Mitochondria Permeabilization by a Novel Polycation Peptide BTM-P1*

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Victor V. Lemeshko‡§, Mauricio Arias‡, and Sergio Orduz¶
From the ‡Escuela de Física, Facultad de Ciencias, Universidad Nacional de Colombia, Sede Medellín, AA 3840 Medellín, Colombia, ‡Unidad de Biotecnología y Control Biológico, Corporación para Investigaciones Biológicas, AA 7378, Medellín, Colombia, and ¶Facultad de Ciencias Basicas, Universidad de Pamplona, Pamplona, Colombia

Bacillus thuringiensis subsp. medellin is known to produce the Cry11Bb protein of 94 kDa, which is toxic for mosquito larvae due to permeabilization of the plasma membrane of midgut epithelial cells. Earlier we found that a 2.8-kDa novel peptide BTM-P1, which was artificially synthesized taking into account the primary structure of Cry11Bb endotoxin, is active against several species of bacteria. In this work we show that BTM-P1 induces cyclosporin A-insensitive swelling of rat liver mitochondria in various salt solutions but not in the sucrose medium. Inorganic phosphate and Ca²⁺ significantly increased this effect of the peptide. The uncoupling action of BTM-P1 on oxidative phosphorylation was stronger in the potassium-containing media and correlated with a decrease of the inner membrane potential of mitochondria. In isotonic KNO₃, KCl, or NH₄NO₃ media, a complete drop of the inner membrane potential was observed at 1–2 μg/ml of the peptide. The peptide-induced swelling was increased by energization of mitochondria in the potassium-containing media, but it was inhibited in the NaNO₃, NH₄NO₃, and Tris-NO₃ media. All mitochondrial effects of the peptide were completely prevented by adding a single N-terminal tryptophan residue to the peptide sequence. We suggest a mechanism of membrane permeabilization that includes a transmembrane- and surface potential-dependent insertion of the polycation peptide into the lipid bilayer and its oligomerization leading to formation of ion channels and also to the mitochondrial permeability transition pore opening in a cyclosporin A-insensitive manner.

Mitochondria play a crucial role in the life and death of aerobic eukaryotic cells. They are a principal energy source in these cells (1) and also are responsible for some mechanisms of apoptosis and necrosis (2–6) as well as for ischemic damage of tissues (4, 7–10). Mitochondrial energy metabolism is very specific for cancer cells, which are characterized by the Crabtree effect (11–14), by a very high hexokinase activity of mitochondria (15, 16), and by high resistance to permeabilization of mitochondrial membranes by various pro-apoptotic peptides (2, 17–20). Nevertheless some peptides have been found to be toxic for cancer cells (21–28).

Mitochondrial voltage-dependent anion channel and its interaction with other proteins seem to be directly related to the metabolic specificity and permeabilization resistance of mitochondria in tumor cells. It has been reported that oncogenic Crk kinase inhibits cytochrome c release from mitochondria in growth factor-starved cells, and it has been hypothesized that the voltage-dependent anion channel mediates the switch to aerobic glycolysis (29). This hypothesis is consistent with theoretical models predicting a higher probability of the Crabtree effect in the case of decreased permeability of the mitochondrial voltage-dependent anion channel and a high amount of hexokinase II attached to the outer mitochondrial membrane (30, 31). Lithium chloride, which has an anti-proliferative effect on melanoma cells (32), induces detachment of hexokinase from mitochondria (33), and vice versa, the interaction of hexokinase II with voltage-dependent anion channel inhibits the release of cytochrome c from mitochondria induced by pro-apoptotic protein Bax (34). In this sense some synthetic polycation peptides are also able to inhibit the mitochondrial permeability transition (35).

