Biochimica et Biophysica Acta 1788 (2009) 532–537

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Potential-dependent permeabilization of plasma membrane by the peptide BTM-P1 derived from the Cry11Bb1 protoxin

Mauricio Arias [a], Sergio Orduz [b,c], Victor V. Lemeshko [a,*]

[a] Escuela de Física, Facultad de Ciencias, Universidad Nacional de Colombia, Sede Medellín, Calle 59A, No 63-20, Medellín, Colombia
[b] Escuela de Biociencias, Facultad de Ciencias, Universidad Nacional de Colombia, Sede Medellín, Calle 59A, No 63-20, Medellín, Colombia
[c] Unidad de Biotecnología y Control Biológico, Corporación para Investigaciones Biológicas, Medellín, Colombia

ARTICLE INFO

Article history:
Received 12 August 2008
Received in revised form 2 December 2008
Accepted 12 December 2008
Available online 25 December 2008

Keywords:
Plasma membrane
Pore size
Cry11Bb protoxin
Polycation peptide
Hemolysis
Light dispersion

ABSTRACT

The peptide BTM-P1, which is derived from the amino acid sequence of the Cry11Bb1 protoxin, is able to permeabilize mitochondrial membranes and reveals antimicrobial activity. In this work we demonstrated that the permeabilizing activity of BTM-P1 for the plasma membrane of rat red blood cells increased in a dose-dependent manner for the concentration range of 1–4 μg/ml. Using osmotic protectants, the radius of pores formed at 4 μg/ml BTM-P1 was determined as 0.8 nm for 5 min hemolysis data, 0.7 nm for 5 min decrease in light dispersion of the cell suspension and 0.5 nm for the light dispersion slope measurements. The permeabilizing activity of 1 μg/ml peptide was increased by valinomycin-induced plasma membrane potential, especially under moderately hypotonic conditions. These results might explain the antimicrobial activity of BTM-P1 and support the hypothesis of potential-dependent and pro-apoptotic character of toxicity of naturally proteolysed Cry11Bb1 protoxin for epithelial cells of mosquito larvae midgut.

1. Introduction

The polycation peptides form part of the host defense system of many organisms, including insects, plants, and animals [1,2]. Most of them demonstrate activity against bacteria, fungi and protozoa [2–5]. The increasing number of pathogenic organisms with resistance to conventional antibiotics has raised interest in applications of antimicrobial peptides to treat infections [6]. There are several polycation peptides that are currently being investigated for this pharmaceutical propose [7–9].

The design of synthetic amphipathic peptides based on known structures of discovered natural peptides with antimicrobial activity has a great potential in order to improve the efficacy and selectivity of their action [10]. Some peptides of the same class have shown promising antitumor activity [11]. The polycation peptides such as mastoparan [12] or BMAP-28 [13], for example, have been shown to permeabilize mitochondrial membranes suggesting a possible mechanism of pro-apoptotic and pro-necrotic actions in eukaryotic cells.

The pore-forming protein toxins were found not only in eukaryotic, but also in prokaryotic cells [14]. The bacterial protein toxins have received far more attention than those from any other kind of organism, as a concern for their disease-causing abilities and for possible practical applications [15]. *Bacillus thuringiensis* serovar. *medellin*, for example, produces the protoxin Cry11Bb1 (94 kDa), which reveals toxic effects against different species of mosquito larvae like *Culex quinquefasciatus* and *Aedes aegypti* [16]. The action of Cry11Bb1 is dependent of the solubilization and a proteolytic processing in the midgut of the susceptible larvae, where shorter segments of the protein are produced. At least two different fragments of Cry11Bb1 (30 and 35 kDa), to which the toxic effect could be attributed, are generated by the proteolysis [17]. The possibility that shorter fragments with membrane permeabilizing activity could be generated during Cry11Bb1 proteolysis has not been reported in the literature.

Based on the amino acid sequence of the domain I of the Cry11Bb1 protoxin, we recently designed a novel polycation peptide, BTM-P1, composed of 26 amino acid residues [18–20]. This polycation fragment is sufficiently long to traverse a membrane. It contains 4 hydrophobic amino acid residues at the N-terminus, which should facilitate the incorporation of the peptide into a lipid bilayer, and 22 amino acid residues of the domain I α2 helix. Three lysine residues and one hydroxyl-containing threonine, each in the i+4 position of the α2a helix [21], should be important for forming an amphipathic helix with an aligned polar side and an opposing, relatively hydrophobic side. The presence of a double AXXXA motif (AXXXAXXXA) in the α2a helix sequence could be favorable for peptide oligomerization in a lipid environment [22,23], allowing membrane permeabilization as was suggested earlier [19]. The possibility of formation of typical helical structure in BTM-P1 between residues K6-A22 was demonstrated by ^{1}H NMR [20]. In addition, a 68% helical structure of the peptide was

* Corresponding author. Tel.: +57 4 4309378; fax: +57 4 2604489.
E-mail address: vvasilie@unal.edu.co (V.V. Lemeshko).

0005-2736/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.12.009

observed by circular dichroism in 40% aqueous 2,2,2-trifluoroethanol [20]. These data supported the hypothesis that BTM-P1 forms ion channels due to its transmembrane oligomerization [19,20]. The particular mechanism, "barrel-stave" or "toroidal pore" [24 and references therein], or a clustering of membrane negatively charged lipids by polycation peptides [25], has not yet been established.

BTM-P1 has been shown to induce mitochondrial swelling and to cause uncoupling of the oxidative phosphorylation system [19]. It also demonstrated high activity against Gram-positive and Gram-negative bacteria [18,20]. Except for an evaluation of hemolysis [20], its capacity to permeabilize the plasma membrane of eukaryotic cells has not yet been studied in detail and it seems to be important to investigate the mechanism of toxicity of Cry protoxins.

