5- and 4′-Hydroxylated flavonoids affect voltage gating of single alpha-hemolysin pore

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A B S T R A C T

Molecular mechanisms of the influence of flavonoids on the voltage gating of a single alpha-hemolysin channel in planar lipid membranes are studied. It is shown that the addition of flavonoids hydroxylated in position 5 of the A-ring and in position 4′ of the B-ring into bilayer bathing solution shifts the voltage dependence of channel switching from high- to low-conductance states to voltages nearer zero. It is concluded that the effect is likely to be attributed to a specific interaction of at least three flavonoid molecules with the voltage sensor of an alpha-hemolysin pore. Possible flavonoid binding sites and identification of amino acid residues included into the voltage sensor domain of the alpha-hemolysin channel are discussed.

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1. Introduction

Staphylococcus aureus α-hemolysin (α-HL) is a cytolysic exotoxin, which is secreted as a single water-soluble polypeptide containing 293-residues with a molecular weight of 33.2 kDa [1,2]. Alpha-HL is lytic to a variety of cell types including human monocytes, lymphocytes, erythrocytes, platelets, and endothelial cells [3,4]. In the plasma membrane seven toxin protomers assemble to form a 232-kDa mushroom-shaped heptamer comprising three distinct domains [5,6]: cap, rim, and stem. The cap and rim domains of the α-HL heptamer are located at the surface of the membrane, while the stem domain serves as a transmembrane channel. Crystallographic data and data obtained from polymer exclusion testified to 10-nm-long pore with cis and trans entrances of about 3 and 2.5 nm in diameter, respectively, and two constrictions, near cis entrance and between cap and stem domains of about 2 and 1.5 nm, respectively. Widest bubble-like cavity of more than 4 nm in diameter in its center region is flanked by these constrictions [5,7]. Different organic molecules could be detected by individual events of blockade of single α-HL-pore [8–14].

At high transmembrane potentials \(V^+ \geq \pm 100 \text{ mV}\) α-HL-pore fluctuates between high- (HCS) and low-conductance states (LCS) [15–17]. Korchev et al. [16] showed that fluctuations between these conductance states were not accompanied by large alterations in pore size (37%) despite more than 10-fold difference in the conductance between the states. In the cited study two possible models of the channel structural transformations during transition between HCS and LCS were discussed, namely a “local” gating related to a movement of a single group and the global rearrangement of channel walls. Relatively small changes in the size of the channel favored the first model. However, the authors showed that the osmotic stress caused by the presence of nonpenetrating polymers in the membrane bathing solution shifts the voltage-dependence of the channel switching to voltage close to zero. These results were consistent with the second model. Later, Krasilnikov and Bezrukov [18] showed that the channel conductance reduction in the presence of large polymers is not caused by the channel elastic deformation under the osmotic stress. Song et al. [5] discussed the transition in terms of varying degrees of conformational rearrangement in a response to the applied transmembrane potential and subsequent partial or complete closing of the pore. The voltage-dependent transition of α-HL-channel between HCS and LCS is influenced by divalent cations or by protons [19–21]. Song et al. [5] proposed that possible mechanisms by which di- and trivalent cations affect partial or complete reduction in the pore conductance may involve a steric blockage of the channel by an ion binding to Glu111 at the stem top or include an ion binding to Asp127 and Asp128, resulting in the collapse of the barrel at the glycine-rich stem base. Protonation of

Abbreviations: α-HL, alpha-hemolysin; HCS, high-conductance state; LCS, low-conductance state; PC, 1,2-dioleoyl-sn-glycero-3-phosphocholine

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Glut111-residues may lead to a disruption of Glut111–Lys347 ion pairs and to an enlargement of the pore neck by the rearrangement of amino-acid side chains [5]. In the recent study Mohammad and Movileanu [17] found that charge-reversal α-tiHL mutations, substitution of Lys by Asp in 147 and 131 positions of each protomers, that disrupt ion–pair interaction on the cis end of the β barrel and on the solvent exposed trans end of the β barrel, respectively, affect the gating activity of channel.

