency of these TAA compounds inversely correlated with their size (Fig. 7D).

4. Discussion

In the present study we investigated the permeation and inhibition of the PCL channel by monovalent organic amines and TAA cations of various sizes, including MA, DMA, TriEA, TMA, TEA, TPA, TBA and TPeA, using the whole-cell two-microelectrode voltage clamp and single-channel patch clamp. The PCL single-channel conductance decreased as the size of cations increased (Fig. 1). Similar conclusion was previously drawn for ryanodine receptor [23]. TEA, TPA, TBA and TPeA are not permeable through PCL and inhibited whole-cell Ca-induced activated currents and basal single-channel activities of PCL. Inhibition potency correlates

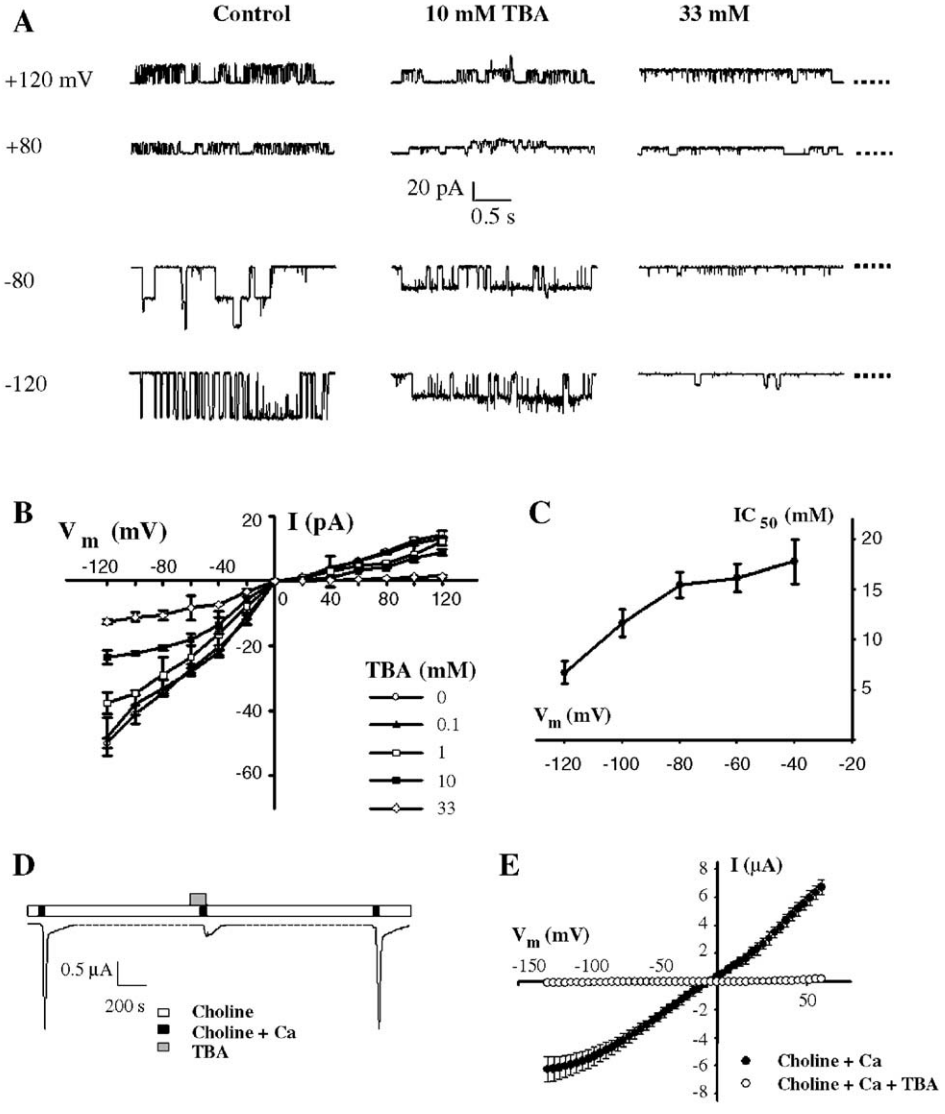

Fig. 5. Inhibition of PCL by TBA. (A) Representative tracings obtained in the presence of 0 (Control), 10 or 33 mM TBA at various membrane potentials as indicated. (B) Averaged I–V relationships (N=25) obtained at various TBA concentrations. (C) Voltage dependence of IC_{50} for TBA inhibition obtained from the data shown in panel B. (D) Representative whole-cell recording in a single oocyte voltage clamped at −50 mV with or without 10 mM TBA. (E) Averaged whole-cell I–V curves before and after addition of 10 mM TBA (N=8).

