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Influence of Tl^+ on mitochondrial permeability transition pore in Ca^{2+}-loaded rat liver mitochondria

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Abstract The Tl^+-induced opening of the MPTP in Ca^{2+}-loaded rat liver mitochondria energized by respiration on the substrates succinate or glutamate *plus* malate was recorded as increased swelling and dissipation of mitochondrial membrane potential as well as decreased state 4, or state 3, or 2,4-dinitrophenol-stimulated respiration. These effects of Tl^+ increased in nitrate media containing monovalent cations in the order of $Li^+ < NH_4^+ \leq Na^+ < K^+$. They were potentiated by inorganic phosphate and diminished by the MPTP inhibitors (ADP, CsA, Mg^{2+}, Li^+, rotenone, EGTA, and ruthenium red) both individually and more potently in their combinations. Maximal swelling of both non-energized and energized Ca^{2+}-loaded mitochondria in rotenone-free media is an indication of Ca^{2+} uptake driven by respiration on mitochondrial endogenous substrates. It is suggested that Tl^+ (distinct from Cd^{2+}, Hg^{2+}, and other heavy metals and regardless of the used respiratory substrates) can stimulate opening of the MPTP only in the presence of Ca^{2+}. We discuss the possible participation of Ca^{2+}-binding sites, located near the respiratory complex I and the adenine nucleotide translocase, in inducing opening of the MPTP.

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Keywords $Tl^+ \cdot H^+ \cdot Li^+ \cdot Na^+ \cdot K^+ \cdot Ca^{2+} \cdot$ Mitochondrial permeability transition · Complex I · Adenine nucleotide translocase · Mitochondrial respiration · Mitochondrial swelling · Membrane potential

Introduction

Isolated Ca^{2+}-loaded mitochondria bound Ca^{2+} with matrix calcium-specific trigger sites, located near the adenine nucleotide translocase (ANT), and following a decrease of the mitochondrial inner membrane potential ($\Delta\Psi_{mito}$) triggered mechanisms that opened the mitochondrial permeability transition pore (MPTP) in the high conductance state (Zoratti and Szabó 1995; Ichas and Mazat 1998; Bernardi 1999; Halestrap and Brenner 2003; Halestrap 2009). As a result the mitochondrial inner membrane (MIM) became permeable to molecules up to 1500 kDa causing massive mitochondrial swelling, lowering of [Ca^{2+}] and [ATP] in the matrix, dissipation of the $\Delta\Psi_{mito}$, and release of cytochrome *c*. When the Ca^{2+}-binding sites are insufficiently saturated, MPTPs are opened in the low conductance state and small molecules up to 300 kDa or ions (H^+, K^+, and Ca^{2+}) may penetrate the MIM more easily (Ichas and Mazat 1998; Bernardi 1999). The ANT along with cyclophilin D (CyP-D) and the voltage-dependent anion channel (VDAC) were initially believed to be part of the MPTP complex (Zoratti and Szabó 1995; Bernardi 1999; Halestrap and Brenner 2003). However, this complex composition was recently revised after studies on CyP-D-deficient mitochondria and reconstitution experiments (Basso et al. 2008; Leung et al. 2008; Baines 2009; Halestrap 2009; Zorov et al. 2009). Presently, many researchers consider the mitochondrial phosphate carrier and CyP-D to be primary components of the MPTP,

