Synthetic multifunctional pores: deletion and inversion of anion/cation selectivity using pM and pH

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Abstract

We report the characterization of multifunctional rigid-rod [beta]-barrel ion channels with either internal aspartates or arginine–histidine dyads by planar bilayer conductance experiments. Barrels with internal aspartates form cation selective, large, unstable and ohmic barrel-stave (rather than toroidal) pores; addition of magnesium cations nearly deletes cation selectivity and increases single-channel stability. Barrels with internal arginine–histidine dyads form cation selective ($P_{K^+}/P_{Cl^-} = 2.1$), small and ohmic ion channels with superb stability (single-channel lifetime > 20 seconds). Addition of "protons" results in inversion of anion/cation selectivity ($P_{Cl^-}/P_{K^+} = 3.8$); addition of an anionic guest (HPTS) results in the blockage of anion selective but not cation selective channels. These results suggest that specific, internal counterion immobilization, here magnesium (but not sodium or potassium) cations by internal aspartates and inorganic phosphates by internal arginines (but not histidines), provides access to synthetic multifunctional pores with attractive properties.

Reference


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1 Introduction

Rigid-rod β-barrels1,2 like 1 and 2–7 are synthetic barrel-stave supramolecules developed to exploit the functional plasticity of their biological counterparts in biorganic chemistry and beyond (Fig. 1). Their rare characteristics compared to the other synthetic ion channels/pores,8–21 i.e. the possibility to install functional groups along the ion-conducting pathway, encouraged the shift of our interest from biomimicry to practical applications. Toward this end, p-octiphenyl β-barrel ion channels with internal histidine,12–24 arginine,1 lysine,20–22 and aspartate residues4,5 have been synthesized and used to recognize, translocate and, in some cases,24,25 transform a broad variety of hydrophobic guests.

We have already reported the design and synthesis of rigid-rod β-barrel 1 with internal arginine–histidine (RH) dyads and β-barrel 2 with internal aspartates (D).3,5 The formation of multifunctional pores has been demonstrated in spherical bilayer membranes, i.e., EYPC SUVs or LUVs, for both cases. For instance, α-helix recognition by cationic supramolecule 1 in polarized vesicles confirmed the existence of multifunctional pores with internal RH dyads.5 Barrel-stave (rather than toroidal)11,21–25 type pores formed by the anionic supramolecule 2 in spherical bilayers3 were shown to bind first Mg2+ and then various anionic substrates.5 Thus, it was possible to monitor the enzymatic conversion of anionic compounds using inclusion complex 2 ⊃ Mg2+.5 Circular dichroism (CD) spectroscopy was used as a sensitive tool to elaborate on the structural basis of p-octiphenyl β-barrel pores including 2 under relevant conditions.4,5,7 Ion channel characterization of barrels 1 and 2 in a planar bilayer has not been reported so far.

The introduction of arginines at the inner surface of rigid-rod β-barrels1–7 was expected to yield ion channels with some of the characteristics of R-rich peptides in bilayer membranes.36–47 This was of interest because pores formed by R-rich α-helical peptides, unlike lysine-rich peptide pores, exhibit concentration independent partition coefficients36 and cation selectivity.37,44 Moreover, R-rich α-L-peptides,42,43 α-D-peptides,42,44 β-L-peptides45 and peptoids46 derived from the HIV Tat translocation sequence47 deliver hydrophilic organics to the cell nucleus as if the hydrophobic biomembrane barriers do not exist.42–47

