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Effects of Tl^+ on ion permeability, membrane potential and respiration of isolated rat liver mitochondria

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Abstract It is known that permeability of the inner mitochondrial membrane is low to most univalent cations (K^+, Na^+, H^+) but high to Tl^+. Swelling, state 4, state 3, and 2,4-dinitrophenol (DNP)-stimulated respiration as well as the membrane potential ($\Delta\Psi_{mito}$) of rat liver mitochondria were studied in media containing 0–75 mM $TlNO_3$ either with 250 mM sucrose or with 125 mM nitrate salts of other monovalent cations (KNO_3, or $NaNO_3$, or NH_4NO_3). Tl^+ increased permeability of the inner mitochondrial membrane to K^+, Na^+, and H^+, that was manifested as stimulation of the swelling of nonenergized and energized mitochondria as well as via an increase of state 4 and dissipation of $\Delta\Psi_{mito}$. These effects of Tl^+ increased in the order of sucrose $<K^+ <Na^+ \leq NH_4^+$. They were stimulated by inorganic phosphate and decreased by ADP, Mg^{2+}, and cyclosporine A. Contraction of energized mitochondria, swollen in the nitrate media, was markedly inhibited by quinine. It suggests participation of the mitochondrial K^+/H^+ exchanger in extruding of Tl^+-induced excess of univalent cations from the mitochondrial matrix. It is discussed that Tl^+ (like Cd^{2+} and other heavy metals) increases the ion permeability of the inner membrane of mitochondria regardless of their energization and stimulates the mitochondrial permeability transition pore in low conductance state. The observed decrease of state 3 and DNP-stimulated respiration in the nitrate media resulted from the mitochondrial swelling rather than from an inhibition of respiratory enzymes as is the case with the bivalent heavy metals.

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Introduction

Experiments with swelling of nonenergized mitochondria in nitrate media revealed low permeability of the inner membrane to univalent cations such as K^+, H^+, and Na^+ (Mitchell and Moyle 1969; Brierley et al. 1970; Bernardi 1999). The ion permeability can be further decreased by Mg^{2+} or ADP (Brierley and Jurkowitz 1976; Ichas and Mazat 1998; Garlid and Paucek 2003) and stimulated by inorganic phosphate (P_i) or an increase of a buffer pH (Barrera and Gomez-Puyou 1975; Brierley and Jurkowitz 1976). On the other hand, noticeable swelling of non-energized mitochondria in the $TlNO_3$ media showed natural permeability of the membrane to Tl^+ which penetrates electrogenically in the matrix (Saris et al. 1981; Skulskii et al. 1984; Korotkov et al. 2007a; Korotkov et al. 2008a). Subsequent energization of the mitochondria stimulated their massive contraction which occurred by means of a Tl^+/H^+ exchange mechanism (Saris et al. 1981; Korotkov et al. 2008a). Nonenergized mitochondria in Tl acetate media showed massive swelling that was realized by means of Tl^+/H^+ exchange (Saris et al. 1981; Korotkov et al. 2007a). Further energization of the mitochondria stimulated additional swelling due to an electrophoretic uniport of Tl^+ into the matrix (Melnick et al. 1976; Skulskii et al. 1978; Saris et al. 1981; Skulskii et al. 1984; Korotkov et al. 2007a). These features of Tl^+ ions resulted both in a high level of futile cycling of Tl^+ ions via the inner mitochondrial membrane and in an increase in state 4 respiration of

mitochondria (Melnick et al. 1976; Diwan and Lehrer 1977; Bragadin et al. 2003; Korotkov et al. 2007a; Korotkov et al. 2008a). Experiments with rats exposed to chronic thallium intoxication *in vivo* (Herman and Bensch 1967; Woods and Fowler 1986), and studies *in vitro* of hepatocytes (Zierold 2000) showed that Tl^+ stimulated massive mitochondrial swelling which was followed by disruption of mitochondrial and other intracellular membranes.

Functional state of SH groups in respiratory complexes and other mitochondrial proteins plays a significant role in maintaining the low ion permeability of the inner mitochondrial membrane (Riley and Lehninger 1964; Belyaeva and Korotkov 2003). Interaction of the heavy metals (Cd^{2+}, Hg^{2+}, and Pb^{2+}) with SH groups of mitochondrial enzymes accounted for an increase of the ion permeability of the inner membrane (Scott et al. 1971; Miyahara and Utsumi 1975; Rasheed et al. 1984; Skulskii et al. 1988; Korotkov et al. 1998; Belyaeva and Korotkov 2003; Belyaeva et al. 2004; Lee et al. 2005; Korotkov et al. 2007b) and resulted in massive mitochondrial swelling and in subsequent disruption of the matrix structure (Riley and Lehninger 1964; Sanadi et al. 1981; Rasheed et al. 1984; Koike et al. 1991; Rikans and Yamano 2000). Low concentrations of these bivalent heavy metals stimulated state 4 respiration and increased transport of K^+ and protons in mitochondria (Scott et al. 1971; Miyahara and Utsumi 1975; Rasheed et al. 1984; Diwan et al. 1990; Belyaeva et al. 2002; Belyaeva et al. 2004; Lee et al. 2005). An increase of the heavy metals concentration caused dissipation of the proton gradient on the inner mitochondrial membrane, a decrease of state 4, state 3, or 2,4-dinitrophenol (DNP)-stimulated respiration, opening the mitochondrial permeability transition pore, and complete retardation of contraction of mitochondria, swollen in media with NH_4NO_3 (Scott et al. 1971; Skulskii et al. 1988; Zoratti and Szabo 1995; Korotkov et al. 1998; Rikans and Yamano 2000; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Belyaeva et al. 2004). The ability of Tl^+ to react with SH groups of mitochondrial and cellular proteins has been shown experimentally (Herman and Bensch 1967; Skulskii et al. 1984; Hanzel and Verstraeten 2006). However, the affinity of Tl^+ to molecular SH groups is lower than that of bivalent heavy metal ions (Perrin 1979). Moreover, no considerable inhibition of mitochondrial respiratory enzymes (Melnick et al. 1976; Woods and Fowler 1986) or state 3 and DNP-stimulated respiration has been observed in media containing Tl salts and sucrose (Barrera and Gomez-Puyou 1975; Melnick et al. 1976; Diwan and Lehrer 1977; Korotkov et al. 2007a, 2008a). This suggests that Tl^+-induced mitochondrial dysfunction may occur via different, yet unknown, mechanisms than in the case of the bivalent heavy metals.

The effects of Tl^+ on movement of univalent ions via the inner mitochondrial membrane are not fully understood. It has been found that Tl^+ decreased uptake and efflux of K^+ in rat liver mitochondria, incubated in media with ethylenediaminetetraacetic acid (EDTA) and P_i (Barrera and Gomez-Puyou 1975) or in those of high pH (Diwan and Lehrer 1977). Our earlier studies have shown that Tl^+ increased swelling of nonenergized rat liver mitochondria and decreased state 3 and DNP-stimulated respiration of mitochondria in media with $TlNO_3$ and nitrates (Korotkov and Brailovskaya 2001). The main goal of this research was to study effects of Tl^+ on transport of univalent cations (H^+, K^+, and Na^+) via the inner membrane of rat liver mitochondria in different energetic states. The present research tests the hypothesis that the increase of swelling of nonenergized rat liver mitochondria can result by Tl-induced uptake of the univalent cations into the mitochondrial matrix. In addition, we suggested the correlation between the decrease of state 3 or DNP-stimulated respiration and probable Tl-induced swelling of energized mitochondria in the media with $TlNO_3$ and nitrates. A Tl-induced opening of the mitochondrial permeability transition pore (MPTP) was studied in energized mitochondria in the nitrate media in the low conduction state.