Understanding the mechanisms of action of some types of proteins and peptides on the structural and functional state of mitochondria seems to be important for the development of apoptosis-regulating technologies, particularly of the anticancer therapy, and for the design of new classes of antibiotics, taking into account a certain similarity between mitochondria and bacteria. Among the positively charged peptides, mastoparan, a 14-amino acid amphipathic peptide from wasp venom, has been found to cause mitochondrial permeabilization (36). Mastoparan has been shown to take the α-helical conformation upon binding with phospholipid membrane (37) and to cause liposome permeabilization in a transmembrane potential-dependent manner (36) that seems to be the mechanism for mitochondria as well (38). Recently, it has been shown that the liposome permeation ability of eumenine mastoparan, a novel natural tetradecapeptide with a broad-spectrum inhibitory activity against Gram-positive and Gram-negative bacteria, is correlated with the amount of helical conformation in the membrane (39).

Most of the signal sequences of mitochondrial proteins with a net positive charge have been shown to induce potential-dependent swelling of mitochondria and to activate the mitochondrial multiple conductance channel (38, 40) that has been suggested to be distinct from both the classical permeability transition pore and the mastoparan-induced permeability increase (40). Many membrane-active peptides have been reported to function as a defense system against bacteria in plants and animals (41–43). Among them, the positively charged peptides magainins, secreted by the skin of the am-
A non-insecticidal and non-hemolytic the midgut proteases to yield a membrane-active polypeptide(s). At least some types of Cry family proteins are activated by proteins, particularly Cry11Bb, that is toxic for mosquito larvae.

Here we demonstrate various effects of a new polycation peptide (BTM-P1) on structural and functional state of rat liver mitochondria. The peptide is composed of 26 amino acid residues and derived from the primary structure of Cry11Bb protein of B. thuringiensis subsp. medellin. Its antibacterial activity has been recently demonstrated and related to the membrane toxic permeabilization. In the present work we show that BTM-P1 in low concentrations uncouples respiration and oxidative phosphorylation of rat liver mitochondria, causes the drop of the inner membrane potential, and induces mitochondrial swelling in various salt media. The permeabilization effect of the peptide is potential-dependent, increases with an increase in potassium concentration, and in general depends on the ionic strength and ionic composition of incubation medium. Permeabilizing activity of the peptide can be completely prevented by an additional N-terminal tryptophan residue (the peptide BTM-WP1 of 27 amino acid residues) that allows the suggestion of a possible mechanism of the potential-dependent permeabilization of biomembranes by BTM-P1 and other similar peptides.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sucrose, D-mannitol, Heps, Trizma base (Tris base), bovine serum albumin, (free of fatty acids, fraction V), cyclosporin A, rotenone, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), phosphoric and succinic acids, ADP, KCl, and other salts were purchased from Sigma. All chemicals were of analytical grade. The peptides BTM-P1 and BTM-WP1 were synthesized by Fundación Instituto de Immunología de Colombia (Bogota, Colombia) by the method of solid phase synthesis, according to the amino acid sequences proposed by some of the authors of this work. The peptides were purified by high pressure liquid chromatography, analyzed by mass spectrometry (matrix-assisted laser desorption ionization time-of-flight), lyophilized, and kept in powder at 4 °C until its use.

**Isolation of Mitochondria**—White male rats of 200–250 g starved for 12–14 h were used for isolation of liver mitochondria by the standard procedure as described earlier (48) with some modifications. Mitochondria were finally suspended in the medium composed of 210 mM mannitol, 70 mM sucrose, 20 µM EGTA, 10 mM Hepes-Tris, pH 7.2, with 0.3 mg/ml bovine serum albumin (free of fatty acids). Mitochondrial protein concentration was evaluated by the fast volumetric test (48) and finally determined by the biuret method after finishing all experiments.

**Mitochondria Permeabilization**—Mitochondria were characterized by a higher value of the inner membrane potential in the presence of 5 mM inorganic phosphate and oxidative phosphorylation of rat liver mitochondria almost up to the rate observed for the metabolic state 3 respiration (Fig. 1, a–c). Uncoupling activity of the peptide (Fig. 1c) was slightly less than that observed for 0.1 mM DNP (Fig. 1d). In the range of 1–4 µM, the peptide stimulated mitochondrial respiration in a dose-dependent manner (Fig. 1b). Higher concentrations, 5 µM or above, caused some inhibitory effect (data not shown).