In this work we studied permeabilization of the plasma membrane of rat red blood cells (RBC) by the peptide BTM-P1. On the basis of measurements of hemolysis and light dispersion of cell suspensions versus molecular mass of mannitol, sucrose and polyethylene glycols, used as osmotic protectants, the radius of membrane pores formed by this peptide was determined to be in the range of 0.5–0.8 nm. Valinomycin-induced membrane potential of relatively high magnitude (negative inside) essentially increased the capacity of BTM-P1 to permeabilize the plasma membrane of RBC that was in concordance with the earlier suggested potential-dependent mechanism of permeabilization of the inner mitochondrial membrane by this peptide [19]. The data obtained are in favor of a potential-dependent and apoptosis-like mechanism of toxicity of the naturally proteolysed Cry11Bb1 protoxin for epithelial cells of mosquito larvae midgut.

2. Material and methods

2.1. Materials

Most of the chemicals of analytical grade were purchased from Sigma Chemical Co. (St. Louis, MO, USA), valinomycin was from Merck and polyethylene glycols of highest purity were from FLUKA. The peptide BTM-P1 was synthesized by Fundación Instituto de Inmunología de Colombia (Bogota, Colombia) by the solid phase method [26], according to the amino acid sequences proposed by some of the authors of this work. A p-methyl benzhydrylamine-resin (0.7 Meq/g), t-Boc amino acids (Bachem, USA) and low-high cleavages were used in the process [27]. The peptide was purified by high-pressure liquid chromatography, analyzed by mass spectrometry (Maldi-Tof), lyophilized and kept as a powder and as a water solution at 4 °C before use.

2.2. Isolation of red blood cells

RBC were obtained as described in [28] with slight modification. Approximately 5 ml of Sprague/Dawley rat blood was diluted in 20 ml of medium composed of 120 mM NaCl, 10 mM EDTA, 5 mM sodium citrate and 5 mM Tris–HCl, pH 7.4, and centrifuged at 3000 rpm for 10 min at 15 °C. The pellet was washed twice by gentle resuspension in 20 ml of 150 mM NaCl and 5 mM Tris–HCl, pH 7.4, and subsequent centrifugation at 3000 rpm for 10 min at 15 °C. The final pellet was resuspended in the same medium supplemented with 10 mM glucose to a final hematocrit of 20%.

2.3. Monitoring of plasma membrane permeabilization

The plasma membrane permeabilization accompanied with RBC swelling and light dispersion changes of cell suspension was monitored by measuring the apparent light absorbance at 640 nm (A_{640}) using a Spectronic Genesys-2 spectrophotometer under constant stirring at 25 °C. RBC were added to 1.2 ml of isotonic medium (150 mM NaCl, 5 mM Tris–HCl, pH 7.4) or to 1.2 ml of approximately semi-isotonic medium (85 mM NaCl, 5 mM Tris–HCl, pH 7.4) to a final hematocrit of 0.17%. The BTM-P1 was added at the concentrations of 1–4 μg/ml. To evaluate the influence of the moderately hypotonic conditions and/or the membrane potential on the permeabilizing activity of BTM-P1, the above mentioned isotonic and semi-isotonic media were supplemented with 0.1 mM KCl and 0.5 μM valinomycin. After incubation of the rat RBC with the peptide for 2 or 3 min, hemolysis was evaluated by measuring light absorbance at 541 nm (A_{541}) in the supernatants obtained by centrifugation of samples at 13000×*g* for 2 min at 15 °C. Hemolysis determined in the presence of 0.01% Triton X-100 was taken as 100%.

2.4. Pore size estimation

The size of pores formed by BTM-P1 in the plasma membrane was estimated on the basis of hemolysis and light dispersion changes of RBC suspensions caused by the peptide in incubation media containing 135 mM NaCl, 5 mM Tris–HCl, pH 7.4, supplemented with osmotic protectants such as mannitol, sucrose, PEG-600, PEG-1000, PEG-1500, PEG-2000 and PEG-3000 at the concentration of 30 mM. RBC were added to a final hematocrit of 0.17%. After 5 min of incubation with the 4 μg/ml BTM-P1, the extent of permeabilization was evaluated by hemolysis and by A_{640} change of the cell suspensions, as well as by determining of the A_{640} slope using a Spectronic Genesys-2 spectrophotometer. The effects caused by the peptide in 150 mM NaCl, 5 mM Tris–HCl, pH 7.4, in the absence of osmotic protectants were taken equal to 100%.

3. Results

3.1. Plasma membrane permeabilization by BTM-P1

The measure of light dispersion of the rat RBC suspensions as apparent absorbance A_{640} before and after the addition of 1 μg/ml (Fig. 1, *b*), 2 μg/ml (Fig. 1, *c*), 3 μg/ml (Fig. 1, *d*) and 4 μg/ml (Fig. 1, *e*) of BTM-P1 allowed the observation of a dose-dependent permeabilization of the plasma membrane in isotonic NaCl-Tris media in a scale between the control (without the peptide) (Fig. 1, *a*) and complete hemolysis caused by 0.01% Triton X-100 (Fig. 1, *f*). Light dispersion data were in concordance with hemolysis determined at the end of the 2 min period of incubation with the peptide (Fig. 1, numbers at the end of the curves). The correlation coefficient between these parameters was equal to 0.97. Complete cell swelling was developed in approximately 1 min after the addition of the peptide at 2–4 μg/ml (Fig. 1, *c*–*e*). No detectable cell swelling was registered when only 1 μg/ml of BTM-P1 was added to the RBC suspension in isotonic NaCl-Tris medium (Fig. 2A, *a*). A similar result was observed for moderately hypotonic conditions, when the incubation medium contained 85 mM NaCl, 0.1 mM KCl and 5 mM Tris–HCl (Fig. 2A, *b*).