Fig. 1 Rigid-rod β-barrels 1 and 2 in lipid bilayers, depicted in blue (hydrophilic), gold (hydrophobic), and black (p-octiphenyl and β-sheet scaffolds); α-amino acid residues (one-letter abbreviations) located at the outer surface are black on white, internal ones white on blue. (Without X-ray structure data, the depicted suprastructures may be conceived as, at worst, productive working hypotheses that are consistent with all functions discovered so far.)
Some, if not all, of the unique characteristics of R-rich peptides in bilayer membranes can be rationalized by tight binding of counteranions. For example, immobilization of dianions by guanidinium cations of R-rich peptides can provide the anionic surface expected within cation selective pores. Binding of univalent anions, on the other hand, can produce the anionic surface expected within cation selective pores. The electrostatics of the pore interior should then transform anion-rich to neutral and, eventually, to cation-rich pores formed by D-rich barrel pores. The electrostatics of the pore interior should then be reflected in their ion selectivity. So far, pH-gated inversion of cation selectivity has been reported for R-rich barrel 1, a salt gradient between the cis (1 M KCl) and trans chambers (0.1 M KCl) was applied. Fig. 3 shows macroscopic I–V curves obtained under these asymmetric conditions at pH 6.0 (open) and pH 4.0 (closed). The reversal potential $V_r$ exhibited sign inversion from negative at pH 6.0 to positive at pH 4.0. The permeability ratios calculated from $V_r$ changed from $P_{\text{cis}}/P_{\text{trans}} = 0.5$ at pH 6.0 to $P_{\text{cis}}/P_{\text{trans}} = 3.8$ at pH 4.0. Inversion of anion/cation selectivity of channel 1 occurred at pH ≈ 5 (Fig. 4).

\[ \text{MACROSCOPIC I–V CURVES IN ASYMMETRIC KCI (1.0 M cis, 0.1 M trans) FOR CHANNEL 1 (25 mM PUTATIVE TETRAMER, cis) IN EYPC BLMs AT pH 4.0 (FILLED CIRCLES, 10 mM NaOAc) AND pH 6.0 (OPEN SQUARES, 10 mM MES) WITH LINEAR CURVE FIT AND REVERSAL POTENTIALS.} \]

High intrinsic $pK_a \approx 12.5$ and reduced electrostatic interactions with delocalized charges of guanidinium cations suggested fully protonated arginine residues at pH = 6.0,\textsuperscript{49-51} whereas nearly neutral histidines (intrinsic $pK_a \approx 6.0$) can be assumed.\textsuperscript{7} Cation selectivity of channel 1 with 32 putative internal RH dyads at pH = 6.0 therefore suggested the presence of at least some divalent counteranions. Electrospray ionization mass spectrometry suggested that inorganic phosphates $P_i$ (i.e., $\text{HPO}_4^{2-}$) play this role (Fig. 2). Fig. 5 shows a representative spectrum of the TFA salt of barrel 1 after denaturation with excess AcOH. Despite the presence of triluoroacetic and acetic

Fig. 2 Binding of inorganic phosphates within barrel 1 is expected to give $1 \supseteq (P_i)_n$ with an anionic, small internal space at high pH and a cationic interior at low pH. Putative complex $1 \supseteq (P_i)_n$ is shown at neutral pH as a simplified axial view in gold (hydrophobic residues), black (rigid-rod and $\beta$-sheet scaffolds), blue (basic residues), purple (cationic residues), and red (counteranions); $\beta$-strands are given as solid (backbone) and dotted lines (hydrogen bonds), see Fig. 1. The number $n$ of internal phosphates and $pK_a$ of internal guanidinium–phosphate complexes are unknown and the presence of counteranions other than $P_i$ cannot be excluded.

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2 Results and discussion

2.1 Multichannel characteristics of p-octiphyenyl $\beta$-barrel 1

The activity of rigid-rod $\beta$-barrel 1 with internal arginine–histidine (RH) dyads in black (planar) lipid bilayers (BLMs) formed by egg yolk phosphatidylcholine (EYPC) was studied by conventional conductance experiments.\textsuperscript{49} Conditions comparable to previous BLM-studies of H- and K-rich p-octiphyenyl $\beta$-barrel ion channels were selected.\textsuperscript{24-25,29-31} After the addition of barrel 1 (25 mM putative tetramer) at $V = +50$ mV, macroscopic (“multichannel”) currents could be observed. Roughly ohmic I–V curves were consistent with formation of “dipole-free” $\beta$-sheets in the symmetric supramolecule. To elucidate anion/cation selectivity of pore 1, a salt gradient between the cis (1 M KCl) and trans chambers (0.1 M KCl) was applied. Fig. 3 shows macroscopic I–V curves obtained under these asymmetric conditions at pH 6.0 (open) and pH 4.0 (closed). The reversal potential $V_r$ exhibited sign inversion from negative at pH 6.0 to positive at pH 4.0. The permeability ratios calculated from $V_r$ changed from $P_{\text{cis}}/P_{\text{trans}} = 0.5$ at pH 6.0 to $P_{\text{cis}}/P_{\text{trans}} = 3.8$ at pH 4.0. Inversion of anion/cation selectivity of channel 1 occurred at pH ≈ 5 (Fig. 4).

