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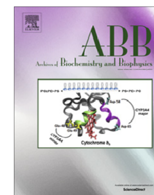

Competitive interactions of amphipathic polycationic peptides and cationic fluorescent probes with lipid membrane: Experimental approaches and computational model

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ABSTRACT

The electrostatic interaction of polycationic peptides with negatively charged biomembranes has been recognized as the first and very important step of their selective binding to many bacteria and transformed cells. In this work we demonstrated the phenomenon of competition of some earlier designed polycationic peptides and fluorescent probes for their binding to the negatively charged inner membrane of mitochondria and to the PC/PG (9:1) liposomes. Rat liver mitochondria swelling induced by the antimicrobial polycationic peptide BTM-P1 (VAPIAKYLATALAKWALKQGFAKLKS) and by the retro-BTM-P1 was significantly diminished in the presence of 10 μM fluorescent probe safranin O. In experiments with liposomes, the polycationic peptides BTM-P1 and P7-5 (IYLATALAKWALKQGF-GG-RRRRRRR) at the concentrations of 2–3 μM completely displaced the membrane-bound fluorescent probe DiSC3(5) in a low ionic strength medium. The developed computational model allowed a mathematical description of such interactions, predicting membrane surface concentrations of bound peptides as the function of the membrane surface charge and lipid quantity in the sample, the peptide charge, hydrophobicity and concentration, the ionic strength of incubation medium and of the presence of a charged fluorescent probe used for monitoring the membrane surface potential under real-time peptide–membrane interactions.

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Introduction

The antimicrobial peptides represent high clinical interest due to their low toxicity to mammalian cells and inherent difficulty to acquire bacterial resistance to them [1–4]. Most of the natural antimicrobial peptides are polycationic due to a presence of lysine and/or arginine residues [5–8]. In addition, they consist of a large proportion of hydrophobic amino acid residues [2,7–9]. The amphipathic character of the polycationic antimicrobial peptides allows their insertion into the lipid bilayer of biomembranes that has been considered a definitive feature of their antimicrobial activity even when the membrane permeabilization does not occur [10,11]. The polycationic peptide insertion into the lipid bilayer and the membrane permeabilization has been shown to be strongly potentiated by high values of the trans-membrane potential of bacteria [12] and of mitochondria [13–15]. The permeabilization of the plasma membrane of red blood cells by such peptides has also been potentiated by a relatively high plasma membrane potential (minus inside) generated in the presence of valinomycin [15–17], or even by the application of external electrical pulses to the cell suspension [17].

The selectivity of action of the polycationic antimicrobial peptides is mainly attributed to a high negative charge of the membrane surface of prokaryotic cells. Cancerous cells have also been reported to possess higher negative surface charges and higher trans-membrane potentials in comparison with normal eukaryotic cells [18–20]. That is why many of the polycationic antimicrobial peptides have also been found to reveal anticancer properties [7,21–23].

The design of new peptides with antibacterial and anticancer activities and the study of the mechanism of their action require development of new experimental approaches to evaluate the peptide interactions with biological membranes, particularly with the charged lipid bilayer. In this respect, the potential-dependent cyanine fluorescent probes, normally used as indicators of the membrane permeabilization by various factors, have also been shown to sense electrical properties of the membrane surface [24]. The charged amphipathic substances interacting with biomembranes might displace membrane-bound dyes. This kind of competitive interaction has been observed, for example, as a displacement of

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the cationic fluorescent probe dancyl-PMBN from the liposomal membrane by various polycationic peptides [25].

Several mathematical models have been developed to describe the peptide–membrane interactions [26–30]. Some of these models have been based on the Gouy–Chapman theory of the electrical double layer to estimate electrostatic interaction of polycationic peptides with the membrane, considering the uniformly distributed fixed charge density of the membrane surface [29,30]. On the other hand, the membrane surface charge and the surface potential might be affected by the peptide adsorption, hence inhibiting further peptide binding to the membrane, like that described for 1-anilino-8-naphthalenesulfonate, the anionic and hydrophobic fluorescent dye [31]. The combined electrostatic and hydrophobic interactions of peptides, dyes and other substances with lipid membrane might result in their competition or in a synergistic increase of the peptide adsorption on the membrane.

In the present work, we first experimentally observed the influence of the widely used potential-sensitive cationic probe safranin O on mitochondria permeabilization by some polycationic peptides, and vice versa, the displacement of the cationic cyanine probe DiSC3(5) from the negatively charged liposomal membrane by the peptides. The experimental data directly suggested the competitive character of the peptide and the fluorescent probe interactions with the membrane. The computational model was developed to explain the observed phenomena in their dependence on the probe and lipid membrane quantities, and on the concentration, net charge and hydrophobicity of a peptide. An effective surface charge density was included in the Gouy–Chapman equation instead of a fixed surface charge. The calculated data demonstrated that the fluorescence intensity of the aqueous phase DiSC3(5) is the most sensitive parameter reflecting peptide interactions with the lipid membranes mimicking eukaryotic or prokaryotic cell surfaces. The model allows the prediction of the optimal experimental conditions to study the membrane–peptide interactions using charged fluorescent probes.