Materials and methods

Chemicals

$Mg(NO_3)_2$, H_3PO_4, $NaNO_3$, KNO_3, NH_4NO_3 and DNP were of analytical grade. Rotenone, oligomycin, cyclosporin A (CsA), safranin, $TlNO_3$, Tris-OH, quinine, ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), ADP, carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP), and succinate were from Sigma (St. Louis, MO, USA). Sucrose as 1M solution was cleaned from cation traces on a column filled with a KU-2-8 ion-exchange resin from Azot (Kemerovo, Russia).

Isolation of mitochondria

Liver mitochondria were isolated from Wistar adult male rats (200–250 g) according to the standard procedure described in detail by Korotkov et al. (Korotkov et al. 2007a). Liver mitochondria were homogenized in a medium containing (mM): 250 sucrose, 3 Tris-HCl (pH 7.3), and 0.5 EGTA; then they were twice washed by resuspension-centrifugation in a medium containing 250 mM sucrose and 3 mM Tris-HCl (pH 7.3) and finally suspended in 1 ml of the latter medium. The protein content in mitochondrial preparations was determined by Bradford's method and amounted to 50–60 mg/ml.

Swelling of mitochondria

Swelling of mitochondria was estimated as a decrease of the apparent absorbance of mitochondrial suspension at 20 °C on an SF-46 spectrophotometer (LOMO, St. Petersburg, Russia) at 540 nm. Mitochondria (1.5 mg/ml of protein) were placed into a 1-cm cuvette with 1.5 ml of media containing 0–75 mM $TlNO_3$ and 0–150 mM sucrose (Figs. 1, 2, 3 and 4). Additionally, these media contained 250 mM sucrose (A), or 125 mM of KNO_3 (B), or $NaNO_3$ (C), or NH_4NO_3 (D), as well as 5 mM Tris-succinate (Fig. 4), 5 mM Tris-NO_3 (pH 7.3), 4 μM rotenone, and 3 μg/ml of oligomycin. Total osmolarity in the media was 400 mOsm. The ability of this technique to detect mitochondrial swelling in 100–500 mOsm media was established by Devin et al. (1996, 1997a, 1997b). Where indicated, the following compounds were added to incubation media before placing there the mitochondria: 1–3 mM Tris-P_i, 5 mM $Mg(NO_3)_2$, 0.5 or 2 mM ADP, 0–1 mM quinine, and 10^{-8}M nonactin. DNP of 30 μM was injected after addition of mitochondria (Fig. 4, traces 9–11). The swelling, the respiration and the mitochondrial membrane potential ($\Delta\Psi_{mito}$) were tested in the 400 mOsm media to check consistency and comparability between the results of different experiments.

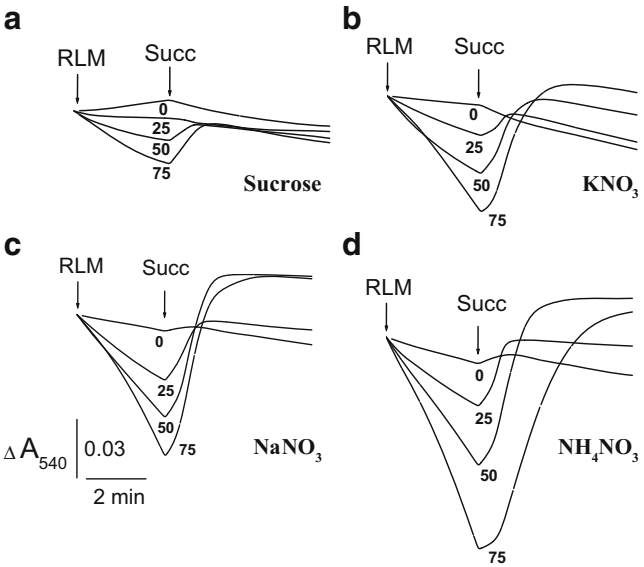

Fig. 1 Effects of Tl^+ on swelling of rat liver mitochondria. Mitochondria (1.5 mg/ml of protein) were added to media containing 0–75 mM $TlNO_3$ and 0–150 mM sucrose. Additionally these media contained 5 mM Tris-NO_3 (pH 7.3), 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**), as well as 4 μM rotenone, and 3 μg/ml of oligomycin. Numbers near the traces show concentration of $TlNO_3$ (mM) in these media. Additions of mitochondria (RLM) and of 5 mM succinate (Succ) are shown by arrows. Typical traces for three different mitochondrial preparations are presented

Oxygen consumption assay

Respiration (oxygen consumption rate) was estimated in an LP-7 polarograph (Czechoslovakia) using a Clark-type oxygen electrode in a 1.5-ml closed thermostatic chamber with magnetic stirring at 26 °C. Mitochondria (1.5 mg/ml of protein) were placed into media containing 0–75 mM $TlNO_3$ and 0–150 mM sucrose. Additionally, these media contained 250 mM sucrose, or 125 mM of KNO_3, or $NaNO_3$, or NH_4NO_3 (Figs. 5, 6, and 7a [traces 1–4]), 5 mM Tris-NO_3 (pH 7.3), as well as 5 mM Tris-succinate, and 4 μM rotenone. Final osmolarity in the media was 400 mOsm. In some incubations, 3 μg/ml of oligomycin (Figs. 5 and 6) or 3 mM $Mg(NO_3)_2$ and 3 mM Tris-P_i (Fig. 7) were added. Sucrose media of 290 mOsm were used in Fig. 7a [trace 5] and b. ADP of 130 μM (Fig. 7) or DNP of 30 μM (Figs. 5, 6 and 7) were added to the media after 2 min recording of state 4 respiration to trigger state 3 or DNP-stimulated respiration. Additions of P_i, Mg^{2+}, ADP, and quinine before or after mitochondria are shown in the Fig. 6 legend. Error bar [X ± ΔX] was calculated by the Muller formula: $\Delta X = C \cdot dx$, where ΔX is the standard squared deviation for the mean; C is the Muller coefficient which was equal to 0.303 (n=3); $dx = X_{max}–X_{min}$, where X_{max} and X_{min} are the maximal and minimal values, respectively, for the used static series of oxygen consumption rates.

Mitochondrial membrane potential

The potential induced by 5 mM succinate on the inner mitochondrial membrane (Fig. 8) was determined according to Waldmeier et al. (2002). We measured intensity of safranin fluorescence (arbitrary units) in the mitochondrial suspension with magnetic stirring at 20 °C using a Shimadzu RF-1501 spectrophotofluorimeter (Shimadzu, Germany) at 485–590 nm wavelength (excitation-emission). Mitochondria (0.5 mg/ml of protein) were placed into a quartz cuvette of four clear walls with 3 ml of 400 mOsm media containing 30 mM $TlNO_3$ (traces 2–6), 5 mM Tris-NO_3 (pH 7.3), 1 mM P_i, 3 μM safranin, 5 μM rotenone, and 3 μg/ml of oligomycin. The media additionally contained 400 (trace 1) or 340 (traces 2–5) mM sucrose (A) or 150 (trace 1) or 90 (traces 2–5) mM sucrose and 125 mM of KNO_3 (B), or $NaNO_3$ (C), or NH_4NO_3 (D). Additions of succinate, FCCP, $Mg(NO_3)_2$, ADP, CsA and quinine to the media are shown in the Fig. 8 legend.