Flavonoids are hydroxylated phenolic substances from plants which impart flavor and color to fruits and vegetables [22]. These plant pigments show antioxidant, hepatoprotective, cardioprotective, neuroprotective, antiinflammatory, antitumoral, and antimicrobial properties [23-27]. Flavonoids may participate in a broad range of events related to cell signaling [28,29]. The influence of flavonoids on cell metabolism is well documented while a concrete mechanism of their functioning has so far remained unclear. It is known that flavonoids are membrane-active agents and some of them are able to reduce the membrane dipole potential. For example, alteration of the membrane dipole potential due to the phloretin or phloridzin insertion into a lipid bilayer significantly modulates the channel forming activity and single channel properties of such membrane-active exogenous compounds, as syringomycin E [30], surfactin [31], gramicidin [32,33], and alamethicin [34].

Our study focuses on the voltage-dependent transition between the conductance states of αHL-channel and the ability of some flavonoids to modify αHL-channel gating. Basing on the results of electrophysiological experiments, we proposed molecular mechanisms of specific interactions of flavonoids hydroxylated in position 5 of the A-ring and position 4′ of the B-ring with a single αHL-pore. Possible binding sites and the localization of the channel voltage sensor are discussed.

2. Material and methods

2.1. Material

All chemicals were of reagent grade. Synthetic 1,2-diphtanoyl-sn-glycerol-3-phosphocholine (PC) was obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxy-phenyl)-1-propanone) (purity ≥99%), phloridzin (1-[2-[β-D-glucopyranosyl]-4,6-dihydroxyphenyl]-3-[4-hydroxyphenyl]-1-propanone) (purity ≥99%), genistein (5,7-Dihydroxy-3-(4-hydroxyphenyl)-4-H-1-benzopyran-4-one) (purity ≥98%), genistin (Genistein-7-O-β-D-gluco-pyanoside) (purity ≥95%), daidzein (7-Hydroxy-3-(4-hydroxyphenyl)-4-H-1-benzopyran-4-one) (purity ≥98%), 2′,4′,6′-trihydroxy-acetophene monohydrate (purity ≥98%), 6-ketocholostanol, quercetin (2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4-H-1-benzopyran-4-one) (purity ≥98%), biochanin A (5,7-Dihydroxy-4′-methoxyisoflavone), (±) taxifolin hydrate ((2R,3R)-3,3′,4′,5′,7-Pentahydroxyflavonane) (purity ≥90%), myricetin (3,3′,4′,5′,7-Pentahydroxyflavone) (purity ≥99%), myricetin (3,3′,4′,5′,7-Pentahydroxyflavone) (purity ≥99%), rose Bengal (4,5,6,7-Tetrachloro-2′,4′,5′,7′-tetraddifluorescein disodium salt) (purity ≥95%) were purchased from Sigma Chemical (St. Louis, MO) and RH 421 (N-(4-sulfobutyl)-4-(4-(4-(4-dipentylamino) phenyl)butadienyl) pyridinium, inner salt) (purity ≥95%) from Molecular Probes (Eugene, OR). Water was distilled twice and deionized. 0.1 or 1 KCl solutions were buffered with 5 mM Heps, pH 7.5. Alpha-hemolysin (αHL) was purchased from Sigma Chemical (St. Louis, MO).

2.2. Bilayer setup, recording system and mode of calculations

Virtually solvent-free planar lipid bilayers were prepared according to a monolayer-opposition technique [35] on a 50-μm-diameter aperture in the 10-μm thick Teflon film separating two (cis and trans) compartments of the Teflon chamber. The aperture was pretreated with hexadecane. Lipid bilayers were made from PC. After the membrane was completely formed and stabilized αHL from stock solution (50 μg/ml 8 M Urea) was added, to one compartment only (cis side) to obtain a final concentration ranging from 10 to 40 nM. Ag/AgCl electrodes with agarose/2 M KCl bridges were used to apply the transmembrane voltage (V) and to measure the transmembrane current. “Positive voltage” refers to a case in which the cis-side compartment is positive with respect to the trans-side. All experiments were performed at room temperature.

The two-side addition of dipole modifiers (phloretin, phloridzin, Rose Bengal, RH 421) and phloretin analogs, plant flavonoids, daidzein, genistein, genistin, 2′,4′,6′-trihydroxy-acetophene, biochanin A, quercetin, taxifolin, myricetin, and catechin to the membrane-bathing solution was used to modulate αHL activity. 6-ketocholesterol was added to the membrane-forming solution to increase the dipole potential of PC-bilayers. Table 1 summarizes the literature data about a structure of modifiers and a value of membrane dipole potential in the presence of dipole modifiers and phloretin analogs.