positively with cation size (Fig. 7D). TEA and TPA reduced NP_o and MOT with low potency, while the inhibition by TPeA was at least three magnitudes more potent. These three inhibitors did not significantly affect single-channel amplitude, suggesting that they bind a site away from the PCL pore pathway. In contrast, TBA reduced single-channel current amplitudes of PCL but did not reduce NP_o and MOT, suggesting binding to a site at the pore entry, which indicates that PCL may possess an extracellular vestibule of about 12 Å in size.

Monovalent inorganic and organic cations have been used as biophysical probes for delineating the pore and vestibule of ion channels. The largest tested cations permeable through PCL were TriEA and TMA and smallest tested cation impermeable through PCL was TEA. The sizes of TriEA, TMA and TEA are in the range of 6–7.2 Å [2,23], 5.5–6.4 Å [11,25] and 6.1–8.2 Å [2], respectively. Thus the PCL pore size can be estimated to be ~7 Å, which is comparable with TRPV6 (~5.4 Å), L-type voltage-gated Ca channel (6.2 Å)[5] and ryanodine receptor (~7 Å) [1,27].

Interestingly, although PCL and PC2 are highly homologous and PC2 has smaller single-channel conductance than PCL, the pore size of PC2 was recently estimated to be at least 11 Å as it is permeable to TPeA [2]. This suggests that other differences in the pore geometry of these two homologous channels, e.g., in selectivity filter and pore depth, may affect cation permeation.

TAA compounds are well-known inhibitors of K channels [17]. TEA and larger TAA compounds, such as TBA and TPeA, are known to block ion permeation through ryanodine receptor, neuronal chloride and anthrax toxin channels [1,4,21]. Using energy-minimized molecular models of TAA cations and independent molecular dynamics analysis, it was found that the diameter of these molecules increased by ~2 Å per symmetrical addition of a methylene group: TMA has one carbon alkyl side chain with a size of 5.5–6.4 Å; TEA, 2 side chains, 6.1–8.2 Å; TPA, 3 side chains, 9.8 Å; TBA, 4 side chains, 11.6 Å; and TPeA, 5 side chains, 13.2 Å [17,24]. In fact, an increase in the molecular diameter of TAA cations due to addition of carbon alkyl side chains is accompanied by a proportional increase in their hydrophobicity. Whether/how the hydrophobicity of these TAA cations influences their potency of inhibition remains to be investigated.

