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Effect of Triclosan on the Functioning of Liver Mitochondria and Permeability of Erythrocyte Membranes of Marsh Frog (*Pelophylax ridibundus* (Pallas, 1771))

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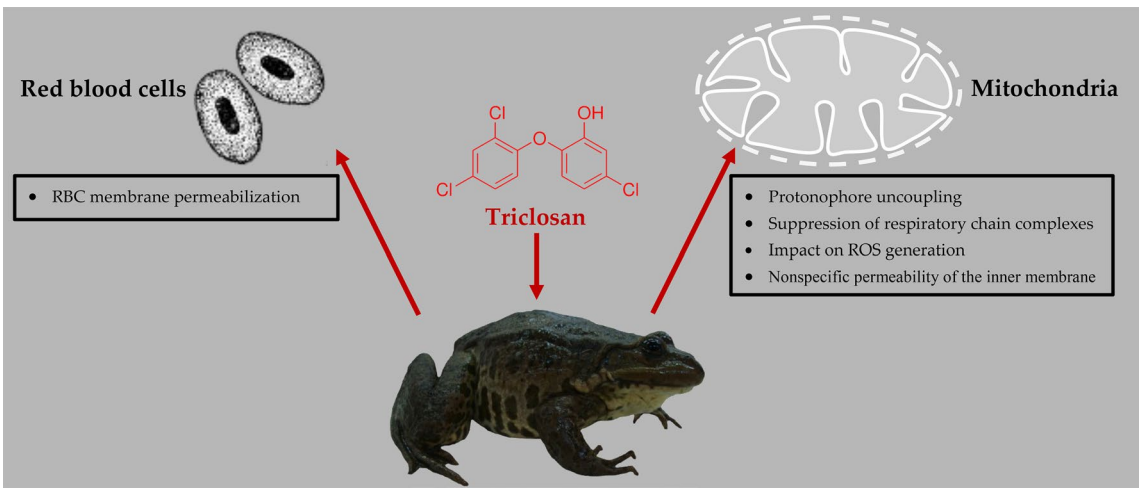
Abstract

The paper examines the effects of the antimicrobial agent triclosan on the functioning of the liver mitochondria of marsh frog (*Pelophylax ridibundus* (Pallas, 1771)). It was established that triclosan inhibits DNP-stimulated respiration of mitochondria and decreases respiratory control ratio. In addition, triclosan causes the collapse of the mitochondrial membrane potential on both types of substrates. Such an action of triclosan can be mediated by both a protonophore effect and suppression of the activity of complex II and combined activity of complexes II + III (and, to a lesser degree, the combined activity of complexes I + III) of the mitochondrial respiratory chain. It is shown that high concentrations of triclosan enhance the production of hydrogen peroxide during the oxidation of substrates of the complex I by mitochondria, and decrease it in the case of succinate oxidation. It is found that triclosan is able to induce nonspecific permeability of the liver mitochondria of these amphibians, as well as the plasma membrane of erythrocytes. The possible mechanisms of triclosan effect on marsh frog liver mitochondria and red blood cells are discussed.

Graphic Abstract



Keywords Triclosan · Toxicology · Mitochondria · *Pelophylax ridibundus* · Erythrocytes · Membrane permeability

Abbreviations

TCS	Triclosan
CsA	Cyclosporine
MPT	Mitochondrial permeability transition
RBC	Red blood cells

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Introduction

Triclosan (5-chloro-2′-(2,4-dichlorophenoxy)phenol) (TCS) is a synthetic antimicrobial agent with a broad spectrum of activity. This drug is used in many personal care products, medical equipment, veterinary medicine, the textile industry, and the production of plastics. The antibacterial effect of triclosan has been well studied and is associated with the suppression of the bacterial enoyl-acyl carrier protein reductase (ENR), a key enzyme in the biosynthesis of fatty acids. As a result, the synthesis of cell membrane phospholipids and further proliferation of bacteria are suppressed (Rubin et al. 1999; Levy et al. 1999).

Eukaryotic organisms do not have the ENR enzyme, so for a long time, it was believed that this agent is harmless to humans and animals. However, evidence has been recently accumulated that triclosan affects many biological processes in eukaryotes. It is shown that at the organism level, triclosan has a powerful toxic effect, and its accumulation in the body can lead to damage to the endocrine system, disruption of muscle contraction, and stimulation of carcinogenesis (Lee et al. 2014; Huang et al. 2014; Dhillon et al. 2015; Kolšek et al. 2015). At the cellular level, this agent affects the functioning of a number of receptors and intracellular Ca^{2+} channels, and also causes oxidative stress and cell death (Cherednichenko et al. 2012; Zorov et al. 2014; Kolšek et al. 2015).

It is assumed that the effects of triclosan may be mediated by its effect on the permeability of biological membranes and the functioning of membrane-associated enzymes. One of the targets of the toxic effect of triclosan is mitochondria, since these organelles are responsible for initiating apoptosis. In particular, today it is known that triclosan causes a decrease in the mitochondrial membrane potential and uncoupling of oxidative phosphorylation in mammalian mitochondria (Popova et al. 2018). It was suggested that this effect of triclosan may be associated with both a specific uncoupling effect and inhibition of the respiratory chain complexes (Teplova et al. 2017; Popova et al. 2018). On the other hand, we have recently shown that this agent is able to induce disruption of the membrane structure of lecithin liposomes and the mitochondrial inner membrane, which leads to its permeabilization. Induction of mitochondrial permeabilization can quite well explain the proapoptotic effect of these agents, since in this case, mitochondria swell and proapoptotic protein cytochrome *c* is released from organelles (Belosludtsev et al. 2018).