Our precautions against contamination in the aqueous and membrane forming solutions and αHL species included ascertaining that: (i) no channels formed in the absence of αHL in a large voltage range from −200 to 200 mV; (ii) no current fluctuations between channel substrates at V(≤)|100| mV were observed in the absence of flavonoids.

Measurements of transmembrane currents were carried out using Axopatch 200B amplifier (Axon Instruments) in the voltage clamp mode. Data were digitized by Digidata 1440A and analyzed using pClamp 10 (Axon Instruments) and Origin 8.0 (Origin Lab).

Conductance transition and lifetime histograms were constructed for tested voltages. Relative frequency n/N was set as the histogram ordinate. The total number of events used for the conductance fluctuation analysis was 50±150. Conductance transition histogram peaks were fitted with the normal density function. For the dwell time the histogram N value was equal to 50±1100 and the distribution was fitted with an exponential density function. The distribution hypothesis was verified by a χ²-minimization (P<0.05).

Transport numbers of K⁺ (t⁺) and Cl⁻ (t = 1–r⁻) were estimated using the general expression [46]: Veff = (RT/F)ln(γz1/γz2), where Veff is the reversal potential; R, T, and F have their usual meanings; γz1 and γz2 indicate, respectively, activity coefficients and KCl concentration in the cis and trans compartments (C1 = 0.1 M KCl, C2 = 1 M KCl).

Analysis of the spatial pattern of proteins from PDB was performed using RasMol.

3. Results and discussion

Fig. 1A presents a typical current response to the application of long-lasting voltage pulses to a membrane containing single αHL-channel. It should be underlined that in the control measurements αHL-pore in PC-bilayers bathing in 1 M KCl fluctuates between high and low-conductance states at transmembrane voltage V(≥)|100| mV. Fig. 2A shows current transition histograms at different voltages for control experiments (Fig. 1A). It is seen that at V = 50 mV the channel conductance is uniform (mean conductance corresponding to HCS is about 800 pS), whereas at V = −150 mV two pools with the mean currents of about 1000 pS (corresponding to HCS) and 100 pS (corresponding to LCS) are observed. The small lower pool probably reflects a noncomplete closing of the channel as a result of the high voltage application. Conductance–voltage curves for both channel states are presented in Fig. 3A. Note that the mean conductance of LCS (−110 pS) does not practically depend on the transmembrane voltage in the range of [100]–[150] mV, whereas the conductance of HCS decreases linearly with the voltage increase (GHCS (V→0)~900 pS).

Different forms of the conductance–voltage curves forced us to examine other characteristics of the αHL-conductance states. Fig. 4 demonstrates different cation/anion selectivity of the states. The reversal potential (Veff) for HCS of 400 pS is Veff = −14±2 mV, whereas Veff for LCS ranges from 20 to 35 mV at the conductance

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<table>
<thead>
<tr>
<th>Modifier</th>
<th>Chemical structure*</th>
<th>Sizes, nm**</th>
<th>Concentration, μM§</th>
<th>φd, mV#</th>
<th>Refs</th>
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<td>Phloretin</td>
<td><img src="image" alt="Phloretin structure" /></td>
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<td>5</td>
<td>190 ± 20</td>
<td>[36]</td>
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<td></td>
<td></td>
<td>10</td>
<td>160 ± 20</td>
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<td>15</td>
<td>150 ± 20</td>
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<td>120 ± 20</td>
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<td>40</td>
<td>115 ± 20</td>
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<td></td>
<td>45</td>
<td>110 ± 20</td>
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<td></td>
<td></td>
<td>50</td>
<td>95 ± 20</td>
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<td>a₁ = 1.3 a₂ = 0.5</td>
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<td>250 ± 40</td>
<td>[33,37]</td>
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<td>Genistin</td>
<td><img src="image" alt="Genistin structure" /></td>
<td>a₁ = 1.8 a₂ = 0.6</td>
<td>5 ÷ 90</td>
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<td>Quercetin</td>
<td><img src="image" alt="Quercetin structure" /></td>
<td>a₁ = 1.1 a₂ = 0.6</td>
<td>5 ÷ 120</td>
<td>Ø</td>
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<td>2',4',6'-trihydroxy-acetophenone</td>
<td><img src="image" alt="Triterpenoid structure" /></td>
<td>a₁ = 0.7 a₂ = 0.5</td>
<td>20 ÷ 80</td>
<td>230 ± 40</td>
<td>[37]</td>
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<tr>
<td>Daidzein</td>
<td><img src="image" alt="Daidzein structure" /></td>
<td>a₁ = 1.3 a₂ = 0.4</td>
<td>10 ÷ 50</td>
<td>≤ 200</td>
<td>[37,38]</td>
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<td>Biochanin A</td>
<td><img src="image" alt="Biochanin A structure" /></td>
<td>a₁ = 1.4 a₂ = 0.6</td>
<td>20 ÷ 80</td>
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<td>Taxifolin</td>
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<td>5 ÷ 180</td>
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<td>Catechin</td>
<td><img src="image" alt="Catechin structure" /></td>
<td>a₁ = 1.3 a₂ = 0.7</td>
<td>5 ÷ 120</td>
<td></td>
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</table>

