Characterization of hemolytic activity of 3-alkylpyridinium polymers from the marine sponge Reniera sarai

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Abstract

Polymeric alkylpyridinium salts (poly-APS) isolated from the marine sponge Reniera sarai act as potent anticholinesterase agents; in addition they show moderate hemolytic and cytotoxic activities. The hemolytic activity of poly-APS is due to their detergent-like structure and behavior in aqueous solutions. In this work, the hemolytic activity of poly-APS is analyzed and compared to that of structurally-related monomeric cationic surfactants. The influence of different divalent cations and lipids on poly-APS induced hemolysis is discussed. The dimensions of lesions caused by poly-APS in erythrocyte membranes are determined by the use of osmotic protectants. Finally, the possible role of poly-APS in their natural environment is proposed. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: 3-Alkylpyridinium polymers; Cationic surfactant; Detergent; Hemolytic activity; Marine sponge; Reniera sarai

1. Introduction

Marine sponges produce a variety of antiviral, antibacterial, cytotoxic, cytolytic, and hemolytic compounds. As hemolytic were proved proteins [8,9,17], saponin-like peptides [23], and non-identified compounds [7,19]. Cytolytic agents, water soluble polymeric 3-octylpyridinium salts (poly-APS) (see Fig. 1), were isolated from the marine sponge Reniera sarai [26,27]. Poly-APS are structurally and functionally related to halitoxins, 0.5–25 kDa 3-alkylpyridinium polymers isolated from several sponges of the genus Haliclona [5,24]. Poly-APS were determined to be a mixture of 29 (5520 Da) and 99 (18 900 Da) polymerized 3-octylpyridinium units [27]. In halitoxins, pyridinium rings are connected by methyl-branched alkyl chains composed of 8–11 carbon atoms instead of the octyl chain in poly-APS. To date, 3-alkylpyridinium oligomers and polymers have been reported to be cytotoxic [13,24,26,31], hemolytic [5,24,26], antibacterial [24], antifeeding [1], toxic to animals [5,24,31], antimitotic [5,13], neurotoxic [5], and inhibitory for acetylcholinesterase [27–29], epidermal growth factors [10] and muscarinic receptors [12].

In aqueous solution, poly-APS form larger structures with an average hydrodynamic radius of 23 ± 3 nm (mean ± SEM) and surfactant-like characteristics [27]. Our preliminary study has shown that besides a potent and complex anticholinesterase activity [28], poly-APS are hemolytic and cytotoxic for different cell lines including the transformed ones [26]. This study addresses the characteristics of hemolytic activity of poly-APS as compared to hemolysis induced by the chemically related pyridinium surfactants, cetylpyridinium chloride (CPC), and cetyltrimethylammonium bromide (CTAB). Cationic surfactants are known as hemolytic compounds [15,32,34] and are also used as antibacterial or plaque-inhibiting agents [2]. We suggest that 3-alkylpyridinium polymers, displaying a broad spectrum of biological activities, may serve as unspecific defense agents in marine sponges.

Abbreviations: CMC, critical micelle concentration; CPC, cetylpyridinium chloride; CTAB, cetyltrimethylammonium bromide; HC_{50}, concentration of surfactant causing 50% hemolysis in 2 min; IC_{50}, cation concentration producing 50% inhibition of hemolysis; PEG, polyethylene glycol; Poly-APS, polymeric alkylpyridinium salts.

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2. Materials and methods

2.1. Materials

Poly-APS were purified from the marine sponge R. sarai as described previously [27]. A series of poly-APS solutions were prepared by diluting the 1.3 mg ml$^{-1}$ stock solution in water.

