Mastoparan Dissipates Mitochondrial Transmembrane Potential in the Physiological (ADP-LIKE) Range

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Abstract: Experimental studies of antimicrobial peptides as potential drugs having a pore-forming mechanism of action have attracted increasing interest for clinical applications due to their broad spectrum of activity. Here in a short report, we describe an experimental approach for testing potential drugs using rat liver mitochondria as model and pore-forming peptide mastoparan. We have used tightly coupled mitochondria and a sensitive oximetric cell to study activation of mitochondrial respiration by mastoparan and showed limited dissipation of their transmembrane potential in the presence of this peptide. Mastoparan stabilized this potential synchronously with stabilization of mitochondrial State 4 respiration rate (v_4). It was concluded that mastoparan dissipates mitochondrial transmembrane potential only in the physiological (adenosine diphosphate (ADP) -like) range. The comparison of side effects of pore-forming peptides on mitochondrial transmembrane potential may be useful for toxicity testing of new drugs.

Keywords: Antimicrobial peptides, Mastoparan, Alamethicin, Rat liver mitochondria, Mitochondrial transmembrane potential.

INTRODUCTION

Mitochondria are the main intracellular power stations in the living cell and transmembrane potential generated by proton pumps (Complexes I, III and IV) is an essential component in the process of energy storage during oxidative phosphorylation [1]. The level of this potential and adenosine triphosphate (ATP) in the cell are kept relatively stable although there are limited fluctuations of both factors reflecting normal physiological activity [1]. However radical and sustained changes of these factors and damage of mitochondrial functions by any medicine inevitably leads to metabolic disorders. In recent years extensive experimental and medical experience in the use of pore-forming peptides as antimicrobial agents has been obtained. However, the lack of simple analytical tools for studying mechanisms of interaction of pore-forming peptides with biological membranes remains a problem. The liver is the main organ of detoxification of substances, including drugs that are eliminated from the body. Therefore, liver mitochondria are a good model for studying side effects of drugs and their hepatotoxicity. Mastoparan and its analogs displayed a broad-spectrum antimicrobial activity against bacteria and fungi and were highly potent against antibiotic-resistant bacteria [2]. The mastoparans are 14 amino acids in length with the C-terminus amidated and rich in hydrophobic and basic amino acids, which leads to amphipathic chemical character, adopting α-helical secondary structure under proper conditions [3]. Due to biological activities, showing histamine releasing from mast cells, antimicrobial and hemolytic activities, mastoparans have been developed for pharmacological research and for therapeutic use, in particular, as antimicrobial agents [4-6]. The mastoparan peptide is known as an inducer of the mitochondrial permeability transition. Although mastoparan was suggested to interact with a proteinaceous target in mitochondria to induce this transition, the mechanism of mastoparan action have not yet been investigated [7].

MATERIAL AND METHODS

In the course of the experiment the following reagents were used: rhodamine 123, adenosinediphosphate disodium salt (ADP), 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Hepes) (Sigma), carbonylcyanide(4-trifluoromethoxy)phenylhydrazone

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(FCCP) (Aldrich), – 3,5-di-*tert*-butyl-4-oxybenzylidenemalononitrile (SF) (Sumitomo Chem. Co.), *Trichoderma viride* alamethicin (Fluka), sucrose (Merck), KCl, KH_2PO_4, $MgCl_2$, ethylenediaminetetraacetic acid (EDTA), succinic acid of special pure grade (Reakhim), tris(hydroxymethyl)aminomethane (Tris), rotenone, horse cytochrome c, Serva Blue G (Serva). Mastoparan from *Vespa orientalis* (INLKAIAALVKKVL(NH_2)) was isolated as shown in [12].