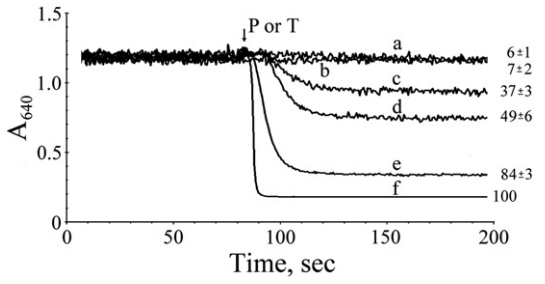

Fig. 1. Swelling and hemolysis of rat RBC induced by the peptide BTM-P1. RBC were incubated in isotonic medium (150 mM NaCl, 5 mM Tris–HCl, pH 7,4) at 0.17% hematocrit; P–the addition of the peptide at concentrations of 1 (*b*), 2 (*c*), 3 (*d*) and 4 (*e*) μg/ml; T–the addition of 0.01% Triton X-100 (*f*); *a*–control without any addition. The numbers at the end of the curves represent hemolysis evaluated at the end of each incubation, means±SEM ($n=3$).

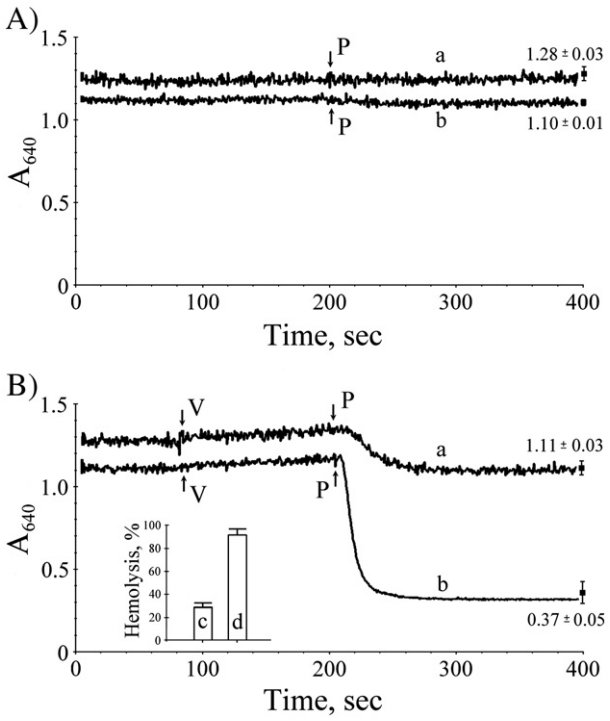

Fig. 2. Influence of osmotic pressure of incubation medium and of membrane potential on the swelling of rat RBC induced by the peptide BTM-P1. RBC were added to isotonic (150 mM NaCl, 5 mM Tris–HCl, 0.1 mM KCl, pH 7.4) (*a*), or semi-isotonic (85 mM NaCl, 5 mM Tris, 0.1 mM KCl, pH 7.4) (*b*) incubation media at 0.17% hematocrit without (A) or with (B) 0.5 μM valinomycin. P–1 μg/ml BTM-P1. The inset figure in panel B represents hemolysis determined at the end of RBC incubation with the peptide in isotonic (*c*) and semi-isotonic (*d*) media containing 0.5 μM valinomycin. The numbers at curves represent A_{640} data at the end of each incubation, means ± SEM ($n=3$).

In previous work we demonstrated that the permeabilizing activity of BTM-P1 in rat liver mitochondria was strongly increased by the inner membrane potential [19]. The membrane potential of normal rat RBC is known to be about −10 mV [29]. In order to test if the artificial increase in the plasma membrane potential might enhance the permeabilizing activity of BTM-P1, 0.5 μM valinomycin was added to the cell suspension before adding the peptide. The addition of valinomycin caused initial shrinkage of RBC that was observed as an increase in A_{640} (Fig. 2B, *a, b*), probably due to the efflux of potassium ions accompanied with a slow release of chloride anions. Valinomycin also increased the permeabilizing activity of subsequently added 1 μg/ml BTM-P1 that was expressed as cell swelling in isotonic (Fig. 2B, *a*) or in moderately hypotonic (Fig. 2B, *b*) media containing 0.1 mM KCl, as well as in a more significant hemolysis that was higher in moderately hypotonic (Fig. 2B, *d*) than in isotonic (Fig. 2B, *c*) media. These results showed a meaningful synergistic effect of the plasma membrane permeabilization by BTM-P1 caused by valinomycin and moderately hypotonic conditions, thus leading to almost complete hemolysis (Fig. 2B, *d*) and to a significant decrease in A_{640} of the cell suspension (Fig. 2B, *b*).

3.2. Pore size estimation

To estimate the size of pores formed by BTM-P1 in the plasma membrane of normal rat RBC, we first determined the dependence of light dispersion decrease and of hemolysis, induced by the peptide, on the molecular weight of osmotic protectants included in isoosmotic incubation media at 30 mM concentration. Mannitol (Fig. 3A, *b*), sucrose (Fig. 3A, *c*), PEG-600 (Fig. 3A, *d*), PEG-1000 (Fig. 3A, *e*), PEG-1500 (Fig. 3A, *f*), PEG-2000 (Fig. 3A, *g*) and PEG-3000 (Fig. 3A, *h*) were used as osmotic protectants. The data obtained were normalized taking as 100% the effects caused by RBC treatment with 4 μg/ml BTM-P1 without osmotic protectants (Fig. 3A, *i*; Fig. 3B, control) and as 0% for the non-treated control (without the peptide and osmotic protectants). As shown in Fig. 3A, curve *a*, no light dispersion decrease was observed for the non-treated control. Taking into account the known hydrodynamic radii of used osmotic protectants [30] and estimating the molecular weight of osmotic protectant that should cause a 50% decrease in A_{640} drop for the cell suspension (Fig. 3B, *b*) or a 50% decrease of hemolysis (Fig. 3B, *a*), the pore radius was determined as approximately 0.7 nm and 0.8 nm, respectively, for 5 min incubation of the rat RBC with 4 μg/ml BTM-P1. An excellent correlation ($r^2=0.98$) was obtained between hemolysis and A_{640} data (Fig. 3C), indicating that the pore size measured by the two different approaches were the same. A smaller pore radius (0.5 nm) was determined according to the 50% decrease in the A_{640} slope (Fig. 3B, *c*).