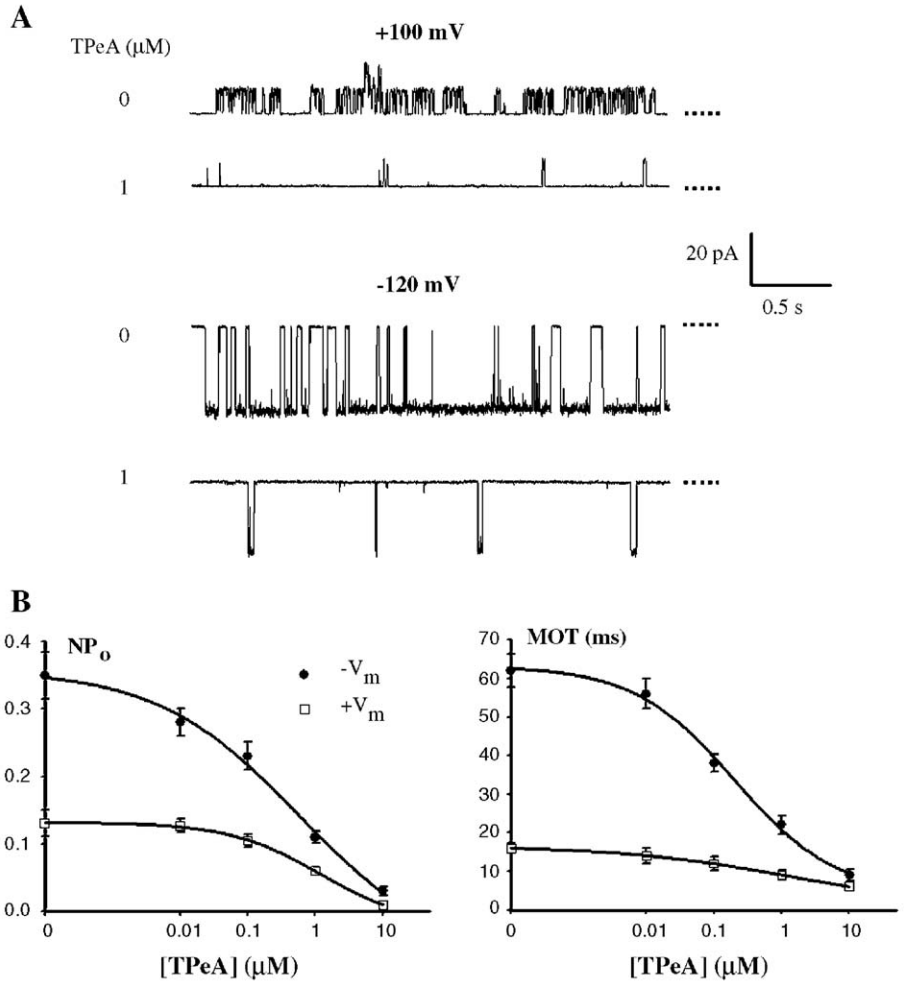

Fig. 6. Effects of TPeA on PCL single-channel currents. (A) Representative recordings in the presence or absence of 1 μM TPeA in the pipette containing 123 mM K. (B) Concentration-dependent effects of TPeA on NP_o (left panel) and MOT (right panel) at $+V_m$ or $-V_m$. Shown data were averaged from 20 measurements.

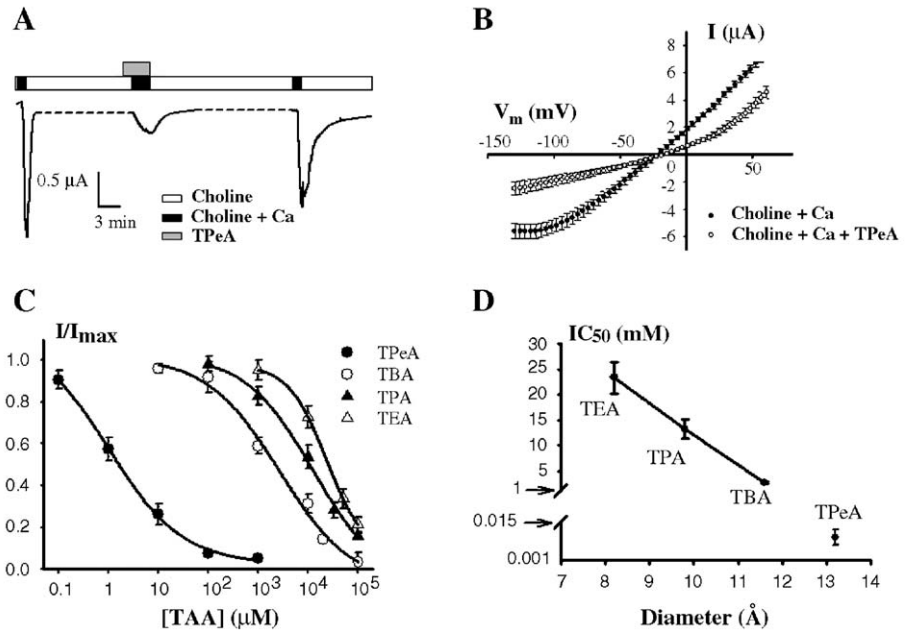

Fig. 7. Inhibition of PCL whole-cell currents by TPeA. (A) Representative whole-cell current recording from an oocyte showing the inhibition of PCL channel activation by TPeA. Currents carried by Ca were measured at −50 mV. (B) Averaged *I–V* relationships ($N=13$) for PCL channels in the presence or absence of 1 μM TPeA. (C) Concentration-dependent inhibition of PCL whole-cell current by the four TAA cations. Each point was averaged from 10 determinations. (D) IC_{50} values for TAA cations inhibition as a function of their molecular sizes.

In summary, we discovered novel biophysical and pharmacological properties of the PCL channel through examining the permeation of and inhibition by monovalent organic amines and TAA cations.

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