The uncoupling activity of the peptide was significantly lower in the sucrose medium (Fig. 1, a and b) in comparison with that in the sucrose-KCl medium (Fig. 1, a and c). It was impossible to observe ATP synthesis with ADP/O = 1.44 (1.87 in the control) even in the presence of the peptide at a concentration of 2 µM in the sucrose medium (Fig. 1e), although the respiratory control ratio was significantly decreased (3.3 in comparison to 6.9 in the control). In the presence of 4 µM BTM-P1 in the sucrose medium, i.e. at 4 µg per 1 mg of mitochondrial protein, the respiratory rate may be further stimulated by 0.1 mM DNP (Fig. 1f) up to the value observed in the presence of DNP without the peptide (Fig. 1g).

The effect of BTM-P1 on the inner membrane potential increased with an increase of concentration of KCl in isotonic incubation media (Fig. 2, A–C). Only a slight decrease of the inner membrane potential was caused by 1 µM BTM-P1 (2 µg per 1 mg of mitochondrial protein) in the sucrose medium with or without inorganic phosphate (Fig. 2A), but almost complete deenergization occurred in the KCl medium (Fig. 2C). A higher effect of the peptide was observed in the presence of inorganic phosphate in the KCl medium, where even 1 µg/ml of BTM-P1 caused biphasic and a complete drop of the inner membrane potential (Fig. 2C, b). In the sucrose medium the effect of BTM-P1 was also higher in the presence than in the absence of inorganic phosphate, but only after a third addition of the peptide, up to final concentration of 3 µg/ml (Fig. 2A, b).

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phosphate in all used media (Fig. 2, A–D). As shown in Fig. 2B (curve b), the addition of inorganic phosphate to the energized mitochondria in the sucrose-KCl medium caused a further increase of the inner membrane potential that resulted from a decrease of the transmembrane $\Delta p$H caused by $\Delta p$H-dependent phosphate transport with subsequent recovery of the proton electrochemical gradient by the respiratory chain function, mainly due to an increase in the transmembrane electrical potential. Without inorganic phosphate, the effect of $1 \mu$g/ml BTM-P1 on the inner membrane potential of mitochondria in the sucrose-KCl medium was only slightly less than in the KCl-medium (Fig. 2, B, a, and C, a, respectively).

Significantly different, biphasic kinetics of the potential drop were observed in these media with inorganic phosphate after the addition of the peptide; the first phase of depolarization of the inner mitochondrial membrane in the KCl medium was followed by a second phase of further autacatalytically increased membrane permeabilization (Fig. 2C, b). In the sucrose-KCl medium, in contrast, the first phase of depolarization was changed by a repolarization process (Fig. 2B, b) that looks like an excitable membrane behavior after under-threshold stimulus.

A depolarization-repolarization process was also observed in the NH$_4$NO$_3$ medium with inorganic phosphate; a small decrease of the inner membrane potential was observed just after the addition of $1 \mu$g/ml BTM-P1, but very soon its value was almost completely recovered (Fig. 2D, b). A higher depolarization effect, with a subsequent tendency to recover the inner membrane potential, occurred in the medium without phosphate (Fig. 2D, a). Although partial (Fig. 2B, b) or complete recovery (Fig. 2D, b) of the inner membrane potential was observed after the first addition of $1 \mu$g/ml of BTM-P1, the second addition of $1 \mu$g/ml of the peptide (i.e. to the final concentration of $4 \mu$g per 1 mg of mitochondrial protein) caused fast and complete depolarization of the membrane.

The inner membrane potential generated by ATP hydrolysis was also altered by the peptide, with almost complete depolarization after the second addition of $1 \mu$g/ml BTM-P1. A somewhat higher sensitivity of mitochondria to the peptide was observed after the first addition of $1 \mu$g/ml of BTM-P1, the second addition of $1 \mu$g/ml of the peptide (i.e. to the final concentration of $4 \mu$g per 1 mg of mitochondrial protein) caused fast and complete depolarization of the membrane.