whereas ANT is viewed as a regulatory part of the MPTP, and VDAC is no longer considered to be a part of the MPTP complex (Leung et al. 2008; Baines 2009; Halestrap 2009; Zorov et al. 2009). It is common knowledge that ADP and bonkrekic acid keep ANT in the "m" conformation, while cyclosporine A (CsA) decreases the reaction of CyP-D with MIM, and that bivalent cations (Mg^{2+}, Sr^{2+}, and Mn^{2+}) and H^+ prevent binding of Ca^{2+} to the matrix specific Ca^{2+}-binding sites, and finally that Ca^{2+}, Mg^{2+} and Li^+ occupy divalent cation binding sites on the external surface of MIM. Eventually, this permits us to attribute all these substances to inhibitors of the MPTP (Novgorodov et al. 1994; Zoratti and Szabó 1995; Ichas and Mazat 1998; Waldmeier et al. 2002; Halestrap and Brenner 2003; Shalbuyeva et al. 2007; Leung et al. 2008; Halestrap 2009; Zorov et al. 2009). Rotenone inhibits opening of MPTP because it blocks electron flow in complex I and retains pyridine nucleotides in the reduced state (Rigobello et al. 1995; Fontaine et al. 1998; Waldmeier et al. 2002; Baines 2009). It has been found that the MPTP-inducing concentrations of Ca^{2+} were lower in experiments with mitochondria energized by complex I substrates or succinate alone in comparison to the concentrations observed when mitochondria were energized with succinate in the presence of rotenone (Fontaine et al. 1998; Belyaeva et al. 2004a; Baines 2009). This phenomenon, referred to as substrate specificity, can be associated with the participation of complex I in the formation of MPTP (Fontaine et al. 1998; Fontaine and Bernardi 1999).

It has been shown that the essence of the deleterious effects of Tl^+ on living organisms is its ability to easily penetrate the inner membrane (Melnick et al. 1976; Saris et al. 1981; Skulskii et al. 1984; Korotkov et al. 2007; Korotkov et al. 2008). This is manifested as notable swelling of non-energized mitochondria and as well as increased state 4 respiration in media containing $TlNO_3$. Tl^+ expresses a considerably lower affinity to molecular SH groups in contrast to bivalent heavy metals (Cd^{2+}, Zn^{2+}, Hg^{2+}, and Pb^{2+}) (Perrin 1979). Thus Tl^+ does not inhibit state 3 or 2,4-dinitrophenol (DNP)-stimulated respiration (Melnick et al. 1976; Korotkov et al. 2007; Korotkov et al. 2008; Korotkov 2009). It has been assumed that the interaction of Tl^+ with SH groups of mitochondria and glutathione depletion, as well as outflow of Ca^{2+} in the cytoplasm and increased production of reactive oxygen species can, at least partly, explain the detrimental effects of Tl^+ on cells and mitochondria (Herman and Bensch 1967; Zierold 2000; Verstraeten 2006; Hanzel and Verstraeten 2006; Pourahmad et al. 2010). Our recent studies of the effects of Tl^+ in media containing nitrate salts of univalent cations and $TlNO_3$ (Korotkov 2009) showed that Tl^+ similar to the bivalent heavy metals (Miyahara and Utsumi 1975; Korotkov et al. 1998; Wudarczyk et al. 1999; Belyaeva et al. 2004b) increased the permeability of the inner membrane to univalent cations (H^+, K^+, and Na^+). This led to increased swelling of both non-energized and energized mitochondria, and resulted in lowered state 3 and DNP-stimulated respiration and in dissipation of $\Delta\Psi_{mito}$ (Korotkov 2009). Contraction of succinate-energized mitochondria, swollen in the nitrate media, was inhibited by quinine, which blocks mitochondrial K^+/H^+ exchange (Korotkov 2009). This demonstrated participation of the exchanger in extruding the Tl^+-induced excess of the univalent cations from the matrix as previously suggested (Korotkov et al. 2008).

It was found earlier that ADP and CsA could inhibit Tl^+-induced opening of MPTP in Ca^{2+}-loaded rat liver mitochondria (CaRLM), which was manifested both as increased mitochondrial swelling and decreased state 3 and DNP-stimulated respiration (Bragadin et al. 2003; Korotkov and Lapin 2003). These findings encouraged us to study the molecular mechanism behind opening of the Tl^+-induced MPTP in CaRLM which has not been sufficiently studied. Our aim was to study the effects of Tl^+ on CaRLM energized by substrates of respiratory complexes I and II (glutamate *plus* malate or succinate, respectively). We checked the hypothesis of the role of participation of the Ca^{2+}-binding sites of complex I or ANT in opening the Tl^+-induced MPTP in CaRLM. In addition, we studied the presence of substrate specificity in the combined effects of Tl^+ and Ca^{2+} on the swelling of energized mitochondria. We examined swelling, state 4, state 3 and DNP-stimulated respiration, as well as $\Delta\Psi_{mito}$ of CaRLM in media containing $TlNO_3$ and the nitrates (individually and in combination) in the presence of MPTP inhibitors (ADP, CsA, EGTA, Mg^{2+}, and Li^+), of MPTP modulators (Ca^{2+} and P_i), and of ruthenium red (an inhibitor of the mitochondrial Ca^{2+} uniporter). Experiments were performed with the nitrate and sucrose media containing $TlNO_3$ and Ca^{2+} to study the MPTP opening in the high and low conductance states.