For isolation of tightly coupled intact rat liver mitochondria the modified Weinbach method was used (centrifugation: 5500g for 10 minutes), while all fractions of these organelles are sedimented at 12000g [8]. Modification of Bradford method was used for determination of mitochondrial protein [9]. Rates of succinate oxidation of rat liver mitochondria (v) and the mitochondria respiratory control (RC) ratio were determined in thermostatic cell at 25ºC with quick response oxymetric electrode [10]. In the presence of succinate in all used incubation media the Chance RC was about 5.0 -5.2. Mitochondrial incubation medium (pH 7.2) contained 4 μM horse cytochrome *c*, 30 mM Tris, 1.5 μM rotenone, 125 mM sucrose, 2 mMEDTA, 10 mM KH_2PO_4, 10 mM sodium succinate, 6 mM $MgCl_2$,. Transmembrane mitochondrial potential was determined in the same media as control oximetric measurements, using Shimadzu RF-5301 PC fluorometeras described in [11]. We showed that rhodamine 123 had no influence on the respiration ratesin the absence and in the presence of mastoparan. The principle of this method is that under the influence of the potential the amphiphilic charged rhodamine 123 enters the membrane and in its hydrophobic environment the dye changes its fluorescence spectrum [11]. The authors gave calibration dependence between the value of artificially created transmembrane potassium potential of rat liver mitochondrial membrane and the value of altered fluorescence. We used this linear relationship in the work because we isolated rat liver mitochondria using the same technique as the authors of the paper [11] and used the same incubation medium. The empirical equation $\Delta F/F = (\Delta\Psi - 60)/323$ [11] was used for calculation of the value of potential ($\Delta\Psi$), where ΔF was the difference between fluorescence values obtained before and after addition of protonophore SF, F was fluorescence in the presence of SF. Oximetric effects of mastoparan and alamethicin were normalized by the initial rate of mitochondrial respiration (v_0). Monocationic media in which all cations except magnesium were replaced by K^+ (potassium medium) or Li^+ (lithium medium) and 10 mM Hepes instead of Tris were used. The scheme of oximetric experiment: we successively added pore forming peptide (mastoparan or alamethicin) in several concentrations and a protonophore (FCCP) to an oximetric cell containing the mitochondrial incubation medium and suspension of mitochondria. The addition of FCCP showed that the transmembrane potential of mitochondria was partially preserved, since this protonophore caused significant activation of respiration. The scheme of fluorometric experiment: to a cell containing the mitochondrial incubation medium and 0.4 μMrhodamine 123 we successively added a suspension of mitochondria and pore forming peptide in several concentrations, measuring the changes in fluorescence.

RESULTS AND DISCUSSION

Earlier, it was shown that the transmembrane cationic current induced in rat liver mitochondria by various inducers of membrane permeability linearly depends on the activation of the mitochondrial respiration [13]. Figure **1A** shows activation of mitochondrial respiration by addition of mastoparan in monocationic potassium and lithium incubation media, Figure **1B** shows changes in relative rhodamine 123 caused fluorescence, proportional to mitochondrial transmembrane potential. In both incubation media mastoparan stabilized transmembrane potential synchronously with stabilization of mitochondrial v4 activation. In many repetitions we observed that this potential stabilized and reached a plateau in the presence of mastoparan in 2-5 (up to 10) minutes. Unlike this another peptide alamethicin continuously decreased transmembrane potential for critical values (see Figure **1B**, the insert) synchronous tomitochondrial v4 activation (not shown). Mastoparan and alamethicin (in the highest concentrations used) activated mitochondrial v4 to a level stable for 20 min and mitochondria appeared to be stable during this interval of time. A close results for physiologically different cations such as potassium and lithium (toxicity of lithium on isolated mitochondria was shown in [14]), indicated that the effect of the pore-forming peptides on mitochondria was associated precisely with induced permeability of mitochondrial inner membrane. In the control measurements the value of activation of mitochondrial v4 in the presence of protonophore SF in potassium medium was equal to the same value in lithium medium (data not shown).