To evaluate the character and the extent of permeabilization effect of BTM-P1 on the inner membrane of mitochondria, the mitochondrial swelling was studied in the media composed of sucrose or various salts buffered with 10 mM Heps-Tris, pH 7.2, and supplemented with $50 \mu$g EGTA to decrease a probability of calcium- and phosphate-induced permeability transition. The obtained data show that $4 \mu$g/ml of BTM-P1 had no appreciable induction of the swelling of energized or non-energized mitochondria in the sucrose medium (Fig. 3, Sucrose, curves c and $a$, respectively). A small induction, higher in the case of energized mitochondria, was observed in the sucrose-KCl medium (Fig. 3, Sucrose-KCl). A significant effect of the peptide was observed in the KCl medium, and the swelling of high amplitude occurred in the KNO$_3$ medium, with the rate of
swelling significantly higher for energized than non-energized mitochondria (Fig. 3, KCl and KNO₃, curves b and a, respectively). The difference in the rate and the extent of the swelling in the KCl and KNO₃ medium may be related to the known higher permeability of the inner membrane to NO₃⁻ than to Cl⁻ ions (1). Cyclosporin A did not influence mitochondrial swelling induced by BTM-P1 in the potassium containing medium, as shown in Fig. 3 for the KNO₃ medium.

An opposite effect of the inner membrane energization on mitochondrial swelling, induced by BTM-P1, was obtained using NH₄NO₃, NaNO₃, and Tris-NO₃ incubation media (Fig. 3). Fast swelling of high amplitude was induced by the peptide in the case of non-energized but not energized mitochondria in the NH₄NO₃ medium (Fig. 3, NH₄NO₃, a and b, respectively). The effect of the peptide was significantly higher than that caused by a completely uncoupling concentration of FCCP under the same conditions (Fig. 3, NH₄NO₃, c). Cyclosporin A diminished but not prevented the swelling of non-energized mitochondria in the NH₄NO₃ medium under the influence of BTM-P1 (Fig. 3, NH₄NO₃, +CsA). High amplitude swelling of non-energized mitochondria induced by the peptide was also observed in the NaNO₃ medium (Fig. 3, NaNO₃, a), and a minor effect was registered in the Tris-NO₃ medium (Fig. 3, Tris-NO₃, a).

BTM-P1 did not induce swelling of energized mitochondria in the NH₄NO₃ medium (Fig. 3, NH₄NO₃, b) at least during 4–5 min of incubation without or with cyclosporin A, except an initial swelling of low amplitude. The peptide induced a fast semi-maximal swelling of energized mitochondria in the NaNO₃ medium (Fig. 3, NaNO₃, b) as well as less profound biphasic swelling in the Tris-NO₃ medium (Fig. 3, Tris-NO₃, b).

The data in Fig. 4A show that Ca²⁺ induces mitochondrial swelling in the sucrose (curve a) or sucrose-KCl (curve d) media with inorganic phosphate. BTM-P1, which was added before Ca²⁺, after phosphate (Fig. 4A, b), or before both Ca²⁺ and phosphate (Fig. 4A, c) in the sucrose medium or after phosphate and Ca²⁺ in the sucrose-KCl medium (Fig. 4A, e) significantly accelerated swelling of energized mitochondria. At a concentration of 2 μg/ml in the sucrose-KCl medium with inorganic phosphate, BTM-P1 induced swelling of energized (Fig. 4A, g) but not deenergized mitochondria (Fig. 4A, f). Note, in the sucrose-KCl medium without phosphate, the peptide, even at a concentration of 4 μg/ml did not cause a significant swelling of energized or non-energized mitochondria (Fig. 3, Sucrose-KCl).

The swelling of energized mitochondria may be also initiated by inorganic phosphate added after Ca²⁺ (Fig. 4B, a). FCCP, added to mitochondria before Ca²⁺ and phosphate, completely prevented not only Ca²⁺ and phosphate-dependent swelling but also the swelling of high amplitude induced by the subsequent addition of 4 μg/ml BTM-P1 (Fig. 4B, c). In contrast to FCCP, BTM-P1, added at 2 μg/ml before Ca²⁺ and phosphate, significantly accelerated the Ca²⁺ and phosphate-induced swelling (Fig. 4B, b), in comparison to the control without the peptide (Fig. 4B, a). FCCP, added together with mitochondria in the BTM-P1-containing medium, was able to completely prevent this type of accelerated swelling (Fig. 4B, d).