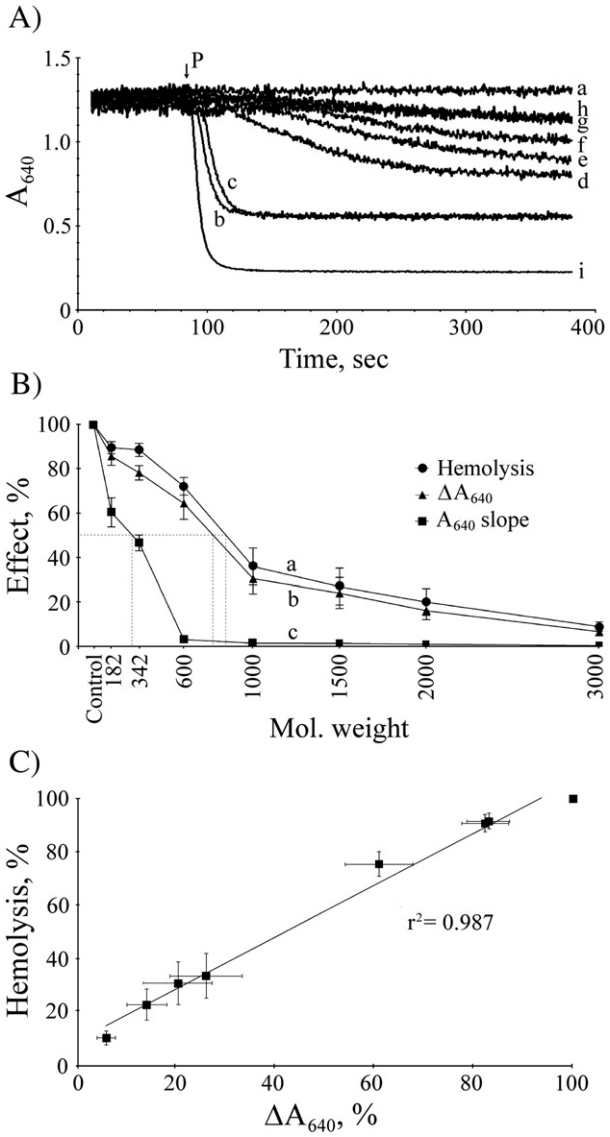

Fig. 3. Evaluation of the size of pores formed by BTM-P1 in the plasma membrane of rat RBC. Panel A: light dispersion changes registered as A_{640} of RBC suspensions of 0.17% hematocrit in 150 mM NaCl and 5 mM Tris–HCl (*a*, *i*) or in 135 mM NaCl and 5 mM Tris–HCl supplemented with 30 mM mannitol (*b*), sucrose (*c*), PEG-600 (*d*), PEG-1000 (*e*), PEG-1500 (*f*), PEG-2000 (*g*) or PEG-3000 (*h*) as osmotic protectants; P–the addition of 4 μg/ml BTM-P1 (*b–i*); *i*–control without osmotic protectants; *a*–non-treated control, without any addition. Panel B: the effect of osmotic protectants on hemolysis (*a*) and A_{640} changes (*b*) determined after 5 min incubation of RBC with the peptide, as well as on the A_{640} slope. Panel C: correlation between hemolysis and A_{640} changes according to the data presented in panel B (*a* and *b*, respectively). The data are means ± SEM ($n=3$).

4. Discussion

To explain the mode of action of Cry toxins produced by *B. thuringiensis*, three hypotheses have been proposed [31]. The first one indicates that the Cry toxins recognize a receptor in the apical microvilli of the midgut of susceptible insects and insert in the cell membrane thus causing formation of pores and cell death by osmotic unbalance [32,33]. A second proposal indicates that in addition to pore formation and osmotic unbalance, bacteria from the midgut microflora are necessary to produce septicemia and insect death [34]. The third hypothesis suggests the activation of intracellular signals mediated by the interaction of the Cry toxins with a receptor [35].

The segment of the Cry proteins responsible for membrane permeabilization is Domain I composed of seven α helices [36]. Gerber and Shai [37] have found that the α4–α5 hairpin of the Cry1Ac toxin is the membrane inserted region in which both helices line the lumen of the pore. In addition, Leetachewa et al. [38] have demonstrated that a hairpin formed by the α4–α5 helices of the Cry4B toxin is active in permeabilization of unilamellar liposomes. Puntheeranurak et al. [39] have observed that the α1–α5 fragment of the Cry4B toxin induces the formation of cation selective pores in planar lipid bilayers suggesting that this fragment can form functional channels.

Some insecticidal *B. thuringiensis* strains display antimicrobial activity after their Cry proteins are proteolytically cleaved [40], and some non-insecticidal *B. thuringiensis* strains produce a new class of crystal proteins, parasporins, which are active against a number of cancer cell lines after proteolysis [41,42]. Recently, we have demonstrated that a 26 amino acid residue peptide BTM-P1, derived from the primary structure of the Cry11Bb1 protoxin of *B. thuringiensis* serovar. *medellin* (BTM-P1) [18,19], adopts a three-dimensional structure as an α-helix [20] and displays antibacterial activity against Gram negative and Gram positive bacteria [18,20]. It can permeabilize rat liver mitochondria [19] at concentrations lower than mastoparan [12].