Materials and methods

Chemicals

$CaCl_2$, $Mg(NO_3)_2$, H_3PO_4, $NaNO_3$, KNO_3, $LiNO_3$, NH_4NO_3 and DNP were of analytical grade. Rotenone, oligomycin, CsA, safranin, ruthenium red (RR), $TlNO_3$, Tris-OH, ethylene glycol-bis(β-aminoethyl ether) N,N,N′, N′-tetraacetic acid (EGTA), ADP, glutamate, malate, succinate, and carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP), were from Sigma (St. Louis, MO, USA). Sucrose as 1 M solution was refined from cation traces on a column filled with a KU-2-8 resin from Azot (Kemerovo, Russia).

Isolation of mitochondria

Liver mitochondria were isolated from Wistar adult male rats (200–250 g) according to a standard procedure described in detail by Korotkov (2009). Liver mitochondria were suspended in a medium containing 250 mM sucrose, 3 mM Tris–HCl (pH 7.3), and 0.5 mM EGTA; next they were rinsed twice 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 tested by Bradford's method and it amounted to 50–60 mg/ml.

Swelling of mitochondria

Mitochondrial swelling was evaluated as a decrease in A_{540} at 20 °C using a SF-46 spectrophotometer (LOMO, St. Petersburg, Russia). Mitochondria (1.5 mg protein/ml) were added into a 1-cm cuvette with 1.5 ml of 400 mOsm medium, composed of two parts. The first one of 150 mOsm contained 0–75 mM $TlNO_3$ and 0–150 mM sucrose (Figs. 1, 2, 3, 4, 5, 6), concentration combinations (mM) of $TlNO_3$ and sucrose in the media are shown in the greater detail in Fig. 1a. The second part of 250 mOsm contained 250 mM sucrose, or 125 mM of KNO_3, or $LiNO_3$, or $NaNO_3$, or NH_4NO_3 (Figs. 1–6), as well as 5 mM Tris-succinate (Fig. 6), 5 mM Tris-NO_3 (pH 7.3), and 1 μg/ml of oligomycin. We previously established the ability of this technique to detect mitochondrial swelling in the medium (Korotkov 2009). Where indicated, the following chemicals were administered into the media before addition of mitochondria: 1–5 μM rotenone, 25–100 μM $CaCl_2$, 3 mM Tris-P_i, 3 mM $Mg(NO_3)_2$, 0.5 or 2 mM ADP, 1 μM CsA, 7 μM RR, and 1 mM EGTA. The swelling, $\Delta\Psi_{mito}$ and the respiration rates were tested in 400 mOsm media in order to check the comparability and consistency between the results in different experiments.

Respiration

Respiration (oxygen consumption rate) was measured polarographically using LP-7 (Czechoslovakia) in a 1.5-ml closed thermostatic chamber with magnetic stirring at 26 °C. Mitochondria (1.5 mg protein/ml) were added to media containing 75 mM $TlNO_3$ (Figs. 7–8). Additionally, these media contained 250 mM sucrose, or 125 mM of KNO_3, or $NaNO_3$, or NH_4NO_3, as well as 5 mM Tris-NO_3 (pH 7.3), 5 mM Tris-succinate, and 4 μM rotenone. In some incubations, 3 μg/ml of oligomycin (Fig. 7) or 3 mM Mg $(NO_3)_2$ and 3 mM Tris-P_i (Fig. 8) were added. ADP of 130 μM or DNP of 30 μM were injected into the media after 2 min recording of state 4 to induce state 3 or DNP-stimulated respiration (Figs. 7–8). Additions of P_i, Mg^{2+}, ADP, CsA, and Ca^{2+} before or after mitochondria are shown in the Figs. 7–8 legends.