Given the widespread use of triclosan in personal care products, the tendency for this agent to accumulate in aquatic ecosystems and sludge deposits is not surprising (Dhillon et al. 2015; Weatherly and Gosse 2017). It is shown that the amount of triclosan found in fish living in water polluted by this agent is significantly higher than its content in water, which indicates a strong bioaccumulation of this compound. A potential mechanism for the absorption of lipophilic pollutants by aquatic organisms is direct absorption from water, mainly through the gills, as well as through the skin and with food. Interestingly, in frogs, the absorption of triclosan by the tissues, as well as the coefficient of bioaccumulation, vary considerably depending on the species and stage of development, with the highest sensitivity observed in the early stages of development (Wang et al. 2018). It is shown that the accumulation of triclosan in aquatic organisms can be accompanied by a number of disorders: deviations in postembryonic development, impaired functioning of the thyroid gland and liver, and damage to the reproductive system. At the same time, it is worth noting that high concentrations of triclosan increased the survival rate of *Bufo americanus* tadpoles, which, apparently, was due to the antimicrobial properties of this agent (Smith and Burgett 2005).

The molecular mechanisms of the toxic effect of triclosan on the cells of aquatic organisms are not fully established. It can be assumed that the toxic effect of this agent may be associated with mitochondrial dysfunction and permeabilization of cell membranes, like in mammals. Therefore, in this work, we studied in vitro the effect of triclosan on the parameters of respiration and oxidative phosphorylation of marsh frog (*Pelophylax ridibundus* (Pallas, 1771)) liver mitochondria, the activity of the respiratory chain complexes of organelles and the production of hydrogen peroxide. In addition, we studied the effect of this agent on the permeability of the inner membrane of organelles, as well as the plasma membrane of red blood cells. The data obtained indicate that triclosan is capable of causing a complex disruption of both the functional activity of marsh frog mitochondrial respiratory chain at the level of complex II and coenzyme Q, and also induce nonspecific permeabilization of the inner mitochondrial membrane and plasma membrane of red blood cells.

Materials and Methods

Experimental Animals and Chemicals

Mature males of the marsh frog (*Pelophylax ridibundus* (Pallas, 1771)) weighing 60–70 g were caught in the Pine Grove forest park in the south-eastern part of Yoshkar-Ola (Russia) in July and August of 2018. The study was carried out in accordance with the European Convention for the Protection of Vertebrates used for experimental and other purposes (1986) and the principles of the Helsinki Declaration (2000).

All the experimental protocols were approved by the Mari State University Ethics Committee (Yoshkar-Ola, Russia).

All chemicals were purchased from Sigma-Aldrich (USA).

Isolation of Mitochondria

Mitochondria from the liver of animals were isolated by the conventional method of differential centrifugation (Roussel et al. 2015). The isolation medium contained 250 mM sucrose, 1 mM EGTA, and 3 mM Tris/HCl buffer (pH 7.3). The protein content of the mitochondrial preparation was assayed at 540 nm using the biuret method with bovine serum albumin used as a standard. The mitochondrial preparation from frog livers contains a dark pigment which absorbs at 540 nm; therefore, the absorbance of the same volume of mitochondria in isolation buffer containing 0.6% potassium sodium L(+)-tartrate and 3% NaOH was subtracted (Roussel et al. 2015). During the experiment, the suspension of mitochondria (20–30 mg of mitochondrial protein in 1 mL) was stored on ice in a narrow plastic tube.

Mitochondrial Respiration and Phosphorylation

The oxygen consumption rate of mitochondria was monitored at 25 °C using a Clark oxygen electrode in a 1-mL thermostatic sealed cuvette under magnetic stirring. Mitochondria (1.5 mg of protein/mL) were added to an incubation medium containing 250 mM sucrose, 3 mM $MgCl_2$, 3 mM KH_2PO_4, and 3 mM Tris/HCl buffer (pH 7.3). The concentrations of substrates were as follows: 2.5 mM potassium malate, 2.5 mM potassium glutamate, 5 mM potassium succinate, 0.2 mM ADP, and 2 μM rotenone. Estimated were the mitochondrial respiration in resting state (i.e., basal mitochondrial respiration in the presence of exogenous substrates), in state 3 (exogenous substrates plus 200 μM ADP), and in state 4 (after ADP exhaustion) in uncoupled state (after addition of 2,4-dinitrophenol (DNP)) (Chance and Williams 1955). The oxygen consumption rates are presented as nmol O_2/min/mg of mitochondrial protein. Respiratory control ratio (RCR), i.e., the ratio of respiratory rate in state 3 to that in state 4.

Measuring Activity of Complexes of the Mitochondrial Electron Transport Chain (ETC)

Activity of ETC complexes of marsh frog liver mitochondria was evaluated spectrophotometrically according to the protocol (Spinazzi et al. 2012) using a plate reader Multiskan GO (Thermo). To disrupt mitochondrial membranes and make respiratory chain complexes accessible for the assay (as well as to maximize their enzymatic activity), isolated liver mitochondria (10–15 mg mitochondrial protein per mL) were subjected to three cycles of freezing/thawing at − 20/+ 30 °C in a hypotonic buffer, containing 10 mM Tris/HCl, pH 7.6. The composition of the buffers used for the analysis of activity of individual complexes is given in protocol (Spinazzi et al. 2012). The activity of complex I was estimated at 25 °C by the efficiency of oxidation of added NADH by the suspension of disrupted mitochondria, which was followed by the decrease of absorbance at 340 nm. The activity of complex II was evaluated at 37 °C (in order to fully activate the enzyme) by the efficiency of reduction of 2,6-dicholrophenol indophenol (sodium salt) by the suspension of disrupted mitochondria in the presence of succinate, which was followed by the decrease of absorbance at 600 nm. The activity of complex III was measured at 25 °C by the efficiency of reduction of added cytochrome *c* by the suspension of disrupted mitochondria, which was followed by the decrease of absorbance at 550 nm. The activity of complex IV was estimated at 25 °C by the efficiency of oxidation of added cytochrome *c* (preliminary reduced by the suspension of disrupted mitochondria as described in protocol (Spinazzi et al. 2012), which was followed by the decrease of absorbance at 550 nm. The activities of the respiratory chain complexes (in the absence and presence of triclosan) were registered within 2–3 min after the beginning of the redox reaction (nmol/min/mg protein).

