Furosemide Blocks the Anion Channel Caused by Staphylococcus Aureus α-Hemolysin

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RESUMO

Investigar o efeito de furosemida no canal α-hemolisina (α-HL) de Staphylococcus aureus em bicamadas lipídicas planares por caracterização eletrofisiológica e estudos de docking molecular. As membranas planares de bicamada lipídica foram preparadas e α-HL (0,07 mg/mL) foi adicionada à solução padrão no compartimento cis da câmara experimental. Todos os experimentos foram realizados em temperatura ambiente usando um amplificador Axopatch 200A no modo voltage clamp. Em pH 7,5, os canais α-HL estavam geralmente em uma alta condutância ~ 4 nS e raramente mudam para estados de baixa condutância. Após a incorporação do canal iônico na membrana bicamada, a furosemida também foi adicionada à solução padrão no compartimento cis. Para os estudos de docking, as coordenadas atômicas para o canal heptamérico α-HL foram recuperadas do PDB ID (7AHL) e a estrutura de furosemida foi obtida do PubChem, suas coordenadas foram elaboradas e minimizadas com o software Avogadro. Os experimentos de docking molecular foram realizados usando o Dockthor online. A furosemida inibiu (P<0,05) a condutância do canal α-HL de maneira voltagem-dependente. Foram avaliadas as duas melhores soluções de docking e o canal α-HL, observou-se que o modo de conexão com maior afinidade de interação possui maior número de ligações de hidrogênio. Os resíduos de ligação foram o 113 e o 147, que formam os remanescentes de constrição do canal α-HL. Em conclusão, a furosemida bloqueia as correntes iônicas na constrição do canal causado pela α-hemolisina de Staphylococcus aureus.

Palavras-chave: Furosemida; Staphylococcus aureus; Canal iônico; Fator de virulência; Agente Antimicrobiano.

ABSTRACT

To investigate the effect of furosemide on Staphylococcus aureus α-hemolysin (α-HL) channel in planar lipid bilayers by electrophysiological characterization and molecular docking studies. Planar lipid bilayer membranes were prepared and α-HL (0,07 mg/mL) was added to the standard solution in cis compartment of the experimental chamber. All experiments were performed at room temperature using an Axopatch 200A amplifier in the voltage clamp mode. At pH 7.5, α-HL channels were usually in a high conductance ~4 nS and rarely switch to low conductance states. After the ion channel was incorporated in bilayer membrane, the furosemide was also added to the standard solution to the cis compartment. To docking studies, atomics coordinates for the α-HL heptameric channel was retrieved from PDB ID (7AHL) and the structure of furosemide was removed from the PubChem, their coordinates were built and minimized with Avogadro software. The molecular docking experiments were performed using the Dockthor online. The furosemide inhibited (P<0.05) conductance α-HL channel and it was a voltage-dependent manner. The two best docking solutions and the α-HL channel were evaluated, it was observed the connection mode with the highest affinity of interaction has a greater number of hydrogen bonding. The residues were 113 and 147.
that form the remainders of the constriction α-HL channel. In conclusion, furosemide blocks ion currents in the constriction of channel caused by *Staphylococcus aureus* α-hemolysin.