Mitochondrial membrane potential

The $\Delta\Psi_{mito}$ induced by 5 mM succinate on the MIM (Fig. 9) was evaluated 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 protein/ml) were placed into a quartz cuvette of four clear walls with 3 ml of a medium containing 30 mM $TlNO_3$, 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 340 mM sucrose (a) or 90 mM sucrose and 125 mM of KNO_3 (b), or $NaNO_3$ (c), or NH_4NO_3 (d). Details of additions of succinate, $Mg(NO_3)_2$, ADP, CsA, Ca^{2+}, and FCCP in the media are given in the Fig. 9 legend.

Results

The swelling technique is often used to study MPTP opening in energized mitochondria exposed to Ca^{2+} (Zoratti and Szabó 1995; Ichas and Mazat 1998) or bivalent heavy metals such as Cd^{2+}, Hg^{2+}, Zn^{2+}, and Cu^{2+} (Wudarczyk et al. 1999; Belyaeva et al. 2002; Dorta et al. 2003; Belyaeva et al. 2004b). We studied the effects of Tl^+ on swelling of CaRLM in the 400 mOsm medium containing 125 mM nitrate salts of K^+, Li^+, Na^+ or NH_4^+ and 0–75 mM $TlNO_3$ (Figs. 1–6, panels b–e). The 125 mM electrolyte medium was replaced by 250 mM sucrose medium (Figs. 1–5, panel a) in order to distinguish between MPTP in the low (nitrates) and high (sucrose) conduction states (Ichas and Mazat 1998; Bernardi 1999). Swelling of non-energized mitochondria in the media free of rotenone was progressively enhanced by increasing the concentration of $TlNO_3$ from 0 to 75 mM (Fig. 1). At equal $TlNO_3$ concentrations, the swelling increased in the order of sucrose$<LiNO_3<NH_4NO_3\leq NaNO_3<KNO_3$ (Fig. 1). The swelling was additionally stimulated by 25–100 μM Ca^{2+} in the media containing 50–75 mM $TlNO_3$. Further energization of the mitochondria by glutamate plus malate caused them to contract slightly, followed by weak mitochondrial swelling (Fig. 1). CsA, Mg^{2+}, and ADP are potent inhibitors of MPTP (Novgorodov et al. 1994; Zoratti and Szabó 1995; Bravo et al. 1997; Ichas and Mazat 1998). It is known that RR competitively inhibits mitochondrial Ca^{2+} uptake and prevents Ca^{2+}- or Cd^{2+}-induced MPTP (Korotkov and Skulskii 1996; Belyaeva et al. 2001; Dorta et al. 2003;