Stock solutions of CPC and CTAB (both Sigma, USA) were prepared in erythrocyte buffer (0.13 M NaCl, 0.02 M Tris/HCl, pH 7.4) to achieve a final concentration of 300 µg ml$^{-1}$. The chemical structure of the surfactants is presented in Fig. 1. For osmotic protection, we used glucose, sucrose (both Kemika, Croatia) and a series of polyethyleneglycols (all Sigma, USA) with molecular weights of 1, 1.5, 2 and 4 kDa, respectively. A stock solution of 600 mM saccharides was prepared in water. Sphingomyelin (Serva, Germany), 1-$\omega$-phosphatidylcholine, gangliosides, cholesterol and phosphatidic acid (all Sigma, USA) were assayed for inhibition of the hemolysis induced by poly-APS. Lipids were prepared at 100 µg ml$^{-1}$ in ethanol or water (gangliosides). To study the effect of divalent cations on surfactant-induced hemolysis, water solutions of CaCl$_2$, CoCl$_2$, HgCl$_2$, MnCl$_2$, NiCl$_2$ and ZnCl$_2$ (all Kemika, Croatia) were used. Their stock solutions were prepared at a concentration of 600 mM in water.

2.2. Hemolytic activity of poly-APS

Hemolytic activity was measured on bovine erythrocytes as described before [4,16]. Typically, 10–50 µl of water-dissolved surfactant was added into a cuvette containing 3.0 ml of erythrocyte suspension in 0.13 M NaCl, 0.02 M Tris/HCl, pH 7.4. The decrease of apparent absorbance was recorded at 700 nm using a Shimadzu 2100 UV/VIS spectrophotometer. All the experiments were performed at 25°C. The suspension in the cuvette was magnetically stirred. The surfactant HC$_{0.5}$ was defined as the concentration causing a 50% decrease of absorbance in 2 min.

2.3. Estimation of pore size

The size of lesions produced by poly-APS in erythrocyte membranes was estimated by using osmotic protectants as described by Belmonte et al. [4]. Osmotic protectants of different sizes were added to erythrocyte suspension at a 30 mM final concentration. After mixing, poly-APS at their HC$_{0.5}$ were added and the time course of hemolysis was followed for 10 min. To assure that the observed protection of hemolysis was not the consequence of vesiculation, we repeated the experiment by adding the protectant at the stage of cell lysis [25]. As a parameter of the time necessary for the sugar molecules to enter the cells through the poly-APS induced pores, we used $t_{1/2} = t_0 - t^0_{1/2}$, where $t^0_{1/2}$ was the time to 50% lysis without, and $t_{1/2}$ was the time to lysis with osmotic protectants [4]. Accordingly, the rate of solute diffusion through the pore was proportional to $1/(t_{1/2} - t^0_{1/2})$. The obtained data were fitted using the Renkin equation [22] to estimate the functional radius of the pore.

2.4. Inhibition of hemolysis with lipids

The effect of different lipids on poly-APS induced hemolysis was studied using a competition method [33]. Four microlitres of the stock solution of different lipids were added to 3.0 ml of erythrocyte suspension to obtain a final lipid concentration of about 10$^{-7}$ M. The mixture was incubated for 1 min, then poly-APS at their HC$_{0.5}$ were added, and the time course of hemolysis was monitored for 10 min.

2.5. Effect of divalent cations

To study the effect of divalent cations on hemolysis induced by poly-APS or CPC, to 3 ml of erythrocyte suspension supplemented with 0–10 mM CaCl$_2$, MnCl$_2$, CoCl$_2$, NiCl$_2$, ZnCl$_2$, and HgCl$_2$, surfactants were added at their HC$_{0.5}$, and the hemolytic activity was measured as described. We determined an IC$_{0.5}$, cation concentration increasing the half time of hemolysis $t_{1/2}$ by two-fold, i.e. producing 50% inhibition. In controls without hemolytic agents no hemolysis occurred within 30 min.

The effect of EDTA on divalent cation-induced inhibition of hemolysis was studied as follows. To an
erythrocyte suspension with an apparent absorbance of 0.5 at 700 nm poly-APS or CPC were added at their HC<sub>0.5</sub>. When absorbance dropped to 0.25, ZnCl<sub>2</sub> was added at a 1 mM final concentration, and after 70 s, a final 5 mM EDTA was added.