**Keywords:** Furosemide; *Staphylococcus aureus*; Ion channel; Virulence factors; Anti-bacterial agent.

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**INTRODUCTION**

The rational design of drugs, in the last decade, has been pointed out by some authors as the most effective to reach incremental innovations with new indications of use for chemical entities already known (new target) and has been consolidating itself as a model to accelerate the times of drug research and development. Considering the *Staphylococcus aureus* α-hemolysin (α-HL) channel importance in pathogenesis, it is regarded as a virulence factor playing an role in infection and multidrug resistant strains, the search for chemical entities already known new target antibiotics, targeting different bacterial machinery, has been emerged. Previous studies have evaluated the activity of several compounds to inhibit α-HL action, by hindering its membrane assembly or by direct blocking α-HL channel. In this context, furosemide bases known to possess a wide range of pharmacological properties, a loop diuretic that induce its effect by competing with chloride to bind to the Na-K-2Cl (NKCC2) cotransporter inhibiting the reabsorption of sodium and chloride. Additionally, the α- hemolysin (α-HL) are exotoxins that create lytic pores in the host cell membrane and have role important for the development of invasive infections. α-HL is a monomer secreted during *Staphylococcus aureus* exponential growth and oligomerizes into the host membranes as an heptameric transmembrane pore, causing osmotic cytolysis. Moreover, molecular docking studies provided atomic level details on protein–ligand interactions. These are expected to expand the knowledge on compounds molecular recognition by the heptameric pore, while furosemide is suggested as possible therapeutic adjuvants for the treatment of infected patients. The furosemide to test the broader applicability of this approach, we sought furosemide capable of inhibiting the activity of Staphylococcus aureus α-hemolysin channel, which is regarded as a major virulence factor playing an antimicrobial effect.

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**MATERIALS AND METHODS**

α-HL protein and other chemicals

The wild type of *S. aureus* α-hemolysin was purchased from List Biological Laboratories. Solvent-free planar bilayer lipid membranes (PLM), with capacitance of 40 pF, were formed by the lipid monolayer apposition technique, using DPhPC in hexane at 25 ± 1 °C. Furosemide (4-chloro-2-(furan-2-ylmethylamino)- 5- sulfamoylbenzoic acid was purchased from Sigma. Diphytanoylphosphatidylcholine (DPhPC) was purchased from Avanti Polar Lipids.

Single channel reconstitution in planar lipid bilayer

Planar lipid bilayer membranes were formed as previously described, where the bilayers were composed of two DiPhyPC monolayers. After membrane stabilization, a 0.1–0.4 μl of the stock solution containing α-HL (0.07 mg/ml) was added to the standard solution (4 M KCl, 5 mM Tris-OH, pH 7.5) in *cis* compartment of the experimental chamber, to provide final concentrations of ~2ng/mL. All experiments were performed at room temperature (25±2°C) using an Axopatch 200A amplifier in the voltage clamp mode. At pH 7.5, α-HL channels are usually in a high conductance ~4nS and rarely switch to low conductance states. After the ion channel was incorporated in bilayer membrane, the anion inhibitors were also added to the standard solution to the *cis* compartment. All steps from measuring current to analyzing data were performed with equipment and software that are described in previous studies.

Multiple channel experiments reconstitution in planar lipid bilayer

Planar lipid bilayer membranes for multi-channel experiments were formed via the painting technique from a mixture of PC/Chol (1:1, w/w) across a hole (~0.3mm diameter) in a 25-mm-thick Teflon partition in a Teflon cell. The current
was converted to voltage, filtered by a low-pass eight-pole Butterworth filter, digitized with a sampling frequency of 0.5 kHz (for multichannel experiments) at Whole Cell Electrophysiology Program (WCP V1.7b) and/or the Electrophysiology software. Channels were formed by adding several microliters of the α-HL stock solution (5–50 mg/mL) to one side of the chamber (herein defined as cis). The mean value of single-channel insertion current was 0.3 pA for 50 mM CaCl$_2$ and 40 mV applied potential. The potential was defined as positive when it was greater at the side of the protein addition. At pH 7.5, α-HL channels are usually in a high conductance state and rarely switch to low conductance states. The conductance was compared statistically by GraphPad Prism, where media difference significant was calculated using t-Test and value $P < 0.05$.