Monitoring of Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\psi$) was estimated by the distribution of tetraphenylphosphonium (TPP^+) in the mitochondrial suspension, which was measured with a TPP^+-sensitive electrode (Nico-Analyt, Russia) (Vedernikov et al. 2015). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 10 μM EGTA, and 10 mM Hepes/KOH buffer, pH 7.4. 2.5 mM glutamate + 2.5 mM malate or 5 mM succinate + 1 μM rotenone were used as respiratory substrates. TPP^+ was added to the incubation medium at the concentration of 1 μM. The concentration of mitochondrial protein was 1 mg/mL.

Production of H_2O_2 by Liver Mitochondria

The rate of H_2O_2 production by the suspension of marsh frog liver mitochondria was measured with the fluorescent indicator Amplex Red (excitation wavelength, 560 nm; emission wavelength, 590 nm) using Fluorat-02-Panorama spectrofluorometer (Lumex Instruments, Russia) (Dubinin et al. 2019). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM KH_2PO_4, 10 μM EGTA, and 10 mM HEPES/KOH, pH 7.4. 2.5 mM glutamate + 2.5 mM malate or 5 mM succinate + 1 μM rotenone were used as respiratory substrates. At the beginning of measurements, horseradish peroxidase (1 U/mL), superoxide dismutase (4 U/mL) and

10 µM Amplex Red were added to the incubation medium. The concentration of mitochondrial protein in the cuvette was 0.15 mg/mL. The amount of the resulting hydrogen peroxide was calculated from the calibration curve. A standard hydrogen peroxide solution was prepared on the day of experiment; its concentration was determined using the molar absorption coefficient $E_{240} = 43.6$/M/cm.

Induction of Nonspecific Permeability of the Mitochondrial Inner Membrane

The opening of the mitochondrial pore was assessed by mitochondrial swelling, which was recorded by the change in the optical density of the mitochondrial suspension (A) at a wavelength of 540 nm using a plate reader Multiskan GO (Thermo, Finland). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 10 µM EGTA, and 3 mM Tris/HCl, pH 7.4. 2.5 mM glutamate + 2.5 mM malate or 5 mM succinate + 1 µM rotenone were used as respiratory substrates. The rate of swelling ($V_{max} = \Delta A_{540}$/min per mg protein) was calculated as a change in absorbance within the first 30 s from the beginning of the high-amplitude swelling.

Isolation of Red Blood Cells

The blood was collected into a tube containing 3 mL 3.5% sodium citrate and 1 mg/mL EDTA just after decapitation (Belosludtsev et al. 2019). The tube was centrifuged 10 min at 500×*g* and 4 °C. The plasma and leukocytes were separated, and the erythrocytes were washed three times in five volumes of phosphate buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 5 mM glucose, pH 7.4) at 500×*g* for 10 min at 4 °C each time with the removal of the leukocyte layer. The obtained erythrocyte suspension was diluted with phosphate buffer, so that the final cell concentration was ~ 5–9 × 10^9 cells/mL.

Erythrocytes were counted in a two-grid Goryaev's chamber. The chamber and the cover glass were washed several times with distilled water; the cover glass was then ground until Newton rings appeared. Separate cells were counted in five large quadrants of the chamber grid. The cell number in a 1-mL suspension was calculated via the equation:

$$C = \frac{a}{20} * 10^6,$$

where C is the cell concentration in a 1-mL suspension, a is the sum of the cells in five large quadrants of a Goryaev chamber, and 20 is the volume of five large quadrants in nL.

RBC Membranes' Permeability Assessment

The effect of triclosan on the permeability of the RBS membranes and their viability were assessed using the vital dye trypan blue. For this purpose, a solution containing 2.5–3 × 10^6 cells/mL was supplemented with a dye (0.2%), and after 5 min, cells containing colored and unstained nuclei were counted. In control experiments, digitonin (5 µg/mL) was used to induce maximum permeabilization of the erythrocyte membrane accompanied by staining of nuclei of > 95% of cells.

Statistical Analysis

The data were analyzed using the GraphPad Prism 5 and Excel softwares and were presented as mean ± SEM of three–seven experiments. Significant differences between data points were determined by a two-tailed *t* test.

Results

Triclosan Uncouples Oxidative Phosphorylation in Marsh Frog Liver Mitochondria and Causes Depolarization of Organelles

The effect of triclosan on the functional state of marsh frog liver mitochondria was evaluated by the rate of mitochondrial respiration with glutamate/malate (substrates of complex I of the respiratory chain) or succinate (a substrate of complex II) in the presence of rotenone. Table 1 shows the effect of 10–20 µM triclosan on the respiration of marsh frog liver mitochondria in different functional states (V_2, V_3, V_4, and V_{DNP}). As seen from table, triclosan dose-dependently accelerates the glutamate/malate-driven respiration of mitochondria in states V_2 and V_4, suppresses DNP-stimulated (V_{DNP}) respiration, but does not affect respiration in ADP-stimulated (V_3) state. Due to the change in respiration rate in state V_4 the parameter of respiratory control in liver mitochondria was reduced. In the case of succinate-fueled respiration, triclosan dose-dependently suppressed mitochondrial respiration in states V_3 and V_{DNP}, and activated respiration in resting state and basal nonphosphorylating rate. This was also accompanied by a decrease of the respiratory control level.