Results

We studied effects of Tl^+ on passive permeability of the inner membrane to K^+, Na^+, or H^+ in swelling of nonenergized rat liver mitochondria in the 400 mOsm

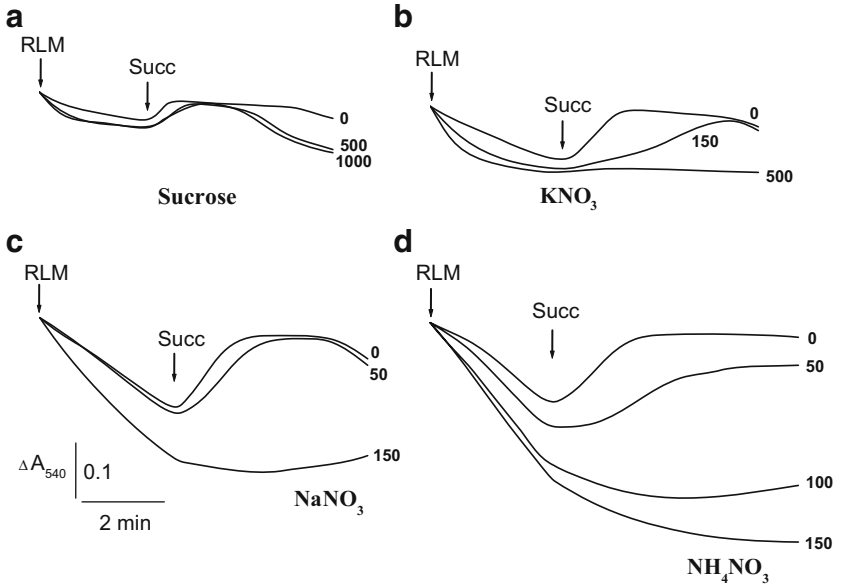

Fig. 2 Effects of quinine on the Tl^+-induced swelling of rat liver mitochondria. Mitochondria (1.5 mg/ml of protein) were added to the media containing 75 mM $TlNO_3$, 5 mM Tris-NO_3 (pH 7.3), 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**), as well as 4 μM rotenone, 3 μg/ml of oligomycin, and 50–1,000 μM quinine. Numbers on the right of traces show concentration of quinine (μM) in the media. Additions of mitochondria (RLM) and 5 mM succinate (Succ) are shown by arrows. Typical traces for three different mitochondrial preparations are presented

media containing 125 mM nitrates of K^+, Na^+ or NH_4^+ and 0–75 mM $TlNO_3$ (Figs. 1, 2 and 3, panels b–d). The replacement of the 125 mM nitrates by 250 mM sucrose was made in the media to distinguish between the degree of swelling directly caused by entry of Tl^+ into the mitochondria and the indirect effects of Tl^+ on ion permeability of the inner membrane to other ions such as K^+, Na^+ and H^+ (Figs. 1, 2 and 3, panel A). Swelling of nonenergized mitochondria was gradually enhanced with increasing concentration of $TlNO_3$ from 0 to 75 mM. At the same $TlNO_3$ concentrations, the swelling increased in the order of sucrose < KNO_3 < $NaNO_3$ < NH_4NO_3 (Fig. 1). Subsequent energization of the mitochondria by succinate stimulated their contraction (Fig. 1). Attenuation or complete inhibition of the contraction in the nitrate media with 75 mM $TlNO_3$ was found in the presence of quinine (Fig. 2b–d), an inhibitor of the mitochondrial K^+/H^+ exchanger (Nakashima and Garlid 1982; Diwan 1986; Garlid et al. 1986). The inhibitory effect of quinine on mitochondrial contraction increased in the order KNO_3< $NaNO_3$ < NH_4NO_3. The contraction was not affected by 1 mM quinine in the sucrose medium with 75 mM $TlNO_3$ (Fig. 2a).

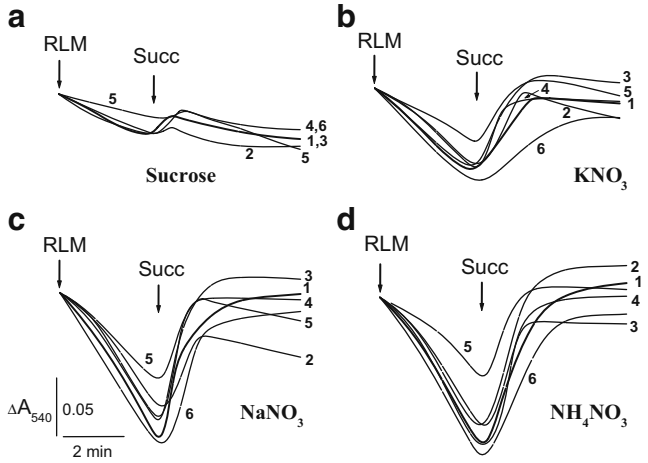

Fig. 3 Effects of Mg^{2+}, P_i, ADP, and nonactin on the Tl^+-induced swelling of rat liver mitochondria. Mitochondria (1.5 mg/ml of protein) were added to the media containing 75 mM $TlNO_3$, 5 mM Tris-NO_3 (pH 7.3), 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**), as well as 4 μM rotenone, and 3 μg/ml of oligomycin. Additions before mitochondria are indicated to right of traces: 1, none (in bold); 2, 1 mM Tris-P_i; 3, 5 mM $Mg(NO_3)_2$; 4, 0.5 mM ADP; 5, 2 mM ADP; and 6, 10^{-8}M nonactin. Additions of mitochondria (RLM) and 5 mM succinate (Succ) are shown by arrows. Typical traces for three different mitochondrial preparations are presented

It is well known that P_i increases, while Mg^{2+} and ADP decrease the ion permeability of the inner mitochondrial membrane (Zoratti and Szabo 1995; Ichas and Mazat 1998), whereas nonactin, a cyclic ionophore, facilitates transport of Tl^+ in mitochondria (Saris et al. 1981; Skulskii et al. 1984; Korotkov et al. 2007a). Therefore, we studied (Fig. 3) effects of P_i (trace 2), Mg^{2+} (trace 3), ADP (traces 4 and 5), and nonactin (trace 6) on swelling of mitochondria in the media containing 75 mM $TlNO_3$ with sucrose (A) or with the nitrates (B–D). Swelling of nonenergized mito-