Molecular docking studies

In order to obtain atomic-level insights into the furosemide mechanism of action, docking experiments were carried out to predict its position and orientation on the surface of α-HL. To docking studies, atomics coordinates for the α-HL heptameric channel was retrieved from PDB ID 7AHL. The structure of furosemide was removed from the PubChem and their coordinates were built and minimized with Avogadro software. The molecular docking experiments were performed using the Dockthor online portal. Molecular docking was performed in the constriction region, as it is the region most likely to have interactions between α-HL and little ligands. The output conformers from program are ranked in order of increasing affinity with the protein. The two conformations with biggest affinity docked were retrieved for further analysis.

RESULTS AND DISCUSSION

Importantly, the large α-hemolysin single-channel conductance ~4 nS in 4 mM KCl, furosemide was assayed for its ability to block ion conductance through the pores formed in artificial membranes α-HL (Fig.1). In addition, the relative conductance of the channel on the presence of the compound is smaller than in the standard solution and decrease with the transmembrane potential, so the effect is voltage-dependent. Addition of 100μM to the cis-side of the membrane switched the channel to a closed state similar to the ‘voltage-gated closed state’ seen commonly for α-HL at +100 mV and higher voltages.

Illustrate typical recordings of an ion current through single α-HL pore (Fig. 2). It is seen that even before the addition of 1, the single α-HL pores conductance level was rather noisy (Fig. 2 A, topmost track). This noisiness represents the well-known poro channels which appears as fast flickering between the open and completely closed conformations. The addition of furosemide to the cis-side of the membrane (the side of toxin addition) caused additional step-wise closures of 6 ms average duration (Fig. 2B, middle). Just as gating, these fluctuations are fast transientes between the fully open and non-conducting channel. Even though the amplitude of these events coincides with the amplitude of the regular α-HL channel (complete channel closure), the difference in the residence times allowed us to sort all observed events into the two different processes. The furosemide concentrations 100 μM channel blockade are more frequent (Fig. 2B, bottom). It was observed that the current through the single α-HL pore was stable; no gating events were seen under applied +100 mV. Even though the amplitude of these events coincides with the amplitude of the regular α-HL channel (complete channel closure), the difference in the residence times allowed us to sort all observed events into the two different processes (Fig. 2A). Rapid events (with an average duration of 6ms) are observed, as well as some lasting events (with an average duration of 2s) (Fig. 2B). Even though the amplitude of these events coincides with the amplitude of the regular α-HL channel (complete channel closure), the difference in the residence times allowed us to sort all observed events into the two different processes: events of shorter duration and greater frequency and events of longer duration and less frequency (Fig. 2B and 2C). Addition of about 100μM to the cis-side of the membrane switched the channel to a closed state similar to the ‘voltage-gated closed state’ seen commonly for α-HL at +50 mV and higher voltages. The residual conductance of the closed state was between 1% and 15% of the total channel conductance. It is remarkable that the introduction
of positive charges to the drugs molecule leads to its ability to block the α-HL channel from the cis-side. It is known that unmodified drugs binds only weakly to the heptameric α-HL channel when added from the trans (intracellular) side of the membrane. Tamoxifen presented similar effects to furosemide.

In order to predict the position and orientation of furosemide on the surface of α-HL and obtain atomic-level insights into drug mechanism of action, docking experiments were carried out to both on the surface of α-HL. The two conformers that showed greater binding affinity with α-HL for furosemide presented -6,766 and -6,634 kcal/mol, respectively. This does not happen with furosemide, as the conformers are positioned in the same region of the constriction, occupying less volume (Fig. 3A).

The furosemide binds to 113 and 147 resides. About interactions, four hydrogen bonding with 147 reside were observed and positioned of the constriction in the channel (Fig. 3B and 3C). Those bonds are able to stabilize the furosemide at lumen of nanopore and are described in literature. Despite that, its polarity substantial support solubilization in the solvent, justifying the fast events observed (Fig. 2B).

The results of the present study make us to conclude for the first time that furosemide blocks ion currents in the constriction of channel caused by Staphylococcus aureus α-hemolysin. In future, furosemide could be suggested as possible therapeutic adjuvants for the treatment of infected patients.

REFERENCES