Figure 1 shows the effect of triclosan on the activity of the mitochondrial respiratory chain complexes. One can see that triclosan practically did not affect the activity of complex I, complex III and complex IV. At the same time, it suppressed the activity of complex II, which was especially evident when the combined activity of complex II + III was assessed (a 90% inhibition by 20 µM triclosan). 20–50 µM triclosan also suppressed the combined activity of complex I + III but not so significantly (a 15% inhibition by 20 µM triclosan).

In the following experiments, we studied the effect of triclosan on the membrane potential ($\Delta\psi$) of marsh frog liver

Table 1 Effects of triclosan on the respiration of marsh frog liver mitochondria

Triclosan (μM)	nmol O_2/min/mg protein				
	V_2	V_3	V_4	V_{DNP}	RCR
Glutamate/malate					
0	3.7 ± 0.2	15.9 ± 0.7	3.4 ± 0.2	22.2 ± 1.2	4.7 ± 0.1
10	6.9 ± 0.5*	16.3 ± 0.5	5.9 ± 0.4*	20.2 ± 0.6	2.8 ± 0.1*
20	12.5 ± 0.8*	16.6 ± 0.9	11.1 ± 1.1*	19.5 ± 0.4*	1.5 ± 0.1*
Succinate/rotenone					
0	7.1 ± 0.1	16.9 ± 0.2	6.3 ± 0.4	22.3 ± 1.2	2.7 ± 0.2
10	10.0 ± 0.3*	13.2 ± 0.4*	9.7 ± 0.2*	15.5 ± 0.4*	1.4 ± 0.1*
20	10.5 ± 0.2*	11.1 ± 0.3*	10.4 ± 0.4*	11.4 ± 0.2*	1.1 ± 0.2*

Medium composition: 250 mM sucrose, 3 mM $MgCl_2$, 3 mM KH_2PO_4, 3 mM Tris/HCl buffer (pH 7.3). Respiration of mitochondria was fueled by 2.5 mM glutamate and 2.5 mM malate or 5 mM succinate. Respiration of mitochondria in the state 3 was initiated by 200 μM ADP. The rate of uncoupled respiration was measured in the presence of 100 μM DNP (V_{DNP}). The results are presented as mean ± SEM ($n = 3$)

RCR respiratory control ratio (V_3/V_4)

*Differences between control (without triclosan) and experiment (with triclosan) were statistically significant ($p < 0.05$)

mitochondria. Figure 2 shows the kinetic curves that demonstrate the ability of triclosan to depolarize the mitochondrial inner membrane. As seen from the figure, the addition of 5 μM triclosan to the mitochondria already leads to a significant decrease in $\Delta\psi$, and the maximum potential decrease was observed after the fourth addition. This uncoupling effect was observed in both the cases of using 2.5 mM glutamate/2.5 mM malate and 5 mM succinate as substrates of respiration.

Effect of Triclosan on H_2O_2 Production by Mitochondria

It was previously shown that triclosan is able to induce oxidative stress in mammalian cells mediated by mitochondria (Ajao et al. 2015; Teplova et al. 2017). In this work, we examined the effect of triclosan on the rate of H_2O_2 production by marsh frog liver mitochondria. As seen in Fig. 3a, high concentrations of triclosan (50–100 μM) enhanced the production of hydrogen peroxide by mitochondria in the case of glutamate/malate-driven respiration. At the same time, such an effect was not observed in the succinate-fueled mitochondria (Fig. 3b). Moreover, in this case, triclosan reduces H_2O_2 production by marsh frog liver mitochondria.

Triclosan Induces Permeabilization of Liver Mitochondria

In the next part of this work, we studied the ability of triclosan to cause permeabilization of marsh frog liver mitochondria. Figure 4 demonstrates that triclosan has the ability to induce mitochondrial swelling. It occurred immediately after the addition of 40 μM triclosan, without a lag-period (Fig. 4a). The maximal swelling rate was observed at TCS concentrations above 50 μM (Fig. 4b).

It is worth noting that, unlike rat liver mitochondria, swelling of frog liver mitochondria induced by triclosan was much lower (the rate and amplitude of swelling of frog liver mitochondria is much lower compared to rat liver mitochondria). The TCS-induced swelling of frog liver mitochondria was insensitive to the specific inhibitor of mitochondrial permeability transition (MPT) pore cyclosporin A (CsA) (Fig. 5a). This suggests that the TCS-induced mitochondrial swelling is not due to the opening of an MPT pore and occurs by another mechanism. As shown earlier, small concentration of TCS caused uncoupling of mitochondrial respiration and oxidative phosphorylation (Teplova et al. 2017). Figure 5b presents data on the effect of mitochondrial energization on triclosan-induced swelling. The figure shows that the maximum effect of triclosan was observed in the presence of substrates of the respiratory chain (glutamate/malate and succinate).

The Effect of Triclosan on the Permeability of the Frog RBC Membrane

As shown earlier, triclosan permeabilized both artificial (liposomal) and natural (mitochondrial) membranes (Belosludtsev et al. 2018). In this connection, we examined the possibility of triclosan to cause changes in the number of frog red blood cells, as well as their lysis. As can be seen from Fig. 6, the addition of triclosan to the RBC suspension did not lead to a change in their number.