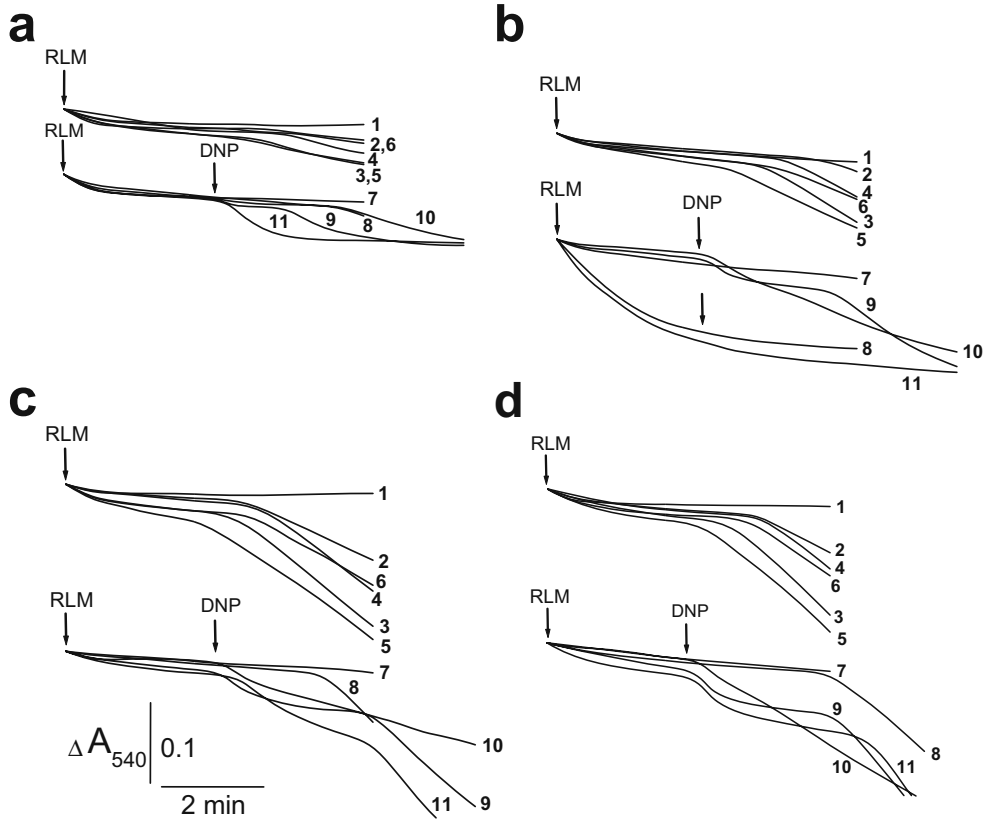

Fig. 4 Effects of Tl^+ on swelling of energized rat liver mitochondria. Mitochondria (1.5 mg/ml of protein) were added to the media containing 0–75 mM $TlNO_3$ and 0–150 mM sucrose. Additionally these media contained 5 mM Tris-NO_3 (pH 7.3), 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**), as well as 5 mM succinate, 4 μM rotenone, and 3 μg/ml of oligomycin. Concentrations of $TlNO_3$ (mM) in the media were correspondingly as follows: 1, none; 2, 50; 3–10, 75. Additions before mitochondria are indicated to right of traces: 4, 5 mM $Mg(NO_3)_2$; 5, 3 mM Tris-P_i; 6, 3 mM $Mg(NO_3)_2$ and 3 mM Tris-P_i; 7 and 10, 2 mM ADP; 8 and 11, 1000 (**a**), or 500 (**b**), or 150 (**c**), or 75 (**d**) of μM quinine. Additions of mitochondria (RLM) and of 30 μM DNP after mitochondria [traces 8–10] (DNP) are shown by arrows. Typical traces for three different mitochondrial preparations are presented

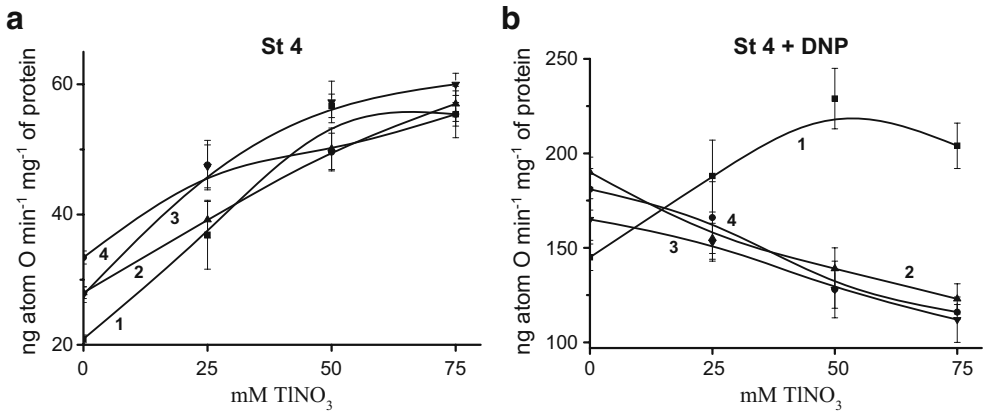

Fig. 5 Effect of $TlNO_3$ on oxygen consumption rates (ng atom O min/mg of protein) in energized rat liver mitochondria. Mitochondria (1.5 mg/ml of protein) were suspended in the media containing: 0–75 mM $TlNO_3$ and 0–150 mM sucrose. Additionally these media contained, 5 mM Tris-NO_3 (pH 7.3), 250 mM sucrose (1), or 125 mM of KNO_3 (2), or $NaNO_3$ (3), or NH_4NO_3 (4), as well as 5 mM succinate, 4 μM rotenone, and 3 μg/ml of oligomycin. DNP of 30 μM (B) was added to the media to trigger DNP-stimulated respiration after 2 min recording of state 4 (**a**). Error bars were calculated by the Muller's formula (see the "Materials and methods") from rates found for three different mitochondrial preparations