The presence of phosphate in the KNO₃ incubation medium increased the sensitivity of mitochondria to a small concentration of BTM-P1, 0.5 μg/ml (0.18 μM), resulting in a biphasic
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Fig. 5. Influence of the peptides BTM-P1 and BTM-WP1 on the inner membrane potential (a and b) and swelling (c and d) of rat liver mitochondria. Mitochondria, 0.5 mg of protein/ml (Mce), and 2.5 μM rotenone (R) were added to the incubation medium composed of 125 mM KNO₃, 50 μM EGTA, 10 mM Hepes-Tris buffer, pH 7.2, and supplemented with 5 mM succinate-Tris, pH 7.2, and 10 μM safranin O was added to monitor the inner membrane potential; 3WP and 5WP, 2 and 3 μg/ml BTM-WP1, respectively; FCCP, 1 μM FCCP; 2P and 3P, 2 and 3 μg/ml BTM-P1, respectively.

mitochondrial swelling (Fig. 4B, f) in comparison to the control without phosphate (Fig. 4B, e). The first phase of swelling seems to be due to direct permeabilization of the inner mitochondrial membrane to potassium; meanwhile, the second phase seems to be related to the permeability transition pore opening (Fig. 4B, f).

To directly form channels in biological membranes, the peptide BTM-P1 should cross the lipid bilayer by its N- or C-terminal sides in a potential-dependent manner according to the obtained data. We assumed that such probability is higher for the N-terminal side because of its higher hydrophobicity. To decrease the probability of a transmembrane movement of the N-terminal side of the peptide, a tryptophan residue was added to a new peptide BTM-WP1. The tryptophan indole rings have been found to be preferentially positioned near the lipid carbonyl moieties in the membrane-water interface (51, 52). The interaction of synthetic tryptophan-flanked peptides with the phosphatidylcholine bilayer is energetically stronger than the hydrophobic matching, although generally it also depends on the membrane lipid composition (53). On the other hand, the flanked tryptophan residues of those peptides were not the first or the last, i.e. neither an extreme N-terminal side nor an extreme C-terminal side. We assume that the strength of the interfacial anchoring of the N-terminal tryptophan in the peptide BTM-WP1 might be even higher than due only to the preferred position of the indole ring near the lipid carbonyl moieties because the terminal amino group of the same tryptophan residue might be electrostatically attracted by a nearest glycerophosphocholine group in the membrane-water interface (54), where the dielectric constant is lower than in the water. Even a free tryptophan has been shown to dramatically affect the structural state of biomembranes and lipid bilayer of liposomes during freezing (55) that might be caused by a tandem action of its indole ring and the amino group. The results of the comparative study of the BTM-P1 and BTM-WP1 influence on the inner membrane potential and on the swelling of energized mitochondria in the KNO₃ medium are shown in Fig. 5.

No effect of BTM-WP1 on the inner membrane potential has been observed even at the concentration of 5 μg/ml peptide, whereas the addition of only 2 μg/ml BTM-P1 or 1 μM FCCP (Fig. 5, a and b, respectively) caused complete depolarization of the membrane. BTM-WP1, at concentrations up to 6 μg/ml, did not influence mitochondrial swelling in the KNO₃ medium (Fig. 5c). In contrast, the peptide BTM-P1, at a concentration of 3 μg/ml, was able to cause a fast and high amplitude swelling of mitochondria in the same conditions (Fig. 5d). Both results indicate that a very simple modification of BTM-P1 can dramatically change its permeation activity, although the peptide remains as an amphiphatic polycation.