However, triclosan is able to induce permeabilization of the plasma membrane of erythrocytes. As shown in Fig. 7, triclosan dose-dependently reduced the number of vital (not

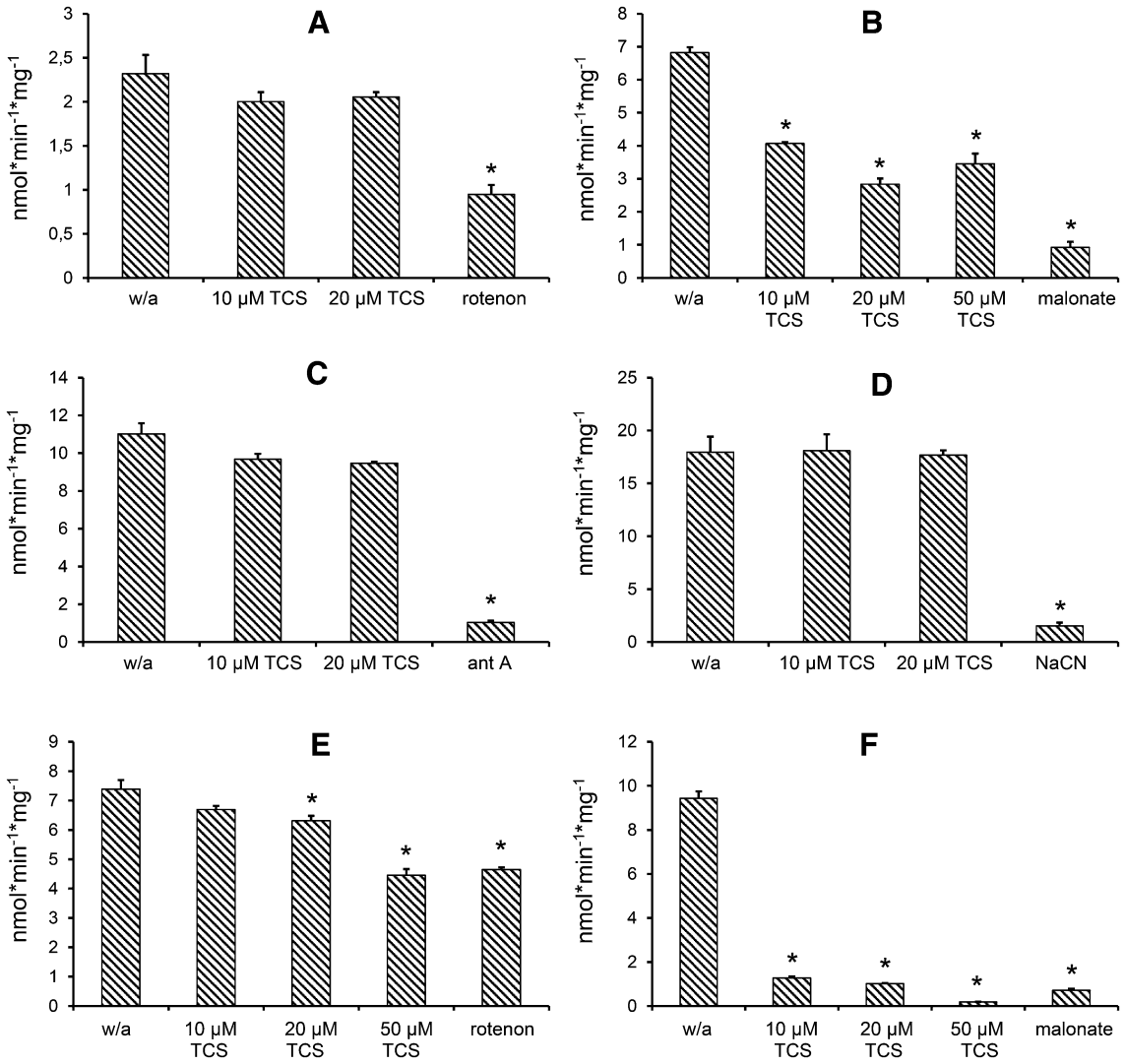

Fig. 1 Effects of triclosan on the activity of the respiratory chain complexes of marsh frog liver mitochondria: complex I (**a**), complex II (**b**), complex III (**c**), complex IV (**d**), complexes I + III (**e**), and complexes II + III (**f**). The experimental conditions are described in "Materials and Methods". Additions: Triclosan (TCS), 10 μM rotenone, 10 mM malonate, 10 μg/mL antimycin A (ant A), 300 μM NaCN. Mean values ± SEM are represented ($n = 4$). Differences between control (without additions, w/a) and experiment (with triclosan or a specific inhibitor) were statistically significant (*$p < 0.05$)

stained by the trypan blue) frog erythrocytes. The addition of 100 μM triclosan to the frog RBC suspension resulted in 95% of erythrocytes staining with trypan blue. Thus, it can be concluded that triclosan causes permeabilization of the plasma membrane of erythrocytes, but cell destruction does not occur.

Discussion

Today, it is generally accepted that triclosan has the property of accumulation in environmental objects. Triclosan widely used in personal care products, flows into natural water bodies, bypassing wastewater treatment systems, and interacts with their inhabitants. As noted in the introduction, the concentration of triclosan found in aquatic organisms significantly exceeds the concentration of this agent in water (Smith and Burgett 2005; Weatherly and Gosse 2017). This fact indicates the ability of triclosan to bioaccumulate. It is known that triclosan may delay the embryonic and postembryonic development of some fish, impair the functions of the thyroid gland, liver, and reproductive system of these animals (Wang et al. 2018). Since mitochondria are one of the main intracellular targets of triclosan, in this work we examined the effect of this agent on the functional activity of marsh frog (*Pelophylax ridibundus* (Pallas, 1771)) liver mitochondria.