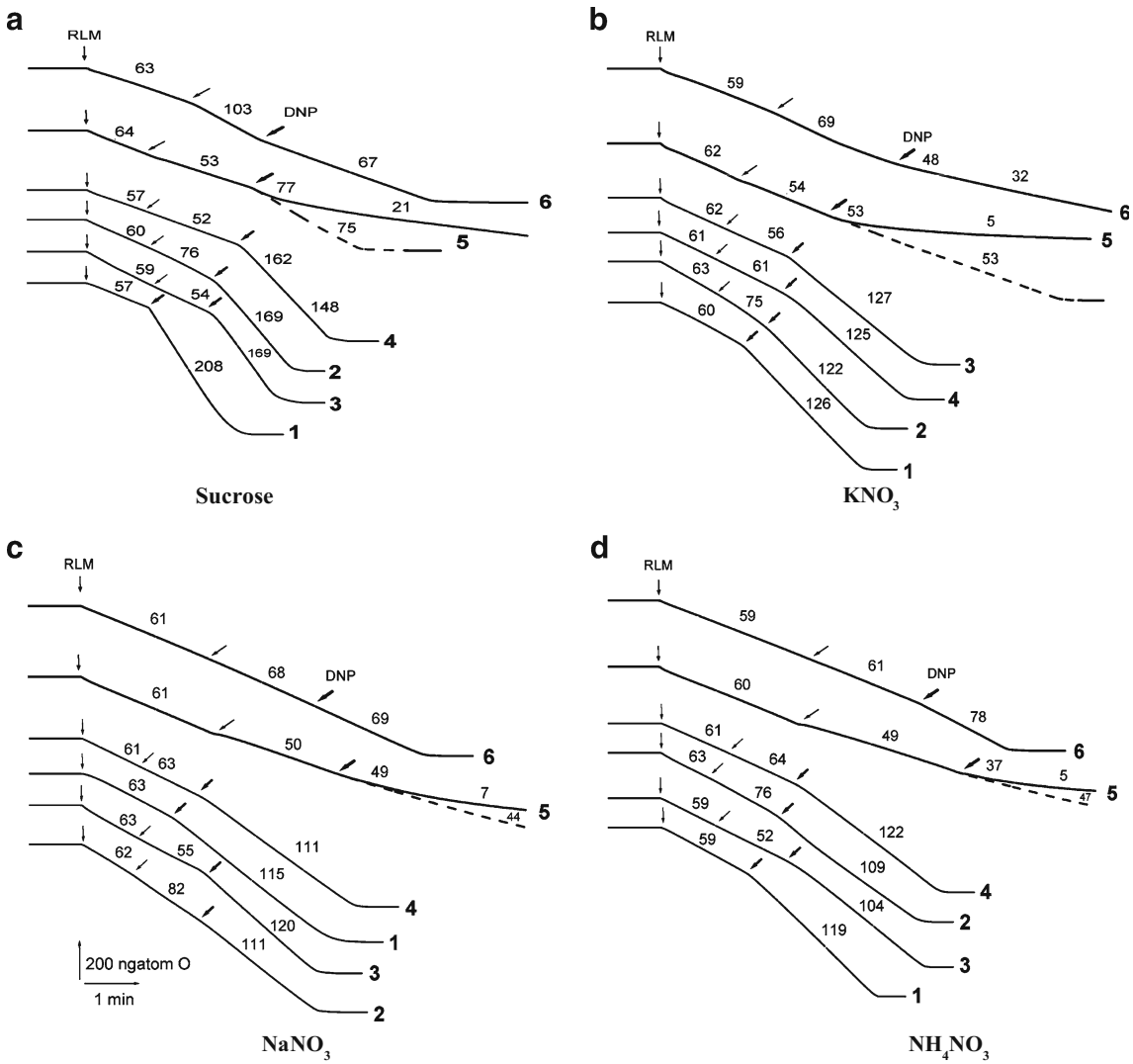

Fig. 6 Effects of 75 mM $TlNO_3$ on oxygen consumption rates (ng atom O min/mg of protein) in energized rat liver mitochondria in the presence of Mg^{2+}, P_i, ADP, and quinine. Mitochondria (1.5 mg/ml of protein) were suspended in the 400 mOsm media containing 75 mM $TlNO_3$, 5 mM Tris-NO_3 (pH 7.3), 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**), as well as 5 mM succinate, 4 μM rotenone, and 3 μg/ml of oligomycin. Additions of mitochondria (RLM) and of 30 μM DNP (DNP) are correspondingly shown by vertical and inclined bold arrows. Other additions are shown by sloping arrows and are indicated to the right of traces: 1, none; 2, 1 mM Tris-P_i; 3, 5 mM $Mg(NO_3)_2$; 4, 0.5 mM ADP; 5, 2 mM ADP; 6, 1000 (**a**), or 500 (**b**), or 150 (**c**), or 75 (**d**) of μM quinine. Oxygen consumption rates (ng atom O min/mg of protein) are presented as numbers placed above the experimental traces. Typical traces for three different mitochondrial preparations are presented

chondria markedly decreased only in the media with 2 mM ADP (Fig. 3a–d, trace 5). On the other hand, the contraction of energized mitochondria showed maximum in the nitrate media with Mg^{2+} (Fig. 3b–d, trace 3) and minimum in all used media with P_i (Fig. 3a–d, trace 2). Nonactin markedly retarded the contraction in the $NaNO_3$ or NH_4NO_3 media (Fig. 3c–d, trace 6), and especially in the KNO_3 medium (Fig. 3b, trace 6). Otherwise effect of 0.5 or 2 mM ADP on the contraction was negligible in all used media (Fig. 3a–d, traces 4 and 5) as compared with control experiments (Fig. 3a–d, trace 1). Swelling of the succinate-energized mitochondria in all used media increased in a dose-dependent manner with increasing $TlNO_3$ (Fig. 4a–d, traces 1–3). The swelling markedly decreased in the presence of 5 mM Mg^{2+} irrespective of the presence of P_i (Fig. 4a–d, traces 4 and 6) and was accelerated in the nitrate media with 3 mM P_i (Fig. 4b–d, trace 5). The swelling in all used media was slightly affected by quinine or by ADP (Fig. 4a–d, traces 7 and 8) with exception of some swelling of mitochondria in the KNO_3 medium with 0.5 mM quinine (Fig. 4b, traces 8 and 11). Administration of 30 μM DNP resulted in a rapid swelling of energized mitochondria in all used media (Fig. 4a–d, trace 9). The DNP-induced swelling was notably accelerated in the presence of 2 mM ADP (Fig. 4a–d, trace 10). Found acceleration of the swelling (observed in the last quarter of the process) (traces 1–6) or

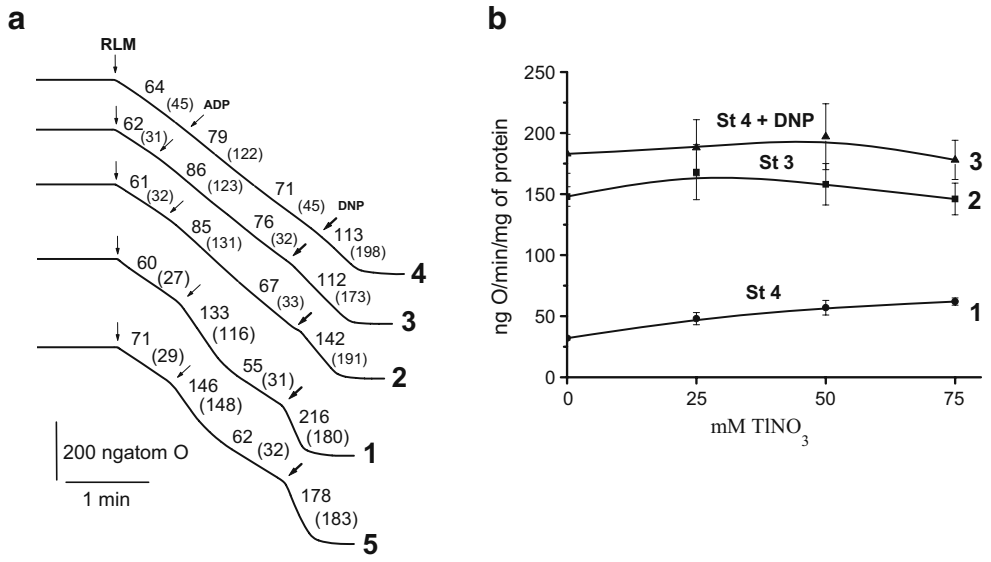

Fig. 7 Effect of $TlNO_3$ on oxygen consumption rates (ng atom O min/mg of protein) in the energized rat liver mitochondria that are in different energy states. Mitochondria (1.5 mg/ml of protein) were suspended in 400 (Panel A [traces 1–4]) or 290 (Panels A [trace 5] and B) mOsm media with 75 (**a**) or 0–75 (**b**) mM $TlNO_3$, and 0–150 mM sucrose (**b**). Additionally these media contained 5 mM Tris-NO_3 (pH 7.3), 100 (A [trace 5]) or 250 (A [trace 1]) mM sucrose, 125 mM of KNO_3 (trace 2), or $NaNO_3$ (trace 3), or NH_4NO_3 (trace 4), as well as 3 mM $Mg(NO_3)_2$, 3 mM Tris-P_i, 5 mM succinate, and 4 μM rotenone. Additions of mitochondria (RLM), 130 μM ADP (ADP), and 30 μM DNP (DNP) are shown by arrows (**a**). Oxygen consumption rates (ng atom O min/mg of protein) are shown as numbers above the experimental traces. Numbers in brackets were found from experiments with the media where 75 mM mM $TlNO_3$ was substituted by 150 mM sucrose. Typical traces (**a**) for three different mitochondrial preparations are presented. Error bars (**b**) were calculated by the Muller's formula from rates found for three different mitochondrial preparations

the additional acceleration of the swelling (observed some time after addition of DNP) (traces 8–11) was caused by deenergization of mitochondria.

In the absence of $TlNO_3$, state 4 and DNP-stimulated respiration of mitochondria were lower in the sucrose medium (Fig. 5a and b, trace 1) than in the nitrate media (Fig. 5a and b, traces 2–4). State 4 of the succinate-energized rat liver mitochondria was steadily stimulated in the 400 mOsm media by increasing concentration of Tl^+ from 0 to 75 mM (Fig. 5a, traces 1–4), and the effect of Tl^+ increased in the order of sucrose < KNO_3 < $NaNO_3$ < NH_4NO_3. After elevation of concentration of $TlNO_3$ from 25 to 75 mM, DNP-stimulated respiration increased in the sucrose medium (Fig. 5b, trace 1) and decreased in nitrate media (Fig. 5b, traces 2–4). State 4 respiration of mitochondria in all used media (Fig. 6a–d) was decreased by 5 mM Mg^{2+} (trace 3) or 2 mM ADP (trace 5) and stimulated by P_i (trace 2) or quinine (trace 6), whereas 0.5 mM ADP (trace 4) did not affect the respiration. A decrease in DNP-stimulated respiration was found in the medium with $TlNO_3$ and sucrose in the presence of Mg^{2+}, or P_i, or 0.5 mM ADP (Fig. 6a, traces 2–4). The decrease in all used media was maximal in the presence of 2 mM ADP or quinine (Fig. 6a–d, traces 5–6, accordingly). In contrast, DNP-stimulated respiration in the nitrates media was not affected by Mg^{2+}, P_i, or 0.5 mM ADP (Fig. 6b–d, traces 2–4). Decrease in DNP-stimulated respiration was less pronounced in the presence of 3 mM Mg^{2+} or 3 mM P_i (Fig. 6a–d, trace 5 in dash).