**DISCUSSION**

Cry11Bb crystal protoxin is synthesized by *B. thuringiensis* subsp. *medellin* as a 94-kDa protein composed of 782 amino acid residues (56), and its three-dimensional structure with nine α helices in the domain I has been proposed earlier (57). This protein is toxic for mosquito larvae (44, 56) after activation by mosquito larvae midgut proteases (58) and further membrane binding and permeabilization of the midgut epithelial cells (59). It has not been clear up to now which are the smallest fragments of the Cry11Bb protoxin that can permeabilize biomembranes, although Segura et al. (58) proposed that the 94-kDa protoxin is activated to form 30- and 35-kDa fragments. In the case of a 70-kDa Cry11Aa protoxin, for instance, the coexistence of the 32- and 36-kDa fragments has been recently reported to be essential for toxicity (60).

We assumed that a very short fragment, VAPIAKYLATALKWALKQGGFAKLLKS (2.8 kDa), provided from the primary structure of Cry11Bb protein and containing the α2a helix of its domain I, might be membrane-active. This fragment has been artificially synthesized as the peptide BTM-P1, and its antimicrobial activity and a capacity to induce mitochondrial swelling have been demonstrated.²

The data obtained in this work show that BTM-P1 uncouples respiration and oxidative phosphorylation of rat liver mitochondria. The uncoupling activity was significantly higher in the sucrose-KCl than in the sucrose medium (Fig. 1), which might be related to higher ionic strength of the sucrose-KCl medium or/and to the presence of potassium, which could penetrate the inner membrane in a voltage-dependent manner if the peptide forms potassium-permeable channels. Interestingly, at small concentrations of BTM-P1 (2 μg per 1 mg of mitochondrial protein), it was still possible to observe ATP synthesis in the sucrose medium with a relatively high coefficient ADP/O and a significantly decreased respiratory control ratio (Fig. 1e).

The peptide BTM-P1 demonstrated higher uncoupling activity than some of the magainins. For comparison, a 23-amino acid residues magainin II amid, a synthetic carboxyamidated derivative of a natural polycation peptide, has been reported to completely uncouple oxidative phosphorylation at a concentration of 20–30 μg/ml and to cause nearly 50% inhibition of the FCCP-uncoupled respiration of rat liver mitochondria in sucrose medium with 5 mM succinate as substrate of oxidation (41). Magainins and their active analogues are known to form an amphiphilic α helix and to permeabilize biological membranes as well as to induce channels in phospholipid membranes with slight specificity for anions (see Ref. 41 and references therein).

BTM-P1 at low concentrations dissipated the inner membrane electrical potential generated by the respiratory chain or by H⁺-ATPase in the rat liver mitochondria. This effect of BTM-P1 is increased with an increase in concentration of KCl in isotonic incubation medium by a partial or complete substitution of sucrose with a corresponding amount of KCl. The ionic strength of the incubation medium seems to be one of the factors favoring ionophoric activity of the peptide because the high depolarization effect of BTM-P1 was also observed in isotonic choline-chloride (data not shown) and KNO₃ media (Fig. 5). Such behavior of BTM-P1 differs from that reported for some of positively charged signal sequences of mitochondrial proteins, which activated the mitochondrial multiple conductance channel and induced cyclosporin A-insensitive, potential-
dependent permeabilization of mitochondria at concentrations of KCl lower than 50 mM; higher concentrations of KCl inhibited all these effects (40).

The dependence of the permeabilization effect of positively charged peptide BTM-P1 on the ionic strength of incubation medium might be explained taking into account the electrical field between the water bulk phase and the membrane surface, resulting from the electrical double layer of biomembranes. A surface electrical potential of approximately −60 mV was estimated for a bilayer lipid membrane containing 25–30 mol% of anionic lipids (61). According to the Guoy-Chapman theory, the thickness of the electrical double layer strongly depends on the ionic strength of the medium. The thickness of the electrical double layer in the 125 mM KCl solution is more than 3 times shorter than in 10 mM KCl (62). The average tension of the electric field near the membrane surface, within the calculated Debye-Hückel length $\delta_1 = 0.84$ nm for the KCl-medium, is 3.7 times higher than in the sucrose-medium ($\delta_2 = 3.09$ nm), both buffered with 10 mM Hepes-Tris (Fig. 6, qualitative curves a and b, respectively).