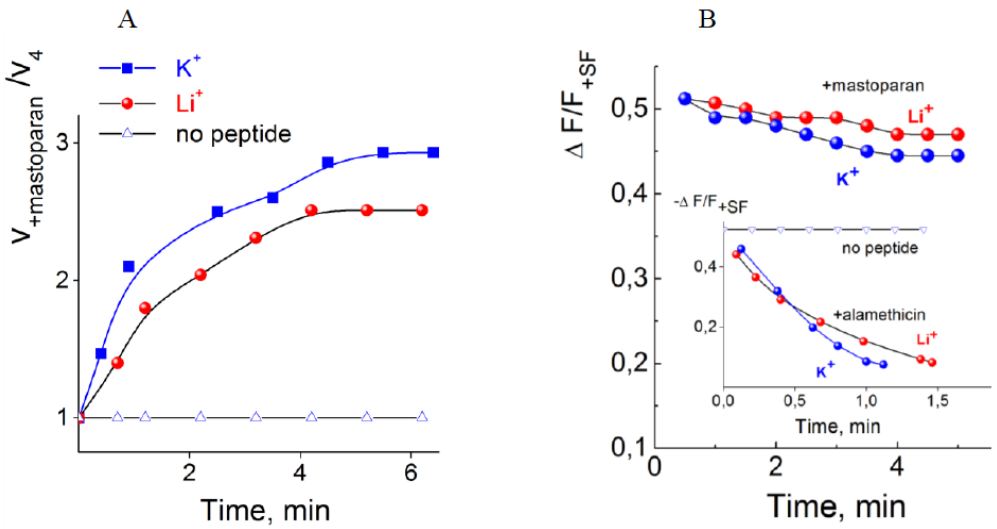

Figure 1: A. Time-course of activation of mitochondrial v4 respiration by 500 nM mastoparan in potassium medium (K^+) and lithium medium (Li^+) (A), and time course of relative rhodamine 123 fluorescence changes (proportional to mitochondrial trans membrane potential), the inset demonstrates data in the presence of 100 nM alamethicin in potassium medium (K^+) and lithium medium (Li^+) (**B**). The concentration of mitochondria was 0.25 mg/ml. F - fluorescence in the presence of SF. Plots were representative for three independent preparations of mitochondria.

As was shown earlier the value (v+peptide/v4) was proportional to cationic current induced by pore-forming peptides at stabilized potential using mitochondrial preparations [13]. In comparison: complex procedures were required for maintenance of constant liposomal transmembrane potential [15]. In contrast to liposomal the corresponding potential generated by mitochondrial respiratory chain existed for several minutes. Under these conditions, the peptide self-association order was measured only for peptides in transmembrane conformation. The effect of pore-forming peptides was accompanied by decrease of mitochondrial transmembrane potential. However, the magnitude of activation of mitochondrial v4 by different peptides did not correlate with this effect on transmembrane potential.

Mitochondria play an important role in cell homeostasis. Mitochondrial transmembrane potential is a driving force for transport of ions and proteins which are necessary for mitochondrial functioning. It is difficult to determine the “optimal” values of transmembrane potential for the cells and mitochondria, however a long-lasting drop or rise of its normal levels may induce unwanted loss of cell viability and be a cause of various pathologies [1]. Physiological activator of respiration – ADP also dissipates mitochondrial transmembrane potential in the normal conditions of the living cell. A degree of this dissipation as compared with protonophore FCCP varies from 0.2 to 0.25 % accordingly to the method of transmembrane potential determination [16, 11]. However such dissipation is not critical for the steady internal physical and chemical conditions maintaining by the living cell.

CONCLUSION

It was earlier shown that in the presence of melittin mitochondria retained stable transmembrane potential in contrary to alamethicin and 1,7,21,23-tetraacetylmelittin [13]. Here we have shown that in the presence of mastoparan transmembrane potential stabilized and reached a long plateau in a few minutes. Mastoparan dissipated mitochondrial potential only in the physiological (ADP-like) range and for this reason this peptide may be less harmful in therapeutic use than peptides radically dissipating potential. Pore-forming peptides are promising drugs. In the course of comparative testing of pore-forming peptides on their potential toxicity, the active membrane concentration of the peptide, activating mitochondrial v4 by 200% and concentration of peptide causing lysis of mitochondria must also be taken into account.

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