Effects of 75 mM $TlNO_3$ in the presence of Mg^{2+} and P_i (Fig. 7a) on state 3, state 4, or DNP-stimulated respiration of mitochondria are shown in experiments with 400 mOsm media containing sucrose (trace 1) or nitrates (traces 2–4). State 4 was strongly activated by 75 mM $TlNO_3$ (Fig. 7a) compared to Tl-free experiments (Fig. 7a [figures in brackets]). At the same time, state 3 or DNP-stimulated respiration were not affected by $TlNO_3$ in the sucrose medium (trace 1). On the other hand, a notable decline in state 3 and DNP-stimulated respiration was found in the nitrate media (Fig. 7a, traces 2–4) compared to the Tl^+ free experiments. This effect increased in the order of KNO_3 < $NaNO_3$ or NH_4NO_3. Effects of $TlNO_3$ on the mitochondrial respiration in 290 mOsm sucrose medium (Fig. 7a [trace 5] and b) are shown for comparison. An increase of the $TlNO_3$ concentration in the medium in the presence of Mg^{2+} and P_i also stimulated state 4 and did not affect state 3 or DNP-stimulated respiration of mitochondria (Fig. 7b). Rat liver mitochondria created $\Delta\Psi_{mito}$ after their energization by succinate in all used media (Fig. 8). Effect of quinine on $\Delta\Psi_{mito}$ was negligible (Fig. 8a–d, trace 1). However, $\Delta\Psi_{mito}$ was clearly dissipated in the presence of 30 mM $TlNO_3$ (Fig. 8a–d, trace 2) compared to the Tl^+-free experiments (Fig. 8a–d, trace 1). The Tl^+-induced dissipation of $\Delta\Psi_{mito}$ was markedly inhibited by 2 mM ADP in all

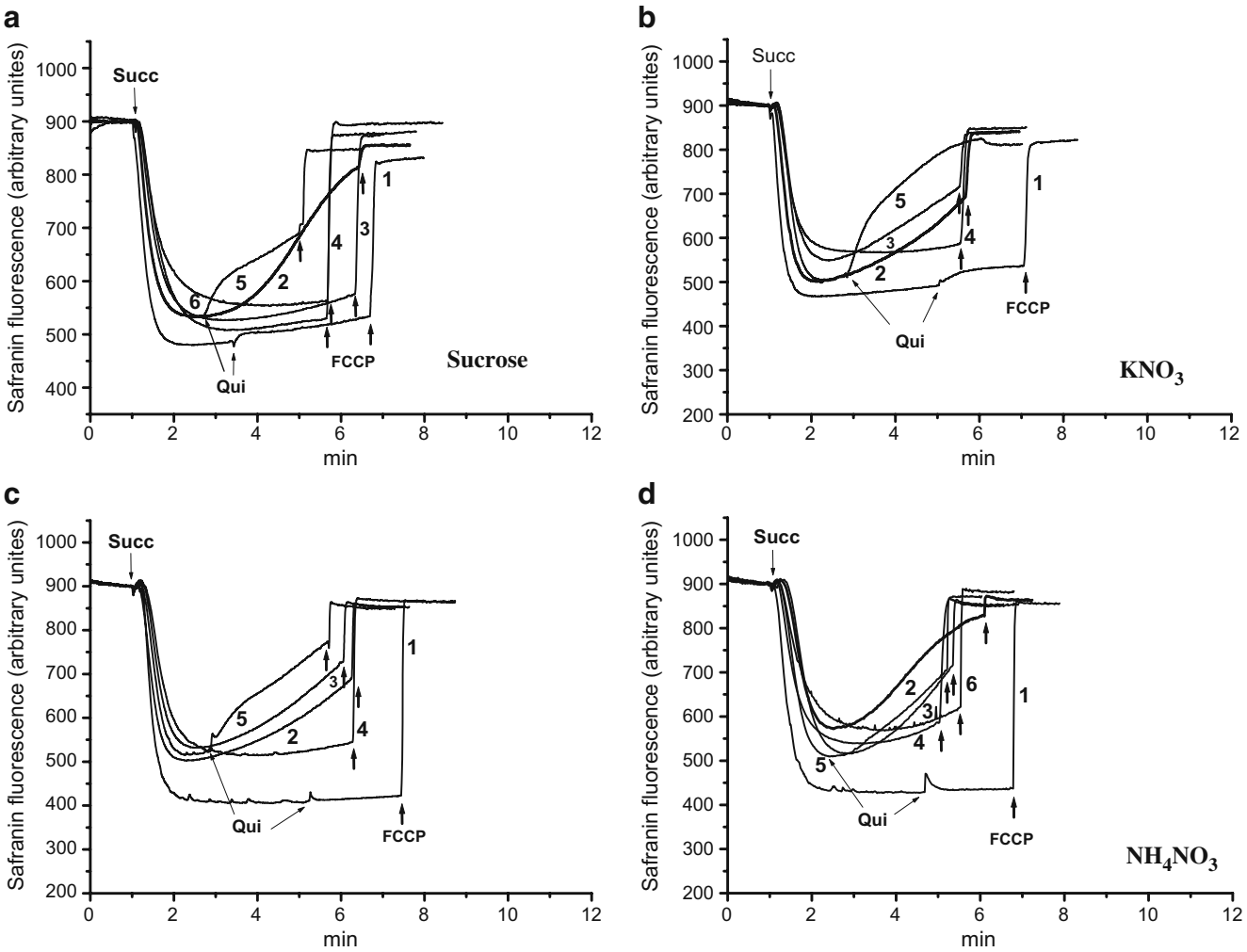

Fig. 8 Effects of Tl^+ on the succinate-induced potential in rat liver mitochondria. Mitochondria (0.5 mg/ml of protein) were added to the 400 mOsm medium containing 400 (1) or 340 (2–5) mM sucrose (**a**), or 150 (1) or 90 (2–5) mM sucrose as well as 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**), as well as 5 mM Tris-NO_3 (pH 7.3), 30 mM $TlNO_3$ (2–5), 1 mM Tris-P_i, 3 μM safranin, 5 μM rotenone, 3 μg/ml of oligomycin. Additions of 5 mM succinate (Succ), quinine (Qui), and 1 μM FCCP (FCCP) are shown by ordinary and bold short arrows. Additions before mitochondria were as follows: 1, none (free of $TlNO_3$); 2, none (marked in bold); 3, 5 mM $Mg(NO_3)_2$; 4, 2 mM ADP; 5, 75–1000 μM quinine; 6, 0.5 mM ADP, 1 mM $Mg(NO_3)_2$, and 1 μM CsA. The additions [traces 1 and 5] of quinine (μM) were the next: 1,000 (**a**), or 500 (**b**), or 150 (**c**), or 75 (**d**). Typical traces for three different mitochondrial preparations are presented

used media (Fig. 8a–d, trace 4), or by 0.5 mM ADP, 1 mM Mg^{2+}, and 1 μM CsA in sucrose and NH_4NO_3 media (Fig. 8a and d, trace 6), or by 5 mM Mg^{2+} in the sucrose medium (Fig. 8a, trace 3). The dissipation of the membrane potential in the nitrate media was not affected by 5 mM Mg^{2+} (Fig. 8b–d, trace 3).