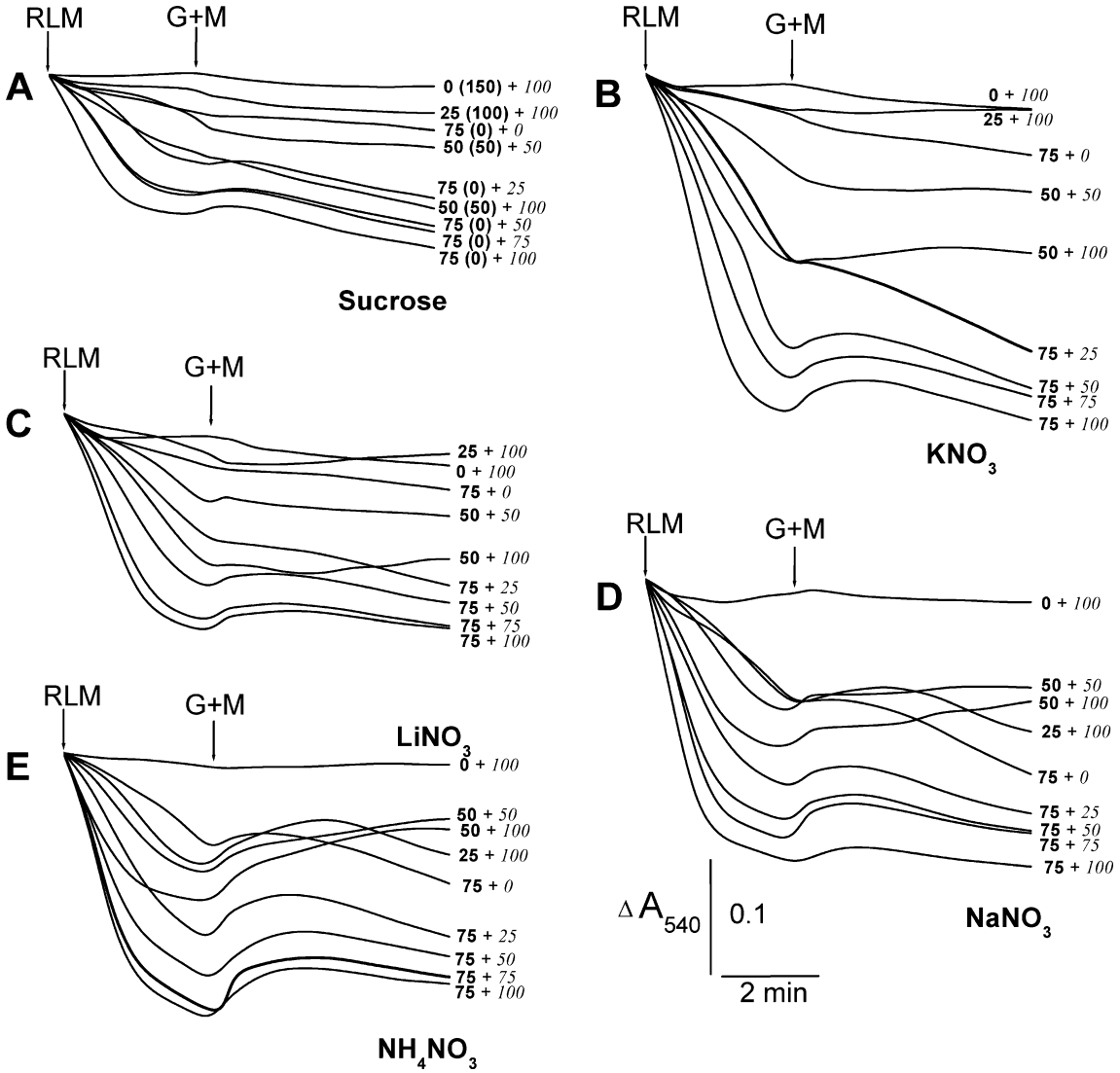

Fig. 1 Effects of Tl^+ and Ca^{2+} on swelling of rat liver mitochondria in the presence of glutamate and malate. Mitochondria (1.5 mg protein/ml) were added to the 400 mOsm medium containing 0–75 mM $TlNO_3$ and 0–150 mM sucrose. Concentration combinations of $TlNO_3$ (mM) [in bold] and sucrose (mM) [in bold parentheses] are shown on the right of the traces (Fig. 1a). The combinations were the same both in Figs. 1b–e and in Figs. 2–6. These media contained additionally 5 mM Tris-NO_3 (pH 7.3), 0–100 μM Ca^{2+}, and 1 μg/ml of oligomycin, as well as 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $LiNO_3$ (**c**), or $NaNO_3$ (**d**), or NH_4NO_3 (**e**). The numbers on the right of the traces show concentrations of $CaCl_2$ (μM) [in italics] in these media. Additions of mitochondria (RLM) and of 5 mM of glutamate and malate (G+M) are shown by arrows. Typical traces for three different mitochondrial preparations are presented

Lee et al