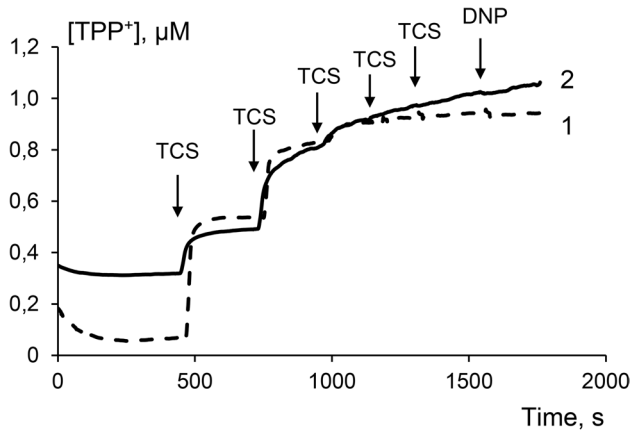

Fig. 2 Effects of triclosan on membrane potential of marsh frog liver mitochondria. The experimental conditions are described in Materials and Methods. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 10 μM EGTA, and 10 mM Hepes/KOH buffer, pH 7.4. 2.5 mM glutamate + 2.5 mM malate (curve 1) and 5 mM succinate + 1 μM rotenone (curve 2) were used as respiratory substrates. Additions: 5 μM triclosan (TCS), 50 μM DNP

As demonstrated in the present work, the effect of triclosan on frog liver mitochondria is similar to what was obtained previously on mammalian organelles. Examination of the activity of mitochondrial respiratory complexes shows that triclosan mainly inhibits the activity of complex II and combined activity of complexes II + III (and, to a lesser degree, the combined activity of complexes I + III) (Fig. 1). Apparently, this causes the suppression of succinate-fueled mitochondrial respiration. In addition, it can be assumed that along with the inhibition of succinate dehydrogenase, triclosan is able to block the electron transfer mediated by coenzyme Q as was suggested in previous studies (Teplova et al. 2017).

The results of our experiments also show that triclosan is able to increase the permeability of the inner mitochondrial membrane. This may be due to both the protonophore effect of this compound (which leads to a drop in $\Delta\psi$ at low concentrations of triclosan) and to the permeabilizing effect leading to swelling of the organelles. It should be noted that this swelling is insensitive to the well-known specific inhibitor of the MPT pore opening, CsA, indicating a different mechanism of mitochondrial membrane permeabilization. A similar effect of triclosan was previously obtained on rat liver mitochondria (Belosludtsev et al. 2018). We have suggested that triclosan is able to induce a disturbance of phospholipid membrane packing, which may be accompanied by the appearance of lipid pores and the corresponding changes in the permeability of the membrane to ions and larger molecules (Belosludtsev et al. 2018). It should be noted that frog liver mitochondria appear to be more resistant to triclosan as a permeabilizing agent, since the rate and amplitude of triclosan-induced swelling of frog liver mitochondria were lower compared to those of rat liver mitochondria. We assume that this resistance may determine the survival of frogs under the conditions of accumulation of triclosan in the tissues of these amphibians.

It is worth noting that the effect of triclosan depends on the presence of respiratory substrates in the incubation medium. Apparently, the effect of triclosan is manifested on the matrix side of the inner mitochondrial membrane, since triclosan, as a protonophore, accumulates more easily in this region in the presence of a membrane potential (Popova et al. 2018).

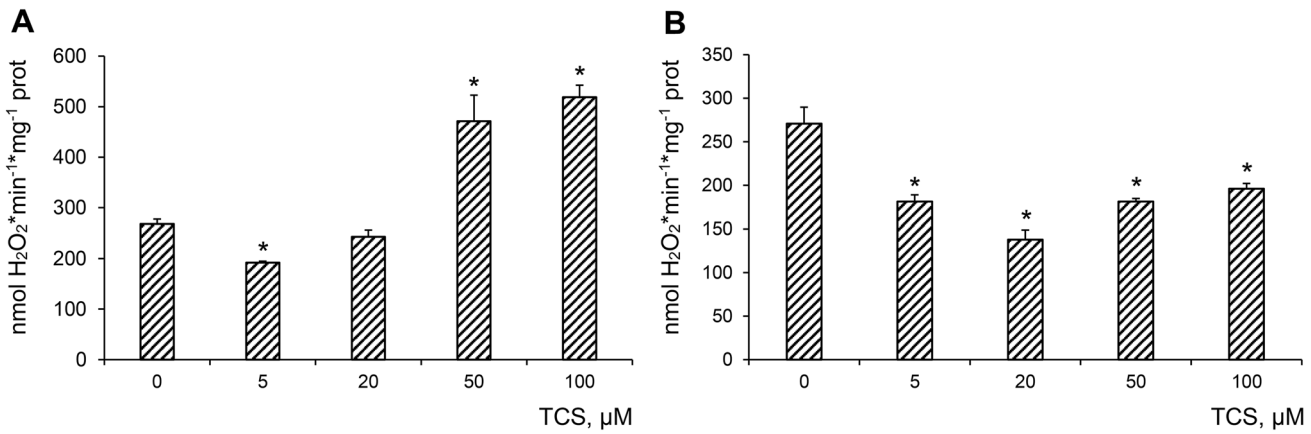

Fig. 3 Effects of triclosan on the rate of H_2O_2 production by marsh frog liver mitochondria. The experimental conditions are described in Materials and Methods. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM KH_2PO_4, 10 μM EGTA, and 10 mM HEPES/KOH, pH 7.4. 2.5 mM glutamate + 2.5 mM malate (**a**) or 5 mM succinate + 1 μM rotenone (**b**) was used as respiratory substrate. Mean values ± SEM are represented ($n = 4$). Differences between control (without triclosan) and experiment (with triclosan) were statistically significant (*$p < 0.05$)