Discussion

A rather low passive permeability of the inner mitochondrial membrane to K^+, Na^+, and H^+ was found in studies of swelling of nonenergized mitochondria in media with 125 mM of KNO_3, $NaNO_3$ and NH_4NO_3 (Mitchell and Moyle 1969; Brierley et al. 1970; Brierley and Jurkowitz 1976; Brierley et al. 1977; Diwan 1986). Swelling of nonenergized mitochondria in the 20–80 mM $TlNO_3$ media revealed a high passive permeability of the membrane to Tl^+ ions (Saris et al. 1981; Skulskii et al. 1984; Korotkov et al. 2007a, 2008a). It was found that Cd^{2+} induced swelling of nonenergized mitochondria in nitrate or chloride salts media was increased in the order of $Na^+ < K^+ < NH_4^+$ (Skulskii et al. 1988; Korotkov et al. 1998; Lee et al. 2005). On the other hand, swelling of the mitochondria in the nitrate media with $TlNO_3$ was increased in the order of $KNO_3 < NaNO_3 < NH_4NO_3$ (Fig. 1) (Korotkov and Brailovskaya 2001). We have earlier proposed that these differences in the effects of Tl^+ and of Cd^{2+} on the swelling in KNO_3 media (Korotkov and Brailovskaya 2001) might reflect the fact that transport of K^+ in mitochondria was

inhibited by Tl^+ (Barrera and Gomez-Puyou 1975; Diwan and Lehrer 1977) but stimulated by Cd^{2+} (Skulskii et al. 1988; Korotkov et al. 1998; Lee et al. 2005). These findings suggested that Tl^+ like Cd^{2+} increased passive ion permeability of the inner mitochondrial membrane (Korotkov and Brailovskaya 2001).

Other possible reason of the increase of swelling of nonenergized mitochondria in the nitrate media (Fig. 1) could be enhanced electrogenic transport of Tl^+ into the matrix. To distinguish between these two alternative hypotheses, we studied the effects of quinine, a blocker of the mitochondrial K^+/H^+ exchanger, which inhibits contraction of energized mitochondria swollen in media containing KNO_3, $NaNO_3$, or NH_4NO_3 (Nakashima and Garlid 1982; Jung et al. 1984; Diwan 1986). Given that quinine inhibits the contraction of energized mitochondria swollen in media with nitrates and $TlNO_3$ (this study, Fig. 2) and that Tl^+ has only weak effects on respiratory enzymes (Melnick et al. 1976; Woods and Fowler 1986), this suggests with a high probability that contraction of the energized mitochondria, swollen in the nitrate media (Fig. 1), occurs with participation of the K^+/H^+ exchanger as we hypothesized earlier (Korotkov et al. 2007a; Korotkov et al. 2008a). On the other hand, mitochondria swollen in the medium with $TlNO_3$ and sucrose (Fig. 2a) contracted despite the presence of 1 mM quinine. This finding suggests that extrusion of Tl^+ in this case occurred by the Tl^+/H^+ exchange mechanism, postulated earlier by Saris et al. (Saris et al. 1981), rather than via the K^+/H^+ exchanger (Brierley and Jurkowitz 1976; Bernardi 1999; Garlid and Paucek 2003). Thus, one can suggests the increase of swelling of nonenergized mitochondria in the nitrate media (Figs. 1 and 2) is actually due to the Tl^+ induced increase of ion permeability of the inner membrane to univalent cations rather than acceleration of entry of Tl^+ into the matrix, as we proposed earlier (Korotkov and Brailovskaya 2001).

It is known that state 4 respiration of mitochondria can be stimulated by an increase of the ion permeability or be elevated cation cycling via the inner mitochondrial membrane (Devin et al. 1997b). It was found that low Cd^{2+} stimulated state 4 owing to an increase of the ion permeability of the inner membrane (Skulskii et al. 1988; Korotkov et al. 1998; Belyaeva and Korotkov 2003). High Cd^{2+} decreased state 4 respiration by 20–30% in media containing KCl or NH_4NO_3. In this case, sulfhydryl reagents eliminated Cd^{2+} inhibition of the mitochondrial respiratory chain, and state 4 increased up to 200% of that found in Cd^{2+} free experiments (Korotkov et al. 1998; Belyaeva and Korotkov 2003; Korotkov et al. 2008b). However, an increase of $TlNO_3$ concentration in sucrose media stimulated state 4 of rat liver mitochondria (Figs. 5a and 7b) (Melnick et al. 1976; Diwan and Lehrer 1977; Bragadin et al. 2003; Korotkov et al. 2007a, 2008a). The stimulating effect of Tl^+ on state 4 (Fig. 5a), swelling (Fig. 4), and dissipation of $\Delta\Psi_{mito}$ (Fig. 8) in the energized mitochondria increased in the order of sucrose < KNO_3 < $NaNO_3 \leq NH_4NO_3$. Similarly, the effect of Cd^{2+} on state 4 respiration increased in the order of sucrose < KCl < NaCl (Korotkov et al. 1998). These data suggest that Tl^+ like Cd^{2+} (Sanadi et al. 1981; Rasheed et al. 1984; Korotkov et al. 1998; Belyaeva and Korotkov 2003; Lee et al. 2005), Pb^{2+} (Scott et al. 1971; Miyahara and Utsumi 1975), and Hg^{2+} (Belyaeva et al. 2004) also increased the ion permeability of the inner membrane of energized mitochondria.

It is known that DNP-stimulated respiration is dependent on activity of complexes of the respiratory chain and state 3 respiration is catalyzed by mitochondrial enzymes taking part in oxidative phosphorylation. It was found that inhibition by Cd^{2+}, Pb^{2+}, and Hg^{2+} of state 3, DNP-stimulated respiration or contraction of energized mitochondria, swollen in the nitrate or chloride media, was due to the interaction of these metals with SH groups of mitochondrial respiratory enzymes and could be eliminated by sulfhydryl reagents (Scott et al. 1971; Miyahara and Utsumi 1975; Sanadi et al. 1981; Rasheed et al. 1984; Miccadei and Floridi 1993; Korotkov et al. 1998; Rikans and Yamano 2000; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Belyaeva et al. 2004; Korotkov et al. 2007b, 2008b). On the other hand, Tl^+ only weakly inhibited mitochondrial respiratory enzymes in comparison with other metals (Melnick et al. 1976; Woods and Fowler 1986) and interacted much less with molecular SH groups (Perrin 1979). It is likely for this reason that the inhibition of state 3 or DNP-stimulated respiration of rat liver mitochondria in was not found in the media containing Tl salts and sucrose (Fig. 7) (Barrera and Gomez-Puyou 1975; Melnick et al. 1976; Diwan and Lehrer 1977; Korotkov et al. 2007a, 2008a). State 3 and DNP-stimulated respiration as well as state 4 respiration (Figs. 5 and 7) were simultaneously stimulated by increasing $TlNO_3$ concentrations in the sucrose medium. On this base it can be supposed that the increase in state 3 and DNP-stimulated respiration in the medium is due to the additive effects of $TlNO_3$, DNP or ADP.