A higher tension of the near membrane electric field might be important for an adequate orientation of a peptide to enter into the membrane, presumably with the N-terminal side in the case of the peptide BTM-P1. For example, a simple dipole with the length of 3 nm and charges equal to $+1$ and $-1$, situated in the electric field of the near membrane space in the KCl medium, should be oriented perpendicularly to the membrane with the energy, which is 5.2 times higher than $kT$ ($k$ is Boltzmann constant, and $T = 302$ K). An additional factor favoring the insertion of the polycationic peptide BTM-P1 into a membrane is its net charge of 4$^+$. The peptide-motive force to insert BTM-P1 into a membrane is proportional to the net charge of the peptide and to the near membrane electric field tension.

A lower uncoupling efficiency of BTM-P1 in the sucrose medium could also be related to its electrostatic adsorption on mitochondrial membranes, as it is known for cytochrome c. Membrane adsorption of the positively charged cytochrome c decreased its electron transport activity in a low ionic strength incubation medium (49, 63). Electrostatic binding of various polycations, including peptides, with anionic phospholipids of biomembranes has been suggested to induce the release of pro-apoptotic and other intermembrane proteins from mitochondria by a direct damage of the outer mitochondrial membrane or by opening of the mitochondrial permeability transition pore (64).

The peptide BTM-P1 did not induce mitochondrial swelling in the sucrose medium (Fig. 3) that was different from the effect of 3 µM positively charged peptide mastoparan, which induced cyclosporin A-insensitive swelling of rat liver mitochondria in a similar incubation medium and caused potential-dependent permeabilization of liposomes (36). On the other hand, BTM-P1 induced mitochondrial swelling in high ionic strength media with the rate, amplitude, and even the kinetics of swelling depending on the type of salt used and on the metabolic state of mitochondria. Very high amplitude of swelling was observed in the KNO$_3$, NH$_4$NO$_3$, and NaNO$_3$ media (Fig. 3). The effect was lower in the sucrose-KCl medium and significantly increased when sucrose was completely substituted with KCl. Relatively low amplitude and rates of swelling were observed in the Tris-NO$_3$ medium, indicating that the channels formed by BTM-P1 in the inner mitochondrial membrane have a restricted permeability for the cation Tris$^+$, a protonated form of Tris.

An interesting phenomenon observed in our experiments is that the rate of the BTM-P1-induced swelling was dramatically increased by energization of mitochondria incubated in the KCl or KNO$_3$ media, but an opposed effect was observed in the NH$_4$NO$_3$ or Tris-NO$_3$ media (Fig. 3). The mechanisms of the BTM-P1-induced swelling might be different; the electrogenic transport through the formed channels for K$^+$ ions but not for NH$_4^+$ ions per se. At least the channels formed by BTM-P1 seem to be less permeable for NH$_4^+$ than for potassium ions, because 1 µg/ml BTM-P1 caused complete depolarization of the inner mitochondrial membrane in the KCl medium with or without inorganic phosphate (Fig. 2C) as well as in the KNO$_3$ medium (data not shown), and only slight transient depolarization was observed in the NH$_4$NO$_3$ medium with inorganic phosphate (Fig. 2D, b). Because it is well known, a lipid bilayer is permeable for NH$_4^+$; thus, the flux of NH$_4^+$ through a membrane depends on the permeability of the membrane for H$^+$ (1). The difference of pH between the mitochondrial matrix and the external medium should also affect the NH$_4^+$ flux, because a relatively high pH in the mitochondrial matrix will decrease NH$_4^+$ and increase NH$_3$ concentrations in it. On the other hand the steady state flux of protons from the mitochondrial matrix to the external medium, maintained by the respiratory chain functioning, might result in extrusion of NH$_4^+$ ions back to the incubation medium, thus actively preventing the high amplitude swelling of mitochondria in the NH$_4$NO$_3$ medium. In addition, energization of mitochondria should inhibit in a potential-dependent manner the entrance of the anions NO$_3^-$ into the mitochondrial matrix.