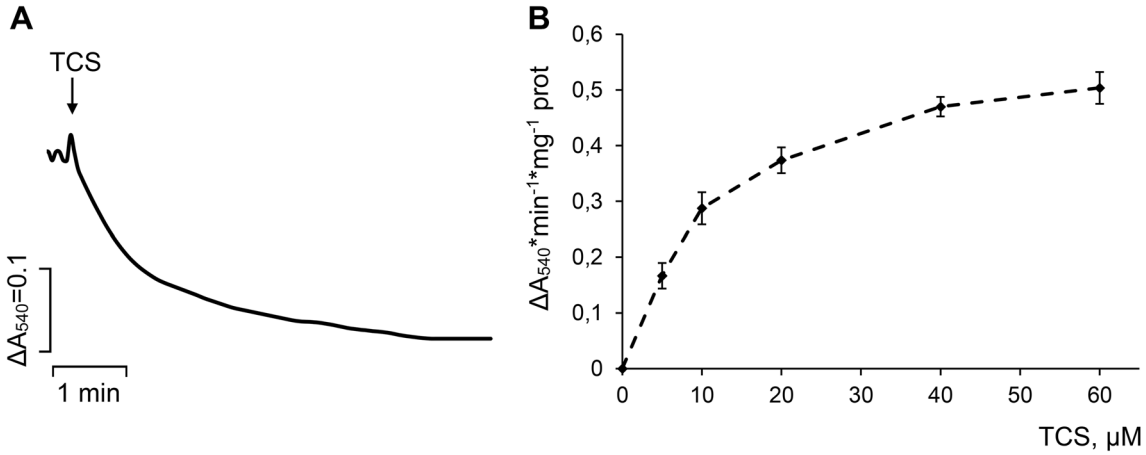

Fig. 4 TCS-induced swelling of marsh frog liver mitochondria. **a** Swelling of marsh frog mitochondria (0.4 mg/mL), measured by absorbance at 540 nm (A_{540}), was induced by 40 μM TCS in 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 1 μM rotenone, 10 μM EGTA, and 3 mM Tris/HCl, pH 7.4. **b** Dependence of the rate of swelling of marsh frog liver mitochondria on the concentration of TCS. Mean values ± SEM are represented ($n = 5$)

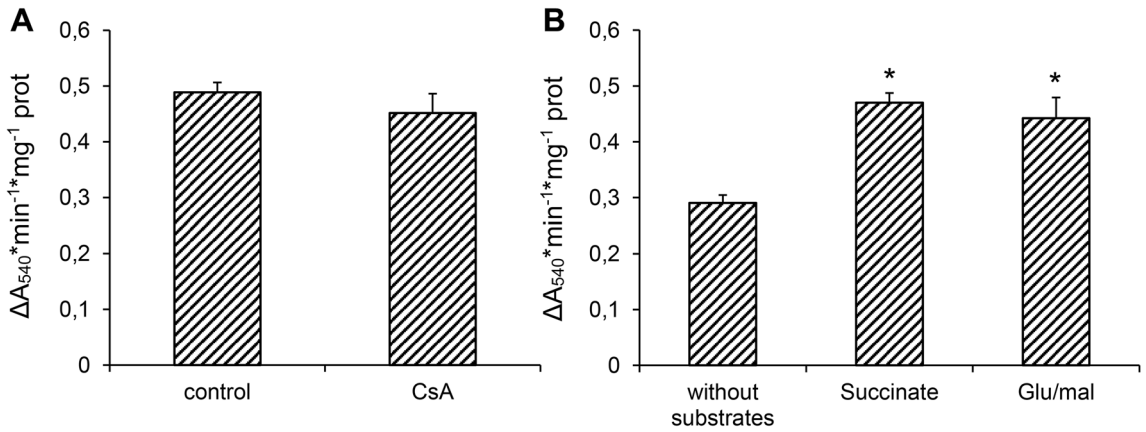

Fig. 5 Induction of mitochondrial swelling by 40 μM TCS under different conditions. **a** Effect of 1 μM CsA on the TCS-induced swelling of marsh frog liver mitochondria. The medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 1 μM rotenone, 10 μM EGTA, and 3 mM Tris/HCl, pH 7.4. Mean values ± SEM are represented ($n = 5$). **b** TCS-induced swelling of marsh frog liver mitochondria in the absence and presence of respiratory substrates: 5 mM succinate or 2.5 mM glutamate plus 2.5 mM malate. The medium contained 210 mM mannitol, 70 mM sucrose, 10 μM EGTA, 1 μM rotenone (only in the absence of respiratory substrates or in the presence of succinate), and 3 mM Tris/HCl, pH 7.4. Mean values ± SEM are represented ($n = 5$). Differences between control (without substrates) and experiment (with substrates) were statistically significant ($^*p < 0.05$)

The data on the effect of triclosan on the ROS production are rather contradictory. On the one hand, it has been shown that it is able to induce superoxide anion production in mitochondria (Teplova et al. 2017). This effect may be due both to the inhibition of mitochondrial respiration, and to the induction of the mitochondrial pore. On the other hand, triclosan, as an uncoupler, should cause the suppression of reactive oxygen species production (Ajao et al. 2015). In addition, it is shown that triclosan can activate the antioxidant system of cells (Popova et al. 2018). Therefore, in this work, we have investigated the effect of triclosan on the production of hydrogen peroxide by marsh frog liver mitochondria. It can be seen that in the presence of succinate and rotenone, triclosan not only inhibits the functioning of complex II and combined activity of complexes II + III of the respiratory chain, but also significantly inhibits H_2O_2 production by mitochondria. At the same time, when mitochondria oxidized glutamate and malate (substrates of complex I of the respiratory chain), low concentrations of triclosan (5 μM) inhibit, and high concentrations (50–100 μM) cause a twofold stimulation of H_2O_2 production by mitochondria. This effect may be due to the inhibition of the combined activity of I + III mitochondrial respiratory chain complexes, which indicates the ability of triclosan to block the electron