Materials and methods

Materials

The polycationic peptides were designed in our laboratory and synthesized by the GenScript Corporation (NJ, USA). The used peptides were BTM-P1 (95.4% purity), with the sequence of VAPIAKYLATALAKWALKQGFAKLKS, derived from the Cry11Bb protoxin [13–15], its retro analog, retro-BTM-P1 (94.2% purity) with the sequence of SKLKAFGQKLAWKALATALYKAIPAV [15], and the peptide P7-5 (95% purity) described earlier [17], with the sequence of IYLATALAKWALKQGF-GG-RRRRRRR, showing anticancer properties. The phospholipids were purchased from Avanti Polar Lipids, Inc. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA),

Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by the method of differential centrifugation as described earlier [17], following the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the USA National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Local Ethics Committee of the National University of Colombia, Medellin Branch. The 10% liver homogenate (5 g of liver) was prepared in medium containing 210 mM mannitol, 70 mM sucrose, 2.5 mM $MgCl_2$, 1 mM EGTA–KOH, 0.3 mg/ml bovine serum albumin (BSA, free fatty acid fraction V), 10 mM HEPES–KOH, pH 7.2, at 0–4 °C. Mitochondria were washed twice in medium containing 210 mM mannitol, 70 mM sucrose, 50 μM EGTA–KOH, 0.3 mg/ml BSA and 10 mM HEPES–KOH, pH 7.2. Finally, the mitochondrial pellet was resuspended in 1 ml of the same medium without BSA.

Monitoring of the inner membrane potential of mitochondria

The inner membrane potential of mitochondria was monitored with the potential-sensitive fluorescent probe safranin O, as described in [32]. The real time fluorescence intensity (580 nm emission, 520 nm excitation) was measured using the Aminco-Bowman Series 2 spectrofluorimeter. Mitochondria, at the final concentration of 0.5 mg protein/ml, were added to the incubation medium composed of 100 mM sucrose, 75 mM KCl, 10 mM potassium phosphate, 5 mM HEPES, 50 μM EGTA, pH 7.2 (SKPH-medium), supplemented with 10 μM safranin O. To energize mitochondria, succinate was added to the final concentration of 2.5 mM. The peptides were added to the energized mitochondria at the final concentrations of 0.5 μM (BTM-P1) or 1.0 μM (retro-BTM-P1), observing subsequent increase in the safranin O fluorescence as a result of the inner membrane permeabilization.

Monitoring of the redox state of mitochondrial pyridine nucleotides

The level of reduced forms of mitochondrial pyridine nucleotides, NAD(P)H, was monitored using the Aminco-Bowman Series 2 Luminescence Spectrometer, as described earlier [33]. Mitochondria, at the final concentration of 0.5 mg protein/ml, were added to the SKPH medium. Where indicated, 2.5 mM succinate was added to energize the mitochondria. After that, the added peptides (0.5 μM BTM-P1 or 1.0 μM retro-BTM-P1) caused a decrease in the NAD(P)H fluorescence due to the inner membrane potential decrease, as explained in [33].

Monitoring of mitochondrial swelling

Mitochondrial swelling was monitored simultaneously with the safranin O or NAD(P)H fluorescence measuring in standard quartz cuvette using a modified cuvette holder for the Aminco-Bowman Series 2 Spectrometer described in [34]. The light, emitted by an infrared light-emitting diode (920 nm) and dispersed at 90° in mitochondrial suspension, was detected by additionally mounted photodiode and amplifier. The amplifier output signal was registered by the data acquisition system of the spectrometer using one of the two auxiliary channels [34].

The samples were constantly stirred with the magnetic stirrer and maintained at the temperature of 30 °C.

Liposome preparation

Small unilamellar lipid vesicles ([1]SUV) were prepared from egg yolk PC:PG (9:1 weight ratio). The lipids dissolved in chloroform were dried under N_2 to form a lipid film on the glass tube wall. After that, the sample was desiccated under a vacuum for 2 h to further remove the solvent. The obtained lipid film, composed of 18 mg PC and 2 mg of PG, was rehydrated with 2 ml of 0.1 M potassium phosphate buffer, 0.1 mM EGTA, pH 7.2 (KPB medium), to the final lipid concentration of 10 mg/ml. After that, the sample was vortexed to obtain multilamellar liposomes. To prepare SUV, the sample was sonicated (tip diameter 3 mM; at 20% power of the Cole-Parmer Ultrasonic Processor CP-500, 500 watts, 20 kHz), pulses "15 s on/45 s off" during 10 min of total sonication time at 4 °C, until the suspension was transparent. Titanium particles were removed by

[1] *Abbreviations used:* SUV, small unilamellar lipid vesicles; PC, phosphatidilcholine; FCCP, carbonyl-cyanide-*p*-trifluoromethoxy phenylhydrazone; PG, phosphatidylglycerol.

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