To test the effect of divalent cations on hemolysis induced by a hyposmotic solution, erythrocyte suspensions supplemented with divalent cations were rapidly mixed with an equal volume of deionized water and the time course of hemolysis was followed as described above.

3. Results

Poly-APS induced hemolysis in a dose-dependent manner. Its time course was sigmoidal (Fig. 2) as were those produced by CPC or CTAB (not shown). In Fig. 3, the $t_{1/2}$ values of hemolysis induced by surfactants are plotted against their concentration, and the estimated values for HC<sub>0.5</sub> are tabulated as compared to critical micelle concentration of surfactants. To compare the hemolytic activity of poly-APS with that of CPC and CTAB on a molar basis, we used the molecular weight of the 3-octylpyridinium moiety (190.16 g mol<sup>-1</sup>) to calculate the concentration of 3-octylpyridinium monomers in erythrocyte suspension. It is obvious that poly-APS are less active than CPC and CTAB. The hemolytic activity of poly-APS was abolished in the presence of phosphatidic acid (Fig. 4) but it was not affected by the other assayed lipids.

Poly-APS mediated hemolysis was apparently attenuated by osmotic protectants with a m.w. higher than 1.5 kDa (Fig. 5a). The high viscosity of solutions precluded the use of osmotic protectants with a m.w. exceeding 4.0 kDa. As well, the protection was preserved if the osmotic protectant was added at the stage of cell lysis, excluding the possibility that a vesiculation process competed with hemolysis [25]. An analysis of hemolysis data using the Renkin plot, i.e. the relative diffusion rate of the osmoticant molecule vs. its size, is shown in Fig. 5b. The best fit to the Renkin equation gave an estimate of a pore-effective radius of about 2.9 nm. In the presence of 1.0 kDa polyethylene glycol, the hemolytic activity of CPC was decreased by 80% in contrast to poly-APS which remained fully active (Fig. 5c). This suggests that poly-APS make larger pores than CPC in erythrocyte membranes.

Zn<sup>2+</sup> and Hg<sup>2+</sup> exhibited the strongest inhibition of the surfactant-induced hemolysis, while Ni<sup>2+</sup>, Mn<sup>2+</sup>,
Fig. 5. Poly-APS-induced lysis of bovine erythrocytes in the presence of osmotic protectants (indicated by arrows), a; Renkin plot reporting the relative diffusion rate of the osmotic protectant molecule vs. its size. Hydrated radii of the osmotic protectants are: 0.42 nm (glucose); 0.54 nm (sucrose); 0.9 nm (1.0 kDa PEG); 1.2 nm (1.5 kDa PEG); 1.4 nm (2.0 kDa PEG) and 2.0 nm (4.0 kDa PEG), b; effect of 1.0 kDa polyethylene glycol on hemolysis caused by poly-APS and CPC, c. Each result is the mean value ± SEM of three experiments.

Ca²⁺ and Co²⁺ were considerably less effective (Table 1). At a 1 mM final concentration, Zn²⁺ was inhibitory even when added after poly-APS or CPC, while the addition of 5 mM EDTA completely restored hemolytic activity as represented for poly-APS in Fig. 6. None of the used cations were able to inhibit hyposmotically induced hemolysis. In fact, Hg²⁺ induced a complete inhibition of hemolysis but only when preincubated with an erythrocyte suspension for more than 5 min (not shown).