In this work, we studied the capability of BTM-P1 to permeabilize the plasma membrane of rat RBC. The obtained results show that the peptide induces swelling and hemolysis of RBC at concentrations higher than 1 μg/ml (Fig. 1). The radius of pores formed at 4 μg/ml BTM-P1 and estimated using 30 mM osmotic protectants was approximately 0.7–0.8 nm according to the light dispersion and hemolysis data and 0.5 nm according to the A_{640} slope changes (Fig. 3B). These values of pore size are lower than the hydrodynamic radius of PEG-1000 and thus cannot be affected by hemoglobin precipitation caused by 30 mM PEGs of molecular weight higher than 3000 (for details see [43]). The determined pore size of 0.5–0.8 nm is very close to the pore sizes reported for other antimicrobial peptides with similar lengths. For comparison, the magainins, 23-residue peptides with charge of +4 (Table 1) from the skin of the *Xenopus laevis* frog, form pores or structural defects of approximately 1 nm in diameter in both, liposomes and mitochondrial inner membrane [44]. The peptide melittin, isolated from the honey bee venom (*Apis mellifera*), formed by 26 amino acid residues with a net charge of +6 (Table 1) [45], generates pores with radius of 0.63–0.67 nm in liposomes [46].

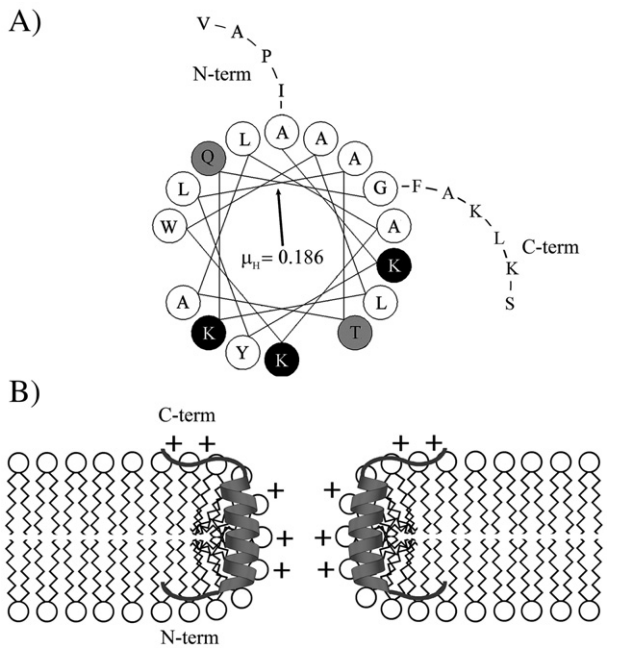

Fig. 4. Helical wheel projection (A) and toroidal-like pore model (B) proposed for BTM-P1. Panel A: Helical wheel projection of α2a helix using the one letter code; the hydrophobic residues are presented in white, polar residues in gray and highly polar cationic residues in black circles in the wheel. The N-term and C-term regions of BTM-P1, which are not part of the α2a helix, are presented as flexible sequences at the beginning and the end of the α2a helix, respectively. The hydrophobic moment (μ_H) of the α2a helix was calculated on the basis of consensus scale of hydrophobicity [47]. Panel B: The N-term VAPI region is embedded into the hydrophobic core of the membrane and the cationic residues (+) of the C-term FAKLKS region and of the α2a helix are interacting with negative head groups of phospholipids thus forming toroidal-like pores.

As a working hypothesis, we assume that highly hydrophobic N-term region (VAPI) of BTM-P1 could function as an anchor facilitating the incorporation of an amphipathic α2a helix (Fig. 4A) into the hydrophobic core of the membrane as shown in Fig. 4B. The hydrophobic moment of BTM-P1 is equal to 0.098 (Table 1) calculated using the consensus scale of Eisenberg [47] and considering the entire peptide as the alpha helix. On the other hand, the hydrophobic moment of BTM-P1 α2a helix is higher and is equal to 0.186 (Table 1, Fig. 4A) that is somewhat less than those calculated by the same method for melittin and magainins (Table 1) [48]. The relatively hydrophilic C-term region (FAKLKS) of the BTM-P1 (Fig 4A) might function as a polar anchor interacting with negatively charged phospholipids of the outer leaflet of the lipid bilayer (Fig 4B). It is very probable that BTM-P1 forms "toroidal pores", as was suggested for magainins and melittin [44,45 and references therein], taking into account the similarity of the length, charge and α-helix structure in hydrophobic environment for these peptides [44,48] and BTM-P1 [20] (Table 1), as well as the similarity of pore sizes [44,46] (Fig. 3B). The mechanism of membrane permeabilization by BTM-P1 could be also

Table 1
Physicochemical parameters of BTM-P1 and representative antimicrobial peptides

Peptide	Origin	Amino acid sequence	Net charge	μH*
BTM-P1	*B.thuringiensis*	VAPIAKYLATALAKWALKQGFAKLKS	5+	0.098
α2a helix [21]	*B.thuringiensis*	AKYLATALAKWALKQG	3+	0.186
Magainin 1 [44]	*X. laevis*	GIGKFLHSAGKFGKAFVGEIMKS	4+	0.310
Magainin 2 [44,48]	*X. laevis*	GIGKFLHSAKKFGKAFVGEIMNS	4+	0.286
Melittin [45,48]	*Apis mellifera*	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	6+	0.266

* Mean hydrophobic moment (μ_H) calculated according to the consensus scale of hydrophobicity [47]. The melittin μ_H was calculated for the α-helical region only (residues 1–21) [48].