(continued on next page)
range from 10 pS (Fig. 4A) to 100 pS (Fig. 4B). These values of the reversal potentials correspond to $t^+ = 0.70 \pm 0.05$ for HCS and $t^-$ from 0.69 to 0.83 for LCS. Thus, the pronounced anion and cation selectivity was revealed for HCS and LCS, respectively. Similar values of $t^-$ for HCS were obtained by Korchev et al.[15] and Menestrina et al.[19], while the selectivity of LCS has not been investigated so far. A broad distribution of the conductance (Fig. 2) and selectivity (Fig. 4) of LCS might stem from the presence of close conformations of LCS with practically the same energy values. The observed inversion of selectivity during the transition between HCS and LCS should involve some voltage-induced movement of a channel voltage “gate” related to the substitution of positive amino acid residues by negative ones in a selective filter of $\alpha$-HL-pore.

We analyzed the relative contribution of the charges of amino acid residues to the voltage gating of the single $\alpha$-HL-pore. To do this, the voltage sensitivity of $\alpha$-HL-channels was also studied in conditions, at which charges of the residues looking toward the pore interior are not screened by a high electrolyte concentration. It appeared that there were no significant changes in the voltage sensitivity of $\alpha$-HL-pore in diluted (0.1 M) and concentrated (1 M) solutions of KCl (see Table 2). These findings argue against the possibility that charges contribute to the voltage gating of $\alpha$-HL-channel.

The other possibility of the voltage sensitive transition of the $\alpha$-HL-channel to a low-conductance state lies in the contribution of dipoles in the channel gating. This assumption provoked us to investigate the effect of agents modifying the membrane dipole potential on the properties of the single $\alpha$-HL-channel. As it is known, the addition of phloretin, phloridzin or Rose Bengal leads to a reduction of the membrane dipole potential ($\phi_d$), while the addition of 6-ketocholestanol and RH 421 results in an increase of this potential (see Table 1).

We have found that the presence of phloridzin (20 ÷ 80 $\mu$M), Rose Bengal (1.3 $\mu$M) or RH 421 (10 $\mu$M) in the PC-membrane bathing solution does not affect the voltage dependent transition of $\alpha$-HL-channel to LCS (see Table 2). Besides, addition of 6-ketocholestanol (50 mol%) to the membrane forming solution does not also influence toxin pore fluctuations (see Table 2). As the introduction of the aforementioned modifiers corresponds to variations of the membrane dipole potential in the range from 90 to 400 mV (see Table 1), one can

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**Table 1 (continued)**

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Chemical structure*</th>
<th>Sizes, nm**</th>
<th>Concentration, $\mu$M</th>
<th>$\phi_d$, mV#</th>
<th>Refs</th>
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<td>Rose Bengal</td>
<td><img src="https://example.com/rose_bengal.png" alt="ChemSketch" /></td>
<td>$a_1 = 1.1 \ a_2 = 0.9$</td>
<td>1.3</td>
<td>$130 \pm 40$</td>
<td>[39,40]</td>
</tr>
<tr>
<td>RH 421</td>
<td><img src="https://example.com/rh_421.png" alt="ChemSketch" /></td>
<td>$a_1 = 2.3 \ a_2 = 1.2$</td>
<td>10</td>
<td>$350 \pm 40$</td>
<td></td>
</tr>
<tr>
<td>6-ketocholestanol</td>
<td><img src="https://example.com/6-keto.png" alt="ChemSketch" /></td>
<td>$a_1 = 1.6 \ a_2 = 0.6$</td>
<td>50 mol%</td>
<td>$400 \pm 20$</td>
<td>[41]</td>
</tr>
</tbody>
</table>

* = Chemical structure was built using ChemSketch. The color indications: C — light blue, O — red, H — light grey, I — green, Cl — brown, Na — dark grey, S — yellow, N — dark blue.