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acid, p-octiphenyls together with up to six inorganic phosphates per monomer were detected. This finding was not surprising considering, for example, the binding of inorganic phosphates ($K_D = 1.7$ mM at $V' = +20$ mV) rather than nitrates, sulfates, citrates, and perchlorates by bioengineered β-barrel pores with internal arginines, or the nanomolar affinity of guanidine-based amphiphiles for AMP at the water–interface. The precise number ($n$) of phosphates within the active ion channel $I \supseteq (P_i)$, remains, however, elusive, particularly since the presence of mixed complexes with additional counteranions can not be excluded. The $pK_a$'s of guanidine–$HPO_4^{2-}$ complexes within $I \supseteq (P_i)$ remain similarly obscure. However, reduction of a $pK_a = 6.8$ of $HPO_4^{2-}$ with tetramethylammonium counterions in water to a $pK_a = 4.8$ in more hydrophobic environments has been reported. It is thus reasonable to assume that neutralization of internal guanidinium–phosphate complexes together with protonation of internal imidazoles contribute to conversion of cation to anion selectivity at pH = 5.

### 2.2 Multichannel characteristics of p-octiphenyl β-barrel 2

Activity and ion selectivity of rigid-rod β-barrel 2 in planar bilayers were determined in a similar way using conditions that assured comparability with previous studies on Mg$^{2+}$-binding in spherical bilayers. The most important difference from the conditions employed for 1 (and other rigid-rod β-barrels) was an approximately 10-fold decrease in ionic strength, and thus increased resistivity of the recording solution (see experimental section). Ohmic channels with a cation selectivity of $P_k/P_Cl = 5.6$ were found at pH = 6.0 (Fig. 6). High cation selectivity in planar bilayers was indicative of the presence of dissociable internal aspartates, and was consistent with previous results in spherical bilayers and characteristics reported for biological “α-barrel” ion channels with chemically introduced internal glutamates.

The presence of Mg$^{2+}$ reduced the cation selectivity of channel 2 by 75% from $P_k/P_Cl = 5.6$ to $P_k/P_Cl = 1.4$. This almost negligible cation selectivity was consistent with our previous findings using spherical bilayers, showing permeability of metallopoire 2 $\supseteq$ Mg$^{2+}$, for anions as large as 6-carboxyfluorescein and binding of various anionic guests to the internal Mg$^{2+}$-aspartate complexes. The overall $K_D = 8.0$ mM approximated for Mg$^{2+}$-aspartate complexes in planar bilayers was in good agreement with $K_D = 2.9$ mM and $K_D = 5.3$ mM found in spherical bilayers using two different assays. The quantitative compatibility of the results on ion selectivity and Mg$^{2+}$ binding obtained in planar and spherical bilayers must be highlighted.

### 2.3 Single-channel characteristics of p-octiphenyl β-barrel 1

Single-channel characteristics of rigid-rod β-barrel 1 were determined under conditions nearly identical to those of the multichannel experiments described above. Lifetimes of seconds were observed for single anion channels at pH = 4.6 (Fig. 7a) as well as for single cation channels at pH = 6.0 (Fig. 7b). Higher stability of the single-channel conductance with mostly neutral histidines at pH = 6.0 compared to partially protonated histidines at pH = 4.6 was noted consistently and attributed to an increase in internal charge repulsion. The superb stability of single anion and particularly cation channels formed by β-barrel 1 was similar to that of contracted β-barrels, i.e., barrels with β-sheets formed by tripeptides rather than pentapeptides. The stability of channel 1 was, however, unprecedented for expanded p-octiphenyl β-barrels.

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*Fig. 5* Electro spray ionization mass spectra of disassembled barrel 1 under denaturing conditions (MeOH–MeCN–H$_2$O–AcOH = 50 : 37 : 12 : 1); (*): Low-intensity tails compatible with more complex counteranion combinations.