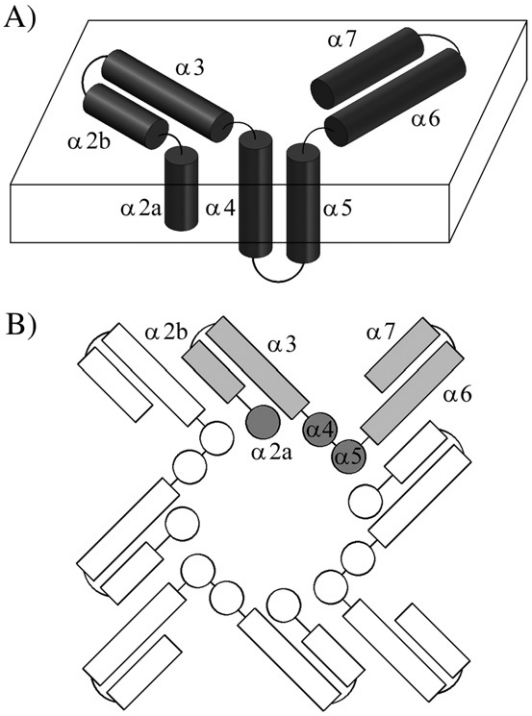

Fig. 5. "Damaged umbrella oligomerization" model of the membrane permeabilization by activated Cry11Bb1 domain I. Panel A: The α2a helix is embedded into the membrane in addition to the α4 and α5 helices, as a modification of the model of Gazit et al. [54]. Panel B: Hypothetic scheme of formation of "damaged umbrella" oligomers that might be tetramers [55].

consistent with a clustering of membrane negatively charged lipids by polycation peptides [25], or with initial formation of transitional disordered torodial-like pores proposed for magainin-2 and melittin [49,50], finally leading to the formation of barrel-stave or toroidal pores as it was suggested by Sengupta et al. [50].

The permeabilizing activity of BTM-P1 was essentially increased by artificially generated high plasma membrane potential in rat RBC (Fig. 2B). These results are in agreement with those indicating that the permeabilizing activity of BTM-P1 is highly expressed in the energized but not in the de-energized rat liver mitochondria [19]. It seems to be very probable that the electrical potential of the plasma membrane of epithelial cells promotes transmembrane movement of positively charged oligomers of the peptides like BTM-P1 inside the cells, which could reach mitochondria and cause mitochondrial membrane permeabilization. This concept is consistent with the relatively high transepithelial potential found in the posterior region of larval *Aedes aegypti* midgut, which is lumen positive in contrast to the lumen negative potential of the anterior "stomach" region [51]. Hyperpolarization of the apical membrane of the posterior larval midgut (negative potential inside the epithelial cells) is attributed to the H^+ V-ATPase, which appears to be localized therein and expels protons from the cells [52 and references therein]. This negative potential might increase the toxicity of membrane active polycation peptides for the posterior "stomach" region of *A. aegypti* larvae. This assumption is in accordance with experimental data showing that the lesion caused by the Cry11Bb1 toxin is most prominent for epithelial cells of the posterior larval midgut [53].

The data obtained in this work support the "damaged umbrella oligomerization" model of permeabilization of the midgut epithelial cells by Cry proteins [19] (Fig. 5). Initially, the "umbrella model" considered the formation of pores by the oligomerization of domain I of the Cry family proteins, where α4 and α5 helices are the only ones inserted into the membrane and the remaining helices lie on the membrane surface [54]. The high membrane permeabilizing activity of BTM-P1, which contains significant part of the α2 helix, allows the suggestion that the α2a helix of Cry11Bb1 is also inserted in the membrane, in addition to the α4 and α5 helices (Fig. 5A), thus forming oligomers of "damaged umbrellas" that might be tetramers [55] as shown in Fig. 5B. This hypothesis is consistent with recent data obtained for other Cry toxins indicating that the α1 is the only α helix of domain I from Cry1Ab that is not inserted in the membrane [56], or that the α2 and α3 helices of the trypsin activated Cry1Aa protein might be either embedded in or strongly interacting with the brush border membrane [57].

The proteolytic processing of Cry11Bb1 protoxin might result in other α2 helix fragments or in larger fragments containing BTM-P1 as their part, which also possess membrane permeabilizing activity [58]. The pore size determined for the plasma membrane permeabilization by BTM-P1 is large enough for the phosphonucleotides to diffuse out of the cell. This fact allows speculation that cytosolic ATP, ADP and mitochondrial energetic substrates can be lost resulting in a decreased ability of mitochondria to maintain an effective oxidative phosphorylation process in addition to the possibility that the peptides can directly permeabilize mitochondrial membranes [19] thus inducing apoptosis of epithelial cells of the larval *A. aegypti* posterior midgut.

Acknowledgments

The authors thank Dr. F. Guzmán and Dr. M. Patarroyo (Fundación Instituto de Inmulogía de Colombia, Bogota) for the synthesis of the peptides. This work was supported by Colciencias (Colombia) Research Grants #11184-08-20380 and #2213-12-17833.