The influence of energization on mitochondrial swelling in the Tris-NO$_3$ medium might be explained in the same manner, assuming that the membrane permeability for Tris$^+$ also depends on the proton permeability of the inner membrane, similar to NH$_4^+$. In the NaNO$_3$ medium the initial rate of swelling was somewhat higher for energized mitochondria, but further swelling was subsequently inhibited, thus resulting in lower amplitude of swelling than the observed for non-energized mitochondria (Fig. 3). This swelling inhibition might be explained if a Na$^+$/H$^+$ antiport is activated during the initial phase of mitochondrial swelling, thus allowing subsequent ΔpH-dependent extrusion of sodium ions and potential-dependent extrusion of NO$_3^-$ anions from the energized mitochondria, as a mechanism preventing the high amplitude swelling.

Mastoparan has been shown to accelerate the phosphate- and Ca$^{2+}$-dependent mitochondrial swelling being added to the energized mitochondria after Ca$^{2+}$ overloading (36). This effect might be related to the inner membrane potential drop in the presence of this peptide (36), like the caused by FCCP, allowing permeability transition pore opening (65). The influence of mastoparan addition before phosphate and Ca$^{2+}$ on mitochondrial swelling has not been studied. FCCP, if added together with mitochondria, prevented permeability transition pore opening by Ca$^{2+}$ and phosphate (Fig. 4B, c) or by BTM-P1, Ca$^{2+}$, and phosphate (Fig. 4B, d) because it prevents mitochon-
Mitochondria sensitivity to a very small concentration of BTM-P1 (0.18 nM) has been demonstrated. Inorganic phosphate increased mitochondrial permeabilization in its presence (Fig. 2) have been demonstrated. Accelerated mitochondrial permeabilization (Fig. 4), in contrast to FCCP, although uncoupling activity of BTM-P1 (Fig. 1) and the inner membrane potential drop in its presence (Fig. 2) have been demonstrated. Inorganic phosphate increased mitochondrial sensitivity to a very small concentration of BTM-P1 (0.18 μM). The peptide seems to act as Ca\(^{2+}\) in the permeability transition pore opening. After the suggestion of Pfeiffer et al. (36) for mastoparan, the polycation peptide BTM-P1 might occupy the transition pore of the permeability transition pore, thus activating permeability transition pore opening like Ca\(^{2+}\) and preventing cyclosporin A binding as reported for very high concentrations of Ca\(^{2+}\) (67).

Contrary to BTM-P1, the peptide BTM-WP1 did not affect the inner membrane potential and did not induce swelling of even energized mitochondria in the K\(_{3}\)HPO\(_{4}\) medium (Fig. 5). The anchoring caused by an additional N-terminal tryptophan residue, as mentioned above, seems to prevent the transmembrane movement of the N-terminal side of BTM-WP1 (Fig. 7D) and subsequent formation of the transmembrane pore.

In addition to demonstrating the biological activity of BTM-P1, our data allow speculation as to a possible mechanism of permeabilization caused by Cry protoxins at the level of the plasma membrane of midgut epithelial cells. According to the “umbrella model” (68), the pore formation by Cry family proteins results from dimerization (oligomerization) of their domains I, where only α4 and α5 helices are inserted in the membrane, and the remaining helices of the domain I are situated in the water space (umbrella). We suggest the hypothesis of “damaged umbrella oligomerization,” according to which the α2 helix or a fragment including the α2 helix, like BTM-P1, could insert into the membrane maybe after α1 helix cleavage (47), thus allowing formation of oligomers with participation of α2, α4, and α5 helices. Also, it seems probable that at certain stage of proteolytic processing of the protoxin, a fragment, which includes the α2 helix, appears in the cytoplasm, transiently passing across the plasma membrane in oligomeric form, and reaches mitochondria to cause the mitochondrial membranes permeabilization, thus inducing apoptosis of the midgut epithelial cells.

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