*Fig. 6* Ion selectivity of channel 2 (20 nM tetramer cis) as a function of the concentration of Mg(0Ac)$_2$ data from macroscopic $I$–$V$ curves in a KCl gradient (0.1 M cis, 0.05 M trans) in EYPC BLMs at pH 6.0 (5 mM MES) using GHK Eqn. (1).

*Fig. 7* Single-channel recordings for channel 1 in EYPC BLMs (1.0 M KCl, symmetric) at (a) pH 4.6 (10 mM NaOAc) and (b) pH 6.0 (10 mM MES); filtered at 20 Hz.
Table 1  Summary of ion channel characteristics of barrels 1 and 2

| pH  | Mg<sup>2+</sup> | P<sub>c</sub>/P<sub>K</sub> | ω/μs<sup>s</sup> | g/nS<sup>s</sup> | d/Å<sup>s</sup>
<table>
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<td>6.0</td>
<td>0.48</td>
<td>&gt;2000&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.30</td>
<td>3.3</td>
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<td>3.75&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>3.3</td>
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<tr>
<td>6.0</td>
<td>0.18</td>
<td>&lt;0.5&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;1&lt;/sup&gt;</td>
<td>10&lt;sup&gt;0&lt;/sup&gt;</td>
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<td>0.70</td>
<td>12</td>
<td>0.26</td>
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*Compositions, see Fig. 1 for structures. <sup>1</sup> = 0 (−) or 50 mM (+) Mg(OAc)<sub>2</sub>. <sup>2</sup> Permeability ratio (calculated from GHK Eqn. (I)). <sup>3</sup> Mean single-channel lifetimes. <sup>4</sup> Single-channel conductances (from I-V curves using Ohm’s law). <sup>5</sup> Inner channel diameter (from g using Hille’s Eqn. (2)). <sup>6</sup> Approximated because of poor detectability of open/close transitions (see Fig. 7b). <sup>7</sup> pH = 4.0 (see Fig. 4). <sup>8</sup> Approximated because of poor detectability of single-channel conductances (see Fig. 8a).

The formation of internal Mg<sup>2+</sup>-aspartate complexes increased the stability of barrel 2 more than 24-times (Fig. 8a versus b). Control experiments with Na<sup>+</sup> in place of Mg<sup>2+</sup> corroborated that this single-channel stabilization indeed originated from specific Mg<sup>2+</sup> binding, i.e. the formation of inclusion complex 2 ⊂ Mg<sup>2+</sup> (Fig. 8c). Different to guanidinium-phosphate ion pairs within barrel 1 ⊂ (P<sub>n</sub>)<sub>Na</sub>, Mg<sup>2+</sup>-carboxylates complexes within barrel 2 ⊂ Mg<sup>2+</sup> did not substantially reduce the single-channel conductance. This finding can be attributed to either the small size or some maintained mobility of internal magnesium cations.

 Charge repulsion between dissociable internal carboxylates has been shown to result in pore destabilization and pore expansion using biological “a-barrels” with chemically introduced internal glutamates. Similar conclusions were drawn from the pH profiles of barrel 2<sup>47</sup> and synthetic “a-barrels” with internal glutamates<sup>48-49</sup> in spherical bilayers. The same explanation can be used to rationalize instability of cation selective channels formed by 2 (high charge repulsion) and stability of nearly non-selective ion channels 2 ⊂ Mg<sup>2+</sup> (low charge repulsion).

2.5 Molecular recognition within p-octiphenyl ion channel 1

The presence of internal immobilized phosphates suggested that molecular recognition of “large” organic anions within the “small” pore 1 ⊂ (P<sub>n</sub>)<sub>Na</sub> occurs by internal ion exchange. To verify this implication from spherical bilayers<sup>4</sup> in planar bilayer conductance experiments, multichannel dose response curves were recorded for 8-hydroxypropene-1,3,6-trisulfonate (HPTS), a known blocker of p-octiphenyl β-barrels with internal histidines.<sup>7,24,25</sup> At pH = 4.6, increasing concentrations of HPTS (added trans) reduced the macroscopic current flowing through channel 1 (added cis) with an apparent K<sub>i</sub> = 30 µM at V = +50 mV (Fig. 9, filled circles). Binding of anions within anion selective channel 1 ⊂ (P<sub>n</sub>)<sub>Na</sub> at pH = 4.6 was consistent with previous studies on p-octiphenyl β-barrels with internal histidines only.<sup>54</sup> Binding of “large” anions within “small” channel 1 ⊂ (P<sub>n</sub>)<sub>Na</sub> confirmed that internal anion exchange from 1 ⊂ (P<sub>n</sub>)<sub>Na</sub> to 1 ⊂ (HPTS)<sub>Na</sub> (P<sub>n</sub>)<sub>Na</sub> is possible. Preliminary results indicate that the esterolytic activity based on molecular recognition of 8-R-pyrene-1,3,6-trisulfonates within p-octiphenyl β-barrels<sup>24-26</sup> is maintained.<sup>68</sup>