References

[1] R.E.W. Hancock, D.S. Chapple, Peptide antibiotics, Antimicrob. Agents Chemother. 43 (1999) 1317–1323.
[2] K.V.R. Reddy, R.D. Yedery, C. Aranha, Antimicrobial peptides: premises and promises, Int. J. Antimicrob. Agents 24 (2004) 536–547.
[3] R.E.W. Hancock, Cationic peptides: effectors in innate immunity and novel antimicrobials, Lancet, Infect. Dis. 1 (2001) 156–164.
[4] O. Toke, Antimicrobial peptides: new candidates in the fight against bacterial infections, Biopolymers 80 (2005) 717–735.
[5] C.K. Moreira, F.G. Rodrigues, A. Ghosh, F. Varotti, A. Miranda, S. Daffre, M. Jacobs-Lorena, L.A. Moreira, Effect of the antimicrobial peptide gomesin against different life stages of *Plasmodium* spp. Exp. Parasitol. 116 (2007) 346–353.
[6] R.M. Epand, H.J. Vogel, Diversity of antimicrobial peptides and their mechanisms of action, Biochim. Biophys. Acta 1462 (1999) 11–28.
[7] E. Andres, J.L. Dimarcq, Clinical development of antimicrobial peptides, Int. J. Antimicrob. Agents 25 (2005) 448–452.
[8] A. Giuliani, G. Pirri, S.F. Nicoletto, Antimicrobial peptides: an overview of a promising class of therapeutics, Cent. Eur. J. Biol. 2 (2007) 1–33.
[9] M. Zaiou, Multifunctional antimicrobial peptides: therapeutic targets in several human diseases, J. Mol. Med. 85 (2007) 317–329.
[10] I. Zelezetsky, U. Pag, H-G. Sahl, A. Tossi, Tuning the biological properties of amphipathic α-helical antimicrobial peptides: rational use of minimal amino acid substitutions, Peptides 26 (2005) 2368–2376.
[11] D.W. Hoskin, A. Ramamoorthy, Studies on anticancer activities of antimicrobial peptides, Biochim. Biophys. Acta 1778 (2008) 357–375.
[12] D.R. Pfeiffer, T.I. Gudz, S.A. Novgorodov, W.L. Erdahl, The peptide mastoparan is a potent facilitator of the mitochondrial permeability transition, J. Biol. Chem. 270 (1995) 4923–4932.
[13] A. Risso, E. Braidot, M.C. Sordano, A. Vianello, F. Macri, B. Skerlavaj, M. Zanetti, R. Gennaro, P. Bernardi, BMAP-28, an antibiotic peptide of innate immunity, induces cell death through opening of the mitochondrial permeability transition pore, Mol. Cell. Biol. 22 (2002) 1926–1935.
[14] A.W. Bernheimer, B. Rudy, Interactions between membranes and cytolytic peptides, Biochim. Biophys. Acta 864 (1986) 123–141.
[15] R.J.C. Gilbert, Pore-forming toxins, Cell. Mol. Life Sci. 59 (2002) 832–844.
[16] N. Restrepo, D. Gutiérrez, M.M. Patiño, I. Thiéry, A. Delécluse, S. Orduz, Cloning, expression and toxicity of a mosquitocidal toxin gene of *Bacillus thuringiensis* subsp. *medellin*, Mem. Inst. Oswaldo Cruz 92 (1997) 257–262.
[17] C. Segura, F. Guzman, M.E. Patarroyo, S. Orduz, Activation pattern and toxicity of the Cry11Bb1 toxin of *Bacillus thuringiensis* subsp. *medellin*, J. Invertebr. Pathol. 76 (2000) 56–62.
[18] V.V. Lemeshko, F. Guzman, M.E. Patarroyo, C. Segura, S. Orduz, Synthetic peptide having an ionophoric and antimicrobial activity, U. S. Patent No. 7,041,647 (2005).
[19] V.V. Lemeshko, M. Arias, S. Orduz, Mitochondria permeabilization by a novel polycation peptide BTM-P1, J. Biol. Chem. 280 (2005) 15579–15586.
[20] C. Segura, F. Guzmán, L.M. Salazar, M.E. Patarroyo, S. Orduz, V.V. Lemeshko, BTM-P1 polycationic peptide biological activity and 3D-dimensional structure, Biochem. Biophys. Res. Commun. 353 (2007) 908–914.