In contrast state 3 and DNP-stimulated respiration of mitochondria was permanently decreased under elevated $TlNO_3$ concentration in the nitrate media (Figs. 5 and 7). The swelling, stimulated both by Tl^+ and by injection of DNP (Fig. 4), and dissipation of $\Delta\Psi_{mito}$ (Fig. 8) in energized mitochondria were more marked in the nitrate media than in sucrose media. It was shown that activity of respiratory complexes of mitochondria is a limiting factor for state 3 and DNP-stimulated respiration in media with KCl or NaCl but not in the sucrose one (Devin et al. 1997b). Since most of respiratory enzymes are membrane

integrated proteins, their enzymatic activity will undoubtedly depend on the structure of the inner mitochondrial membrane. It was earlier found that Tl^+ affected only enzymes which are tightly bound to the membrane (Woods and Fowler 1986). Thus, the revealed decrease of state 3 and DNP-stimulated respiration in the nitrate media (Figs. 5 and 7) can be owing to the considerable swelling of energized mitochondria (Fig. 4). This assumption is confirmed by the observation that a greater swelling (Fig. 4) was accompanied by a more substantial decrease of DNP-stimulated respiration in the nitrate media containing ADP or quinine (Fig. 6). However, other reasons of the decrease of state 3 or DNP-stimulated respiration in the nitrate media could include a greater availability of Tl^+ to the SH groups and loss of K^+ in the matrix due to an increase of the ion permeability. Some retardation of the respiratory decrease in the KNO_3 medium as compared with that found in the $NaNO_3$ or NH_4NO_3 media (Figs. 6 and 7), can argue in favor of the latter hypothesis. Thus, the above-presented data allow us to speculate that the Tl^+ induced swelling of mitochondria and not the interaction of Tl^+ with the SH groups is the main reason of effects of Tl^+ on mitochondrial respiratory enzymes that resulted in a decrease of state 3 or DNP-stimulated respiration of mitochondria in the nitrate media. Notably energized mitochondria maintained their capacity to contract in nitrate media in experiments with Tl^+ (Fig. 1) but not with Cd^{2+} (Belyaeva and Korotkov 2003; Belyaeva et al. 2004).

It was shown earlier that contraction of energized mitochondria, swollen in media with KNO_3 or $NaNO_3$, was markedly accelerated in the presence of Mg^{2+} (Brierley et al. 1970; Brierley and Jurkowitz 1976). Futile cycling of K^+ via the inner mitochondrial membrane was suppressed by Mg^{2+} which inhibited the mitochondrial K^+/H^+ exchanger (Garlid et al. 1986; Bernardi 1999) and decreased the ion permeability of the inner mitochondrial membrane (Brierley et al. 1970; Zoratti and Szabo 1995; Ichas and Mazat 1998). Additionally, Mg^{2+} and ADP have stimulated shrinkage of mitochondria in hypo- and hyperosmotic media (Brierley et al. 1970; Brierley and Jurkowitz 1976; Devin et al. 1996, 1997a; Garlid and Paucek 2003) that in turn brought about decrease of state 3. Experiments with isolated mitochondria showed that Mg^{2+}, ADP, and CsA are inhibitors of MPTP in low conduction state (Zoratti and Szabo 1995; Ichas and Mazat 1998). So, it can be supposed that a stronger contraction of energized mitochondria in the nitrate media (Figs. 3 and 4) and the decrease in DNP-stimulated respiration of mitochondria in the sucrose medium (Fig. 6a) could be due to the inhibition of MPTP by Mg^{2+} or ADP in low conductance state.

It is known that P_i, a modulator of MPTP (Brierley et al. 1970; Brierley and Jurkowitz 1976), retarded contraction of energized mitochondria swollen in media containing KNO_3 or $NaNO_3$ (Brierley et al. 1977; Devin et al. 1997a), $TlNO_3$ (Korotkov et al. 2008a), or KI and valinomycin (Azzone et al. 1976). P_i increased cycling of K^+ (Barrera and Gomez-Puyou 1975; Bernardi 1999) and Tl^+ (Korotkov et al. 2008a) via the inner mitochondrial membrane. Valinomycin (Brierley et al. 1977) and nonactin (Korotkov et al. 2007a) (accelerators of the transport of K^+ or Tl^+ in the matrix, respectively) decreased contraction of the energized mitochondria swollen in the nitrate media (especially in the KNO_3 medium) (Fig. 3). Thus, the minimal contraction of the energized mitochondria swollen in the nitrate media containing P_i or nonactin (Fig. 3), or an increase of swelling of energized mitochondria with P_i (Fig. 4) could be due to an acceleration of the entry of Tl^+ into the matrix in the presence of P_i or nonactin. State 4 (Fig. 6) and swelling of energized mitochondria (Fig. 4) with 75 mM $TlNO_3$ was somewhat decreased by Mg^{2+} or by ADP, but markedly stimulated by P_i in all used media. Tl^+-induced dissipation of $\Delta\Psi_{mito}$ was significantly retarded by 2 mM ADP or by 0.5 mM ADP, 1 mM Mg^{2+}, and 1 μM CsA (Fig. 8). Thus the presented data with Mg^{2+}, ADP, CsA and P_i suggest that increase of state 4 or of the dissipation of $\Delta\Psi_{mito}$ in all used media and the Tl^+ induced decrease of state 3 or DNP-stimulated respiration in the nitrate media resulted from the Tl^+ stimulated swelling of mitochondria due to the induction of MPTP in low conductance state. The induction of MPTP can result both in an increase of uptake of Tl^+ in the matrix and in direct acceleration of Tl^+ cycling via the inner membrane.

In summary, the most significant findings are the following: i. Like Cd^{2+} and other heavy metals, the swelling of nonenergized mitochondria in the nitrate media is due to the Tl^+-induced increase of permeability of the inner membrane to the univalent cations (K^+, Na^+, and H^+). The quinine-induced inhibition of the contraction of energized mitochondria, swollen in the nitrate media, is consistent with the conclusion and indicates participation of K^+/H^+ exchanger in extruding the Tl^+ induced excess of the cations from the mitochondrial matrix; ii. The ion permeability of the inner membrane of energized mitochondria is also stimulated by Tl^+ in the nitrate media; iii. The decrease of state 3 or DNP-stimulated respiration results from the Tl^+-induced swelling of mitochondria in the media with $TlNO_3$ and the nitrates (KNO_3, $NaNO_3$, and NH_4NO_3) rather than by inhibition of respiratory enzymes unlike the situation found with the bivalent heavy metals; iv. Our experiments with Mg^{2+}, ADP, CsA and P_i showed that the Tl^+-induced swelling and dissipation of $\Delta\Psi_{mito}$ in the nitrate media could be caused by opening of MPTP in low conductance state. Electron micrograph studies of liver sections of rats exposed to chronic thallium intoxication (Herman and Bensch 1967; Woods and Fowler 1986), and the effects of Tl^+ on hepatocytes (Zierold 2000) or

mitochondria (Skulskii et al. 1984) *in vitro* have clearly demonstrated that Tl^+ stimulated massive mitochondrial swelling followed by disruption of intracellular and mitochondrial membranes (Herman and Bensch 1967; Barrera and Gomez-Puyou 1975; Woods and Fowler 1986; Douglas et al. 1990; Zierold 2000). The swelling occurred simultaneously with an increase in concentrations of Ca^{2+}, Na^+, or P_i along with a decrease of K^+ concentration in TlCl treated hepatocytes (Zierold 2000), and with ATP deficiency in cells of rats treated with Tl salts (Herman and Bensch 1967; Woods and Fowler 1986). Thus, findings of the present study could provide a better understanding of reasons for massive swelling of mitochondria, numerous disruptions of intracellular membranes, and disturbances in ion concentrations revealed in the studies with Tl salts both *in vivo* and *in vitro* (Herman and Bensch 1967; Woods and Fowler 1986). Of couse, other reasons, non-studied in the research, and especially the participation of SH-groups can not be excluded in taking account of Tl^+-induced disturbances of mitochondrial and cellular functions.

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