An average single-channel conductance of g = 0.26 nS corresponded, according to the Hille model,<sup>20,26,28</sup> to an inner diameter d = 10 Å for Mg<sup>2+</sup>-free channel 2. Considering the underestimates expected for small to medium sized pores,<sup>20,26,28</sup> d = 10 Å corresponded well with the inner diameters expected from molecular models<sup>44</sup> and previous results from spherical bilayers.<sup>42</sup> The inner diameter estimated for barrel 2 was 3-times (or 7 Å) longer than that of barrel 1 ⊂ (P<sub>n</sub>)<sub>Na</sub> (Table 1). In contrast to the stable, small channel 1 ⊂ (P<sub>n</sub>)<sub>Na</sub>, the interior of Mg<sup>2+</sup>-free channel 2 was thus not covered (and stabilized) by immobilized counterions.

Fig. 8  Single-channel recordings for channel 2 in EYPC BLMs (0.1 M KCl, symmetric) at pH 6.0 (5 mM MES) in the absence (a) and presence of (b) 50 mM Mg(OAc)<sub>2</sub> and (c) 100 mM NaOAc; filtered at 1 kHz.

Fig. 9  Relative multichannel currents of channel 1 (80 nM tetramer cis) as a function of HPTS concentration in the trans chamber at pH = 4.6 (filled circles; 10 mM NaOAc, I<sub>max</sub> = 135 pA) and pH = 6.0 (open circles; 10 mM MES, I<sub>max</sub> = 210 pA) with V = +50 mV.

As expected from the ion selectivity, HPTS had little influence on the activity of channel 1 at pH = 6.0 (Fig. 9, open circles). Inversion from anion to cation selectivity of channel 1 ⊂ (P<sub>n</sub>)<sub>Na</sub>, at pH = 5, therefore, significantly lowered blockage activity of anion HPTS. This result suggested that molecular recognition within multifunctional pores can be regulated by the selectivity of molecular translocation across the same pore. However, it must be emphasized that alternative explanations...
should never be excluded with such complex systems as the present one. Interestingly, HPTS blockage at pH = 5.0, i.e., near selectivity inversion of channel 1 \( \equiv (P)_{1} \), was not detectable in a reproducible manner. We also failed to observe reproducible HPTS-binding at the single-channel level, presumably because of too small and/or too complex effects of internal ion exchange on the single-channel "signature".

3 Summary and conclusions

Rigid-rod \( \beta \)-barrels with internal arginine--histidine dyads form cation selective \( (P_{K}/P_{Cl} = 2.1) \), small \( (d = 3.3 \AA) \), and stable \( (r > 20 \text{ seconds}) \) ion channels. Addition of "protons" results in inversion of anion/cation selectivity \( (P_{K}/P_{Cl} = 3.8) \) and the addition of an anionic guest results in partial blockage of anion selectivity \( (K_d \approx 30 \mu M) \), but not cation, selective channels. Barrels with internal aspartates form cation selective \( (P_{K}/P_{Cl} = 5.6) \), large \( (d = 10 \AA) \), and unstable \( (r < 0.5 \text{ ms}) \) ion channels. Addition of magnesium cations \( (K_d = 8 \text{ mM}) \) nearly demolishes cation selectivity \( (P_{K}/P_{Cl} = 1.4) \) and increases stability \( (r = 12 \text{ ms}) \).

Most findings for barrel-stave pores with internal aspartates corroborate previous trends with \( p \)-octiphenyl \( \beta \)-barrel ion channels that do not require further emphasis (e.g., increase of channel diameter with \( \beta \)-sheet length, decrease of channel stability with \( \beta \)-sheet length, barrel stabilization by internal templates). Less obvious exceptions include the variable cation selectivity with \( Mg^{2+} \). This effect was correctly predicted by studies in spherical bilayers and could be understood as originating from immobilization of \( Mg^{2+} \), a divalent counterion, but not univalent counterions such as \( K^+ \) by internal aspartates.

The complementary situation, that is, immobilization of inorganic phosphates, a divalent counterion, by internal arginines but not histidines was predicted to create some of the less usual characteristics of rigid-rod \( \beta \)-barrels with internal arginine--histidine dyads. Seemingly counterintuitive phenomena like a polycationic synthetic pore exhibiting cation selectivity or a "small" ion channel host recognizing "large" anionic guests became accessible using specific counterion immobilization and ion exchange, respectively. The result is the first expanded \( p \)-octiphenyl \( \beta \)-barrel that forms stable ion channels (contracted \( p \)-octiphenyl \( \beta \)-barrel ion channels are "always" stable). This superb stability, coupled with the possibility of controlling molecular recognition and transformation by inversion of cation/anion selectivity of molecular translocation, is promising for practical applications of multifunctional synthetic pores as sensors and catalysts.

4 Experimental section

4.1 Materials

EYPC was purchased from Northern Lipids Inc. (Vancouver, British Columbia, Canada), or from Avanti Polar Lipids (Alabaster, AL), HPTS from Molecular Probes (Eugene, OR), detergents, all salts and buffers of the best grade available from Sigma-Aldrich Corp (St. Louis, MO), and used as received. ES-FS-MS were recorded on a Finnigan MAT SSQ 7000.

4.2 \( 1\),\( 2\),\( 3\),\( 4\),\( 5\),\( 6\),\( 7\),\( 8\)-Octakis(Gla-Leu-Arg-Leu-His-Leu-NH\(_{2}\))\( p \)-octiphenyl 1m

Octiphenyl 1m was synthesized and purified as described in reference 3. Self-assembly of barrel 1 in spherical bilayers is described in reference 3. All indicated concentrations refer to a tetramer.

4.3 \( 1\),\( 2\),\( 3\),\( 4\),\( 5\),\( 6\),\( 7\),\( 8\)-Octakis(Gla-Leu-Asp-Leu-Asp-Leu-NH\(_{2}\))\( p \)-octiphenyl 2m

Octiphenyl 2m was synthesized, purified, and characterized as described in reference 4. Self-assembly of barrel 2 in spherical bilayers is described in references 4–7. All indicated concentrations refer to a tetramer.

4.4 BLM recordings

BLMs were formed by painting a solution of EYPC in \( n \)-decane (42 mg ml\(^{-1}\)) on an aperture \( (d = 150 \mu m) \), pretreated with the same solution) in a delrin cuvette separating two chambers containing 1 ml each of buffer and an agar bridge connection (1 M KCl) to Ag/AgCl electrodes (Warner Instrument Corp., Hamden, CT). Currents were recorded at different holding potentials (trans at ground) in a house-made Faraday cage, amplified (BC-525c, Warner Instrument Corp.), low-pass filtered with an 8-pole Bessel filter at 1–5 kHz (LPF-8, Warner Instrument Corp.), A-D converted (Digipack 1200-2, Axon Instruments, Union City, CA), and sampled at 10 kHz by computer (pClamp 8.0, Axon Instruments). If necessary, the data were further filtered using pClamp 8.0 or Origin (version 7, OriginLab). All the conductance measurements were performed at room temperature (22 ± 1 °C). Overall reliability of the employed system was regularly assessed using alamethicin. Conductances of multilevel channels and voltage dependence were fully reproducible and as in the literature.

Conditions for single- and multichannel experiments with identical cis and trans chambers for I: (a) 1.3 M KCl, 5 mM MES, pH = 5.0, \( K^+ = 6.0 \text{ mM}) \) nearly demolishes cation selectivity \( (P_{K}/P_{Cl} = 1.4) \) and increases stability \( (r = 12 \text{ ms}) \). Conditions for single- and multichannel experiments with different cis and trans chambers for I: 1.0 M KCl in cis, 1.0 M KCl in trans, 10 mM NaOAc (pH < 5.6) or 10 mM MES (pH > 5.6) in both chambers. Conditions for single- and multichannel experiments with identical cis and trans chambers for 2: 0.1 M KCl, 50 mM Mg(OAc)\(_{2}\), 100 mM NaOAc, 5 mM MES, pH = 6.0. Tetramer 2 (20 nM) was added to cis at \( V = +50 \text{ mV} \), anion HPTS (0–400 \mu M) was added to trans at \( V = -50 \text{ mV} \). Resistivity of the recording solution (a) was \( \rho = 7.716 \Omega \text{cm} \). Control experiments indicated decreasing probability of observing stable single channels with decreasing concentration of KCl and poor reproducibility of results around inversion of selectivity at pH 5.1.

Conditions for multichannel ion selectivity experiments with different cis and trans chambers for 1: 1.0 M KCl, 50 mM Mg(OAc)\(_{2}\), 100 mM NaOAc, 5 mM MES, pH = 5.6 in both chambers. Conditions for single- and multichannel experiments with identical cis and trans chambers for 2: 0.1 M KCl, 50 mM Mg(OAc)\(_{2}\), 100 mM NaOAc, 5 mM MES, pH = 5.6. Cation 2 (20 nM) was added to cis at \( V = +50 \text{ mV} \). Besides the dominant g = 0.26 nS with 50 mM Mg(OAc)\(_{2}\), a low conductance level g = 0.16 nS was occasionally observed. Resistivity of the recording solution with 50 mM Mg(OAc)\(_{2}\) was \( \rho = 56.6 \text{ kΩ} \). With 1.0 M KCl, the stabilization of single channels by Mg\(^{2+}\) was not observed. Conditions for multichannel ion selectivity experiments with different cis and trans chambers: 0.1 M KCl in cis, 0.05 M KCl in trans, 0–50 mM Mg(OAc)\(_{2}\), 5 mM MES and pH = 6.0 in both chambers.

4.5 Data analysis

Permeability ratios were calculated from experimentally determined reversal potentials \( V_r \) (Fig. 3) using an equation derived from Goldman–Hodgkin–Katz (GHK) equation

\[
P_{Cl}/P_{K} = \frac{[a_{Kc} - a_{Kc} \exp(-V_r/FRT)]}{[a_{Cl} \exp(-V_r/FRT) - a_{Cl}]} \quad (1)
\]

where \( a_{Kc} \) and \( a_{Kc} \) are the activities of K\(^+\) in the cis and trans chambers respectively, \( a_{Cl} \) and \( a_{Cl} \) the same for Cl\(^-\) and plotted as a function of pH (Fig. 4) or Mg\(^{2+}\) concentration (Fig. 6).

Inner channel diameters \( d \) were approximated with Hille's model

\[
1/g = 1/\pi(d/2)^2 + \rho d \quad (2)
\]

which assumes ion channels are electrolyte filled cylinders with ion channel conductance \( g \) (measured), ion channel length \( l \) (assumed as identical with length of the \( p \)-octiphenyl staves =
34 Å and the resistivity of the recording solution $\rho$ (measured). Note, as this model assumes unhindered movement of electrolyte within the pore, the estimated diameter is often smaller than reality.

Dissociation constants were calculated using

$$\log \left( \frac{Y(1 - Y)}{n} \right) = n \log K_{\text{diss}}$$

where $Y$ is the fractional saturation $\left( \frac{[I]_{\text{max}}}{[I]} \right) / \left( \frac{[I]_{\text{max}}}{[I]_{\text{min}}} \right)$, $n$ the Hill coefficient, and $K_{\text{diss}}$ the dissociation constant.

### 4.6 Abbreviations and symbols used

AMP, adenosine monophosphate; BLM, black (planar) lipid membrane; EYPC, egg yolk phosphatidylcholine; HPTS, 8-hydroxyxypyrane-1,3,6-trisulfonic acid, trisodium salt; LUV, large unilamellar vesicle; MES, 2-morpholinoethanesulfonic acid monohydrate; SIV, small unilamellar vesicle.

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### References

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