[21] P. Gutierrez, O. Alzate, S. Orduz, A theoretical model of the tridimensional structure of *Bacillus thuringiensis* subsp. *medellin* Cry11Bb toxin deduced by homology modelling, Mem. Inst. Oswaldo Cruz 96 (2001) 357–364.
[22] E. Glukhov, M. Stark, L.L. Burrows, C.M. Deber, Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes, J. Biol. Chem. 280 (2005) 33960–33967.
[23] D. Schneider, D.M. Engelman, Motifs of two small residues can assist but are not sufficient to mediate transmembrane helix interactions, J. Mol. Biol. 343 (2004) 799–804.
[24] L. Yang, T.A. Harroun, T.M. Weiss, L. Ding, H.W. Huang, Barrel-stave model or toroidal model? A case of study on melittin pores, Biophys. J. 81 (2001) 1475–1485.
[25] R.M. Epand, R.F. Epand, Lipids domains in bacterial membranes and the action of antimicrobial agents, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamem.2008.08.023.
[26] R.A. Houghten, General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 5131–5135.
[27] J.P. Tam, W.F. Heath, R.B. Merrifield, S_N2 deprotection of synthetic peptides with low concentration of HF in dimethyl sulfide: evidence and application in peptide synthesis, J. Am. Chem. Soc. 105 (1983) 6442–6455.
[28] H. Raghuraman, A. Chattopadhyay, Cholesterol inhibits the lytic activity of melittin in erythrocytes, Chem. Phys. Lipids 134 (2005) 183–189.
[29] R.B. Mikkelsen, K. Tanabe, D.F.H. Wallace, Membrane potential of Plasmodium-infected erythrocytes, J. Cell Biol. 93 (1982) 685–689.
[30] S. Kuga, Pore size distribution analysis of gel substances by size exclusion chromatography, J. Chromatogr. 206 (1981) 449–461.
[31] N. Jimenez-Juarez, C. Muñoz-Garay, I. Gomez, S.S. Gil, M. Soberon, A. Bravo, The pre-pore from *Bacillus thuringiensis* Cry1Ab toxin is necessary to induce insect death in Manduca Sexta, Peptides 29 (2008) 318–323.
[32] E. Schnepf, N. Crickmore, J. Van Rie, D. Lereclus, J.R. Baum, J. Feitelson, D.R. Zeigler, D.H. Dean, *Bacillus thuringiensis* and its pesticidal crystal proteins, Microbiol. Mol. Biol. Rev. 62 (1998) 775–806.
[33] R.A. de Maagd, A. Bravo, C. Berry, N. Crickmore, H.E. Schnepf, Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria, Annu. Rev. Genet. 37 (2003) 409–433.
[34] N.A. Broderinck, K.F. Raffa, J. Handelsman, Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 15196–15199.
[35] X. Zhang, M. Candas, N.B. Griko, R. Taissing, L.A. Bulla Jr., A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 9897–9902.
[36] J. Li, J. Carroll, D. Ellar, Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution, Nature 353 (1991) 815–821.
[37] D. Gerber, Y. Shai, Insertion and organization within membranes of the delta-endotoxin pore-forming domain, helix 4-loop-helix 5, and inhibition of its activity by a mutant helix 4 peptide, J. Biol. Chem. 275 (2000) 23602–23607.
[38] S. Leetachewa, G. Katzenmeier, C. Angsuthanasombat, Novel preparation and characterization of the alpha4-loop-alpha5 membrane-perturbing peptide from the *Bacillus thuringiensis* Cry4Ba delta-endotoxin, J. Biochem. Mol. Biol. 39 (2006) 270–277.
[39] T. Puntheeranurak, P. Uawithya, L. Potvin, C. Asgsuthanasombat, J.L. Schwartz, Ion channels formed in planar lipid bilayers by the dipteran-specific Cry4B Bacillus thuringiensis toxin and its alpha1-alpha5 fragment, Mol. Membr. Biol. 21 (2004) 67–74.
[40] T.G. Yudina, A.L. Brioukhanov, I.A. Zalunin, L.P. Revina, A.I. Shestakov, N.E. Voyushina, G.G. Chestukhina, A.I. Netrusov, Antimicrobial activity of different proteins and their fragments from *Bacillus thuringiensis* parasporal crystals against clostridia and archaea, Anaerobe 13 (2007) 6–13.
[41] Y. Abe, H. Shimada, S. Kitada, Raft-targeting and oligomerization of Parasporin-2, a *Bacillus thuringiensis* crystal protein with anti-tumour activity, J. Biochem. 143 (2008) 269–275.
[42] A. Uemori, A. Ohgushi, K. Yasutake, M. Maeda, E. Mizuki, M. Ohba, Parasporin-1Ab, a novel *Bacillus thuringiensis* cytotoxin preferentially active on human cancer cells in vitro, Anticancer Res. 28 (1A) (2008) 91–95.
[43] J.C. Quijano, V.V. Lemeshko, Hemoglobin precipitation by polyethylene glycols leads to underestimation of membrane pore sizes, Biochim. Biophys. Acta 1778 (2008) 2775–2780.
[44] K. Matsuzaki, Magainins as paradigm for the mode of action of pore forming polypeptides, Biochim. Biophys. Acta 1376 (1998) 391–400.
[45] H. Raghuraman, A. Chattopadhyay, Melittin: a membrane-active peptide with diverse functions, Biosci. Rep. 27 (2007) 189–223.
[46] T. Katsu, T. Imamura, K. Komagoe, K. Masuda, Simultaneous measurements of K^+ and calcein release from liposomes and the determination of pore size formed in a membrane, Anal. Sci. 23 (2007) 517–522.
[47] D. Eisenberg, Three-dimensional structure of membrane and surface proteins, Annu. Rev. Biochem. 53 (1984) 595–623.
[48] M. Dathe, T. Wieprecht, Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells, Biochim. Biophys. Acta 1462 (1999) 71–87.
[49] H. Leontiadou, A.E. Mark, S-J. Marrink, Antimicrobial peptides in action, J. Am. Chem. Soc. 128 (2006) 12156–12161.
[50] D. Sengupta, H. Leontiadou, A.E. Mark, S-J. Marrink, Toroidal pores formed by antimicrobial peptides show significant disorder, Biochim. Biophys. Acta 1778 (2008) 2308–2317.
[51] T.M. Clark, A. Koch, D.F. Moffett, The anterior and posterior 'stomach' regions of larval *Aedes aegypti* midgut: regional specialization of ion transport and stimulation by 5-hydroxytryptamine, J. Exp. Biol. 202 (1999) 247–252.
[52] D.Y. Boudko, L.L. Moroz, P.J. Linser, J.R. Trimarchi, P.J. Smith, W.R. Harvey, In situ analysis of pH gradients in mosquito larvae using non-invasive, self-referencing, pH-sensitive microelectrodes, J. Exp. Biol. 204 (2001) 691–699.
[53] L.M. Ruiz, C. Segura, J. Trujillo, S. Orduz, In vivo binding of the Cry11Bb toxin of *Bacillus thuringiensis* subsp. *medellin* to the midgut of mosquito larvae (Diptera: Culicidae), Mem. Inst. Oswaldo Cruz 99 (2004) 73–79.
[54] E. Gazit, P. La Rocca, M.S.P. Sansom, Y. Shai, The structure and organization within the membrane of the helices composing the pore-forming domain of *Bacillus thuringiensis* δ-endotoxin are consistent with an "umbrella-like" structure of the pore, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 12289–12294.
[55] M.E. Whalon, B.A. Wingerd, Bt: mode of action and use, Arch. Insect. Biochem. Physiol. 54 (2003) 200–211.
[56] M.S. Nair, D.H. Dean, All domains of Cry1A toxins insert into insect brush border membranes, J. Biol. Chem. 283 (2008) 26324–26331.
[57] K. Tomimoto, T. Hayakawa, H. Hori, Pronase digestion of brush border membrane-bound Cry1Aa shows that almost the whole activated Cry1Aa molecule penetrates into the membrane, Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 144 (2006) 413–422.
[58] V.V. Lemeshko, M. Arias, J.C. Quijano Perez, S. Orduz, Permeabilization of biomembranes with peptides derived from Cry11Bb protoxin, 51st Biophysical Society Meeting Abstracts, Biophys. J. Supplement (2007) 316a.