** = The modifier molecule was optimized using HyperChem 7.0 and approximated by the ellipsoid of rotation with major ($a_1$) and minor ($a_2$) axis.

§ = For details see Material and methods.

# = The estimation of value of dipole potential of PC membrane in the presence of different modifiers was made according to literature data. The value of membrane dipole potential in the absence of any agents was an average taken from Refs. [42-45] ($\phi_d = 250 \pm 40$ mV).

Ø = No data.

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Fig. 1. Voltage-dependent transitions between high and low conductance states of the single $\alpha$-HL-channel in the planar lipid bilayers. The membranes were made from PC and bathed in 1 M KCl 5 mM Hepes pH 7.5. (A) — control; (B) — 20 $\mu$M phloretin was added to the bilayer bathing solution after the pore formation. Voltage pulses from $|25|$ mV to $\geq |150|$ mV are shown at the top of current records. The arrows indicate the moments of transmembrane potential changes.
conclude that $\phi_d$ does not affect the $\alpha$-HL-pore voltage sensitivity. These data indicate that gating particles are not sensitive to a dipole potential drop. One of the most probable explanations of this effect is a complete shielding of the membrane dipole potential in the “stem” interior with water dipoles [47] and polar amino acid groups of the protein, as shown for K-Na-ATPase [48].

![Fig. 2. Histograms of the $\alpha$-HL-induced conductance fluctuations in PC-membranes bathed in 1 M KCl 5 mM Hepes pH 7.5. (A) — control; (B) — 20 $\mu$M phloretin was added to the bilayer bathing solution after the pore formation. The value of the transmembrane voltage is indicated at the top of each histogram. Here $n$ is the number of conductance fluctuations corresponding to a particular current level; $N$ is the total number of fluctuations. The solid curves represent the best-fits assuming a normal distribution. The distribution hypothesis was verified by the $\chi^2$-minimization ($P<0.05$).](2055)

![Fig. 3. Conductance–voltage curves of HCS (■) and LCS (○) of the single $\alpha$-HL-channels. (A) — control; (B) — 20 $\mu$M phloretin was added to the bilayer bathing solution after the pore formation. PC-membranes bathed in 1.0 M KCl 5 mM Hepes pH 7.5.](2055)
Surprisingly, the addition of more than 15 μM of phloretin in the membrane bathing solution after channel formation promotes the channel transition to LCS at \( V \geq 5 \) mV (Figs. 1B, 2B). At the same time, phloretin does not practically affect the conductance of HCS \( (G_{\text{HCS}}(V \to 0) \approx 900 \text{ pS}) \) as compared to that takes place in its absence (Fig. 3)), as well as the conductance of LCS (ranges of \( G_{\text{LCS}} \) from 40 to 170 pS after phloretin addition and 50–150 pS in control). Besides, the mean dwell time of LCS is also practically independent on the phloretin addenda (1.5 ± 0.5 ms in the presence vs. 1.2 ± 0.3 ms in the absence of this dipole modifying agent). The cation/anion selectivity of both conductance states in the presence of phloretin was revealed. The transport numbers are equal to \( t^\pm = 0.68 \) for HCS and \( t^\pm = 0.76 \) for LCS. These values are in a good agreement with those in the absence of phloretin.

### Table 2

| Modifier | Concentration, μM | \( |V|, \text{ mV} \) | \( G_{\text{HCS}}(V \to 0), \text{ pS} \) | \( G_{\text{LCS}}, \text{ pS} \) |
|----------|------------------|----------------|----------------|----------------|
| 0.1 M KCl | –                | 125 ± 25       | 100 ± 15       | 5–40          |
| 1 M KCl  | –                | 100 ± 25       | 890 ± 80       | 50–150        |
| Phloretin | 15               | 5 ± 5          | 910 ± 90       | 50–150        |
| Genistein | 10               | 900 ± 110      | 40–170         |                |
| Quercetin | 20               | 890 ± 90       | 45–150         |                |
| Myricetin | 15               | 890 ± 80       | 50–140         |                |
| Taxifolin | 20               | 900 ± 90       | 50–140         |                |
| Catechin | 15               | 900 ± 110      | 60–120         |                |
| Daidzein | 50               | 125 ± 25       | 910 ± 90       | 50–140        |
| Genistein | 90               | 900 ± 90       | 50–120         |                |
| Flavonoid | 80               | 100 ± 25       | 890 ± 90       | 50–100        |
| Biochanin A | 80             | 100 ± 25       | 900 ± 100      | 50–130        |
| 2′,4′,6′-trihydroxy-acetophenone | 80 | 910 ± 90 | 60–120 |
| Rose Bengal | 1.3              | 125 ± 25       | 900 ± 70       | 50–110        |
| RH 421 | 10               | 100 ± 25       | 910 ± 100      | 50–120        |
| 6-ketocholesterol | 50 mol% | 910 ± 80 | 55–140 |