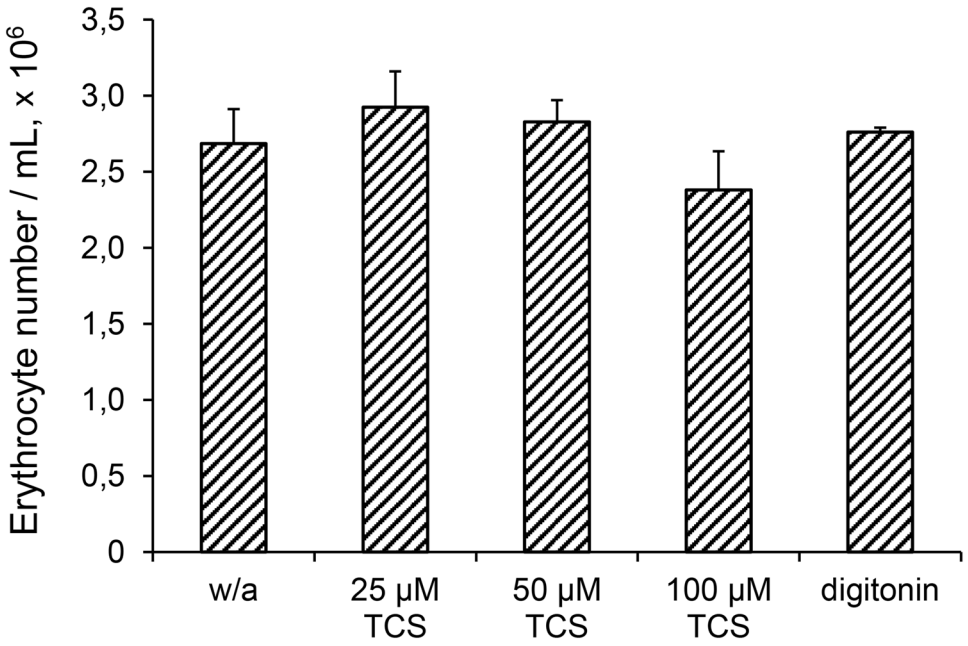

Fig. 6 Changes in the number of red blood cells in the suspension after a 10-min incubation with TCS. The experimental conditions are described in Materials and Methods. Additions: Triclosan (TCS), 5 μg/mL digitonin. Mean values ± SEM are represented ($n = 6$)

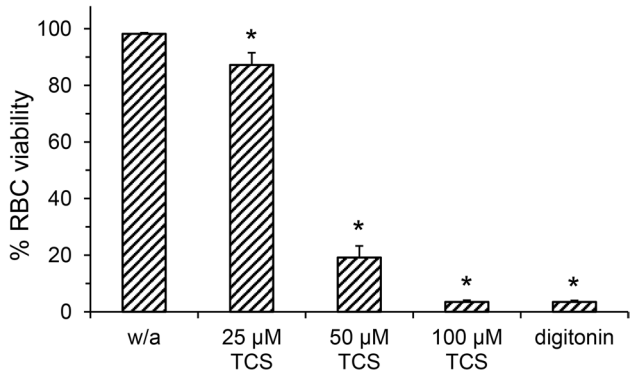

Fig. 7 Changes in the relative number of vital red blood cells in the suspension after a 10-min incubation with TCS. The experimental conditions are described in Materials and Methods. Cell viability in the absence of additions was taken as 100%. Additions: Triclosan (TCS), 5 μg/mL digitonin. Mean values ± SEM are represented ($n = 6$). Differences between control (without additions, w/a) and experiment (with triclosan or digitonin) were statistically significant (*$p < 0.05$)

transfer mediated by coenzyme Q. Indeed, it was previously suggested that triclosan has the unique ability to simultaneously exhibit both a protonophore effect and block electron transfer between ubiquinone at the Q_d-binding site and heme *b* (Teplova et al. 2017). It is quite possible that in high concentrations this inhibition will lead to the generation of reactive oxygen species.

Taking into account that triclosan is able to accumulate in membranes and permeabilize them, we carried out experiments on the effect of this agent on the permeability of the cytoplasmic membrane of frog erythrocytes. As can be seen from the data presented in Fig. 6, triclosan has almost no effect on the number of erythrocytes in suspension. However, triclosan induces permeabilization of the RBC plasma membrane in a dose-dependent manner increasing its permeability to trypan blue dye (Fig. 7). It can be assumed that permeabilization of the erythrocyte is due to the ability of triclosan to cause structural changes in the lipid bilayer of membranes, leading to the formation of nonspecific pores, which was previously shown on membranes formed using natural and synthetic phospholipids (Belosludtsev et al. 2018).

Conclusion

The results obtained show that triclosan can cause a complex dysfunction of marsh frog liver mitochondria. In particular, triclosan reduces the rate of oxygen consumption by mitochondria due to inhibition of the activity of complex II and combined activity of complexes II + III (and, to a lesser degree, the combined activity of complexes I + III) of the respiratory chain. It also leads to the collapse of the mitochondrial membrane potential. In addition, triclosan enhances the production of hydrogen peroxide in glutamate/malate-fueled mitochondria and reduces in succinate-fueled organelles. Finally, triclosan is able to induce permeabilization of mitochondrial membranes, as well as the cytoplasmic membrane of the erythrocytes of these animals, possibly due to the formation of nonspecific pores.

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