4. Discussion

Cytotoxic and cytolytic activities of 3-alkylpyridinium polymers observed so far [5,24,26] at least in part may be explained by their surfactant-like characteristics. Studies of the surfactant/membrane interactions have shown that the hydrophilic/lipophilic balance of the surfactant plays the major role in membrane solubilization, electrostatic factors being less important [11,34]. The hemolytic activity of detergents has been reported to be (i) inversely proportional to their critical micelle concentration (CMC) [20]; (ii) proportional to the length of the alkyl chain [15,34], and (iii) affected by the character of their ionic head [32]. However, the polymeric structure of 3-alkylpyridinium salts do not resemble the classic design of the typical surfactants such as CPC and CTAB with both hydrophilic heads and hydrophobic tails free. Moreover, octyl chains flanked by two pyridinium rings in the case of poly-APS appear considerably shorter than the cetyl group of CPC and CTAB. Despite these differences, poly-APS surprisingly share many functional characteristics with the monomeric surfactants. First, both the characteristics of $t_{1/2}$ vs. concentration plot and the HC₅₀ value for poly-APS are comparable to those of CPC and CTAB (see Fig. 3). Second, experiments with osmotic protectants suggest that poly-APS produce discrete lesions in erythrocyte membranes. The resulting pores of about 5.8 nm in diameter appear slightly larger than those provoked by CPC. In comparison, an estimate for sodium dodecylsulfate and Triton X-100 pores was 4 nm [25]. Furthermore, the divalent cations Zn²⁺ and Hg²⁺ are common inhibitors of poly-APS and CPC (Table 1). These values are in accordance with reports

### Table 1

<table>
<thead>
<tr>
<th>Cation</th>
<th>Poly-APS IC₀.₅ (mM)</th>
<th>CPC IC₀.₅ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn²⁺</td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>0.04 ± 0.009</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>&gt; 3</td>
<td>2 ± 0.24</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
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<tr>
<td>Mn²⁺</td>
<td>&gt; 3</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>&gt; 1</td>
<td>&gt; 1</td>
</tr>
</tbody>
</table>

*a Each result is the mean value ± SEM of three experiments. Poly-APS and CPC were used at their HC₀.₅. IC₀.₅ is the cation concentration increasing the half time of hemolysis $t_{1/2}$ two-fold, i.e. producing 50% inhibition of hemolysis.
Fig. 6. Effect of ZnCl₂ and EDTA on time course of hemolysis induced by poly-APS at HC₀.₅. Additions of ZnCl₂ and EDTA are indicated by the arrows. Numbers in parenthesis are the final concentrations.

of a Zn²⁺ mediated inhibition of hemolysis by both cationic [3,21], anionic, and non-ionic hemolytic agents [25]. A higher inhibitory efficiency of Hg²⁺ with poly-APS may reflect the interaction of different negatively charged forms of mercury chloride (II) in water [HgCl₂⁻, HgCl₂(OH)₂⁻] [18] with a polycationic structure of poly-APS bound into the cell membrane. The polycationic structure of poly-APS could be a reasonable explanation for their preference for the negatively charged lipid as demonstrated for phosphatidic acid.

One reason for Zn²⁺ and other divalent cations mediated inhibition of hemolysis could be their ability to stabilize the erythrocyte membrane [6]. However, in our study Zn²⁺ did not inhibit hyposmotically evoked hemolysis. This fact and the ability of Zn²⁺ to inhibit already progressing lysis suggest that Zn²⁺ neither stabilizes nor prevents the binding of poly-APS to the membrane. Rather, we suggest that divalent cations close the resulted pores.

In conclusion, our results demonstrate that hemolytic activity of poly-APS is comparable to that of cationic surfactants CPC and CTAB despite the polymeric nature of poly-APS and their considerably shorter alkyl chain. As polycationic surfactants poly-APS show strong preference for negatively charged lipids. They induce a colloid–osmotic type of hemolysis by producing discrete lesions, 5.8 nm in diameter, in the cell membrane. Although occurrence of poly-APS in marine sponges is not completely understood, there is emerging evidence that these natural surfactants, possessing a large variety of biological activities, may serve as unspecific antibiotics and repelling agents.

Acknowledgements

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References