| α-HL | – | 125 ± 25 | 100 ± 15 | 5–40 |
| 1 M KCl | – | 100 ± 25 | 890 ± 80 | 50–150 |
| Phloretin | 15 | 5 ± 5 | 910 ± 90 | 50–150 |
| Genistein | 10 | 900 ± 110 | 40–170 |
| Quercetin | 20 | 890 ± 90 | 45–150 |
| Myricetin | 15 | 890 ± 80 | 50–140 |
| Taxifolin | 20 | 900 ± 90 | 50–140 |
| Catechin | 15 | 900 ± 110 | 60–120 |
| Daidzein | 50 | 125 ± 25 | 910 ± 90 | 50–140 |
| Genistein | 90 | 900 ± 90 | 50–120 |
| Flavonoid | 80 | 100 ± 25 | 890 ± 90 | 50–100 |
| Biochanin A | 80 | 100 ± 25 | 900 ± 100 | 50–130 |
| 2′,4′,6′-trihydroxy-acetophenone | 80 | 910 ± 90 | 60–120 |
| Rose Bengal | 1.3 | 125 ± 25 | 900 ± 70 | 50–110 |
| RH 421 | 10 | 100 ± 25 | 910 ± 100 | 50–120 |
| 6-ketocholesterol | 50 mol% | 910 ± 80 | 55–140 |

\( \Omega \) – The threshold concentration of flavonoids affecting voltage gating of α-HL-channel (phloretin, genistein, quercetin, myricetin, taxifolin, catechin) is given. The maximum concentration of other modifiers is indicated. For details see Material and methods.

\( \# \) – The minimal values of transmembrane voltage at which transitions of α-HL-channels from HCS to LCS take place.

\( \$ \) – The conductance of HCS at \( V \to 0 \).

\( \Theta \) – The range of conductance of LCS.

Since the reduction of \( G_\Omega \) induced by phloretin could not be the cause of the observed change of the channel voltage sensitivity and the dipole modifying agent does not influence LCS characteristics of α-HL-pore, we conclude that the effect of phloretin is likely to be attributed to its specific interaction with the voltage sensor of the channel. To rationalize the nature of a specific interaction between phloretin and α-HL-channel, we carried out an estimation of a binding stoichiometry. Fig. 5A presents the dependence of the inverse mean dwell time of HCS on the phloretin concentration in logarithmic coordinates. Linear approximation of the growth region gives the number of phloretin molecules interacting with a channel heptamer equal to 3 ± 1. Taking into consideration that the α-HL-channel consists of seven protomers, these results suggest the involvement of two functional groups of the flavonoid molecule in the interaction with protein. The problem is to identify these functional groups. To solve this problem, we studied the effect on α-HL-channel gating of various flavonoids, namely genistein, genistein, quercetin, 2′,4′,6′-trihydroxy-acetophenone, daidzein, biochanin A, taxifolin, myricetin, and catechin (see Table 1). It was established that the presence of genistin, 2′,4′,6′-trihydroxy-acetophenone, daidzein, and biochanin A in the membrane bathing solution does not affect the channel gating. At the same time, such flavonoids as genistein, quercetin, taxifolin, myricetin, and catechin promote the channel transition to the low-conductance state at \( V \geq 5 \) mV to \( 10 \) mV in a manner similar to that of phloretin (see Table 2). Measurements of the lifetime of HCS of the α-HL-channel as a function of the concentrations of the aforementioned flavonoids have shown that the number of such molecules interacting with the channel \((m)\) varies from 3 to 5 for different analogues (see Fig. 5B). These findings are in agreement with the \( m = 3 \pm 1 \) for phloretin (Fig. 5A). Flavonoids affecting voltage gating of α-HL-channel such as phloretin, genistein, quercetin, taxifolin, myricetin, and catechin are hydroxylated in position 5 of the A-ring and position 4′ of the B-ring. Daidzein is not hydroxylated in position 5 of the A-ring. Biochanin A is methylated in position 4′ of the B-ring (see Table 1). Both compounds do not influence the activity of α-HL-channel. So basing on a comparison between the chemical structures of the aforementioned agents and their effects on α-HL-channel, we propose that the hydroxyl groups in position 5 of the A-ring and position 4′ of the B-ring play a crucial role in the interaction between flavonoid molecules and the voltage sensor of the α-HL-channel. As the smallest analog, 2′,4′,6′-trihydroxy-acetophenone, and the largest ones,
phloridzin and genistin (see Table 1), do not affect the voltage gating of the α-HL-channel, the size of a flavonoid molecule seems to be also important. As flavonoids, affecting voltage gating of α-HL-channel, are also hydroxylated in position 7 of the A-ring, we can not exclude a possibility of participation of this hydroxyl group in the interaction of flavonoids with α-HL-channel.

The 5- and 4'-hydroxylated flavonoid-induced α-HL-channel gating at low transmembrane voltages resembles the transient current blockades of α-HL-pore by α-helical peptides [9], heparins [13], or PEGs [11]. However, there are significant differences: (i) The gating caused by high voltages, low pH [49], charge-reversal mutation [17], and phloretin (Fig. 2B) exhibits the similar wide-range conductance distributions of LCS (in percentages of HCS). Furthermore the characteristics of LCS are independent on the flavonoid addenda (see earlier discussion) while the conductance and lifetime of the blocked state in the presence of various blockers [9,13] differ from the conductance and lifetime of LCS; (ii) In favor of gating vs. current blockade may be also evidenced by the binding stoichiometry. The number of flavonoid molecules interacting with single α-HL-pore is equal to 3 ± 5 (Fig. 5). In the case of blockage it usually observed monomolecular reaction [8,9,11,13]; (iii) The agents reducing the channel conductance by blocking the ion conducting pathway in the pore should bind with the narrowest constriction of the pore or a new constriction should be formed due to blocker interaction with the channel. In both cases the agent should alter the selectivity of the pores. Introduction of phloretin does not alter the predominant cation selectivity of LCS (see earlier discussion), which favors the gating vs. blocking.

We estimated the mean distance between 5-OH and 4'-OH-groups of the flavonoids complexed with different proteins as 8.3 ± 0.5Å [50], PDB ID: 2UXH51, PDB ID: 3KGJ3, PDB ID: 1X7R1, 1X7J52, PDB ID: 2QA854, PDB ID: 1QKM55, PDB ID: 3LYO56, PDB ID: 2J1257, PDB ID: 1G55, PDB ID: 3M53, PDB ID: 3BPT58, PDB ID: 2HCK59, PDB ID: 3HBF60, PDB ID: 1E9061, PDB ID: 20631]. By summing this value with the length of two hydrogen bonds, the distance between two amino acid residues, which can bind one flavonoid molecule, might be about 11Å. According to the literature data (see Introduction) the voltage induced transition between the conductance states of the α-HL-channel might involve conformational rearrangements at the glycinereich stem base and/or at the narrowest constriction in the pore. Using the structure of the α-HL-channel [5, PDB ID: 7AHL], we examined the distance between the polar uncharged amino acid residues (Asn, Gin, Ser, Tyr, Thr) in the same positions of neighboring protoners in the vicinity of the aforementioned regions (106–116, 142–152, 122–133) in order to find a plausible binding sites. The most appropriate candidate is Thr129 because of the distance between two Thr129 of neighboring protoners is about 11–12Å. Basing on this consideration, we assumed that seven Thr in the 129-position of each protoner belong to the voltage sensor of the α-HL-pore and one flavonoid molecule interacts with two Thr129 of neighboring protoners. An alternative explanation can be proposed. The 5-OH and 4'-OH groups of a flavonoid molecule might bind different amino acid residues of one protoner. One of these residues or both might be involved in the voltage gating of the α-HL-pore. In that case a large dipole moment of phloretin (5.6 D) [38] may change the total dipole moment of the channel voltage sensor. This brings about a shift of the transmembrane potential, which results in the transition of the channel to LCS. Within this mechanism one can suggest, taking into account the obtained data concerning different analogs of phloretin, that the voltage-induced conformational rearrangements of at least three of seven protoners are sufficient to switch the channel to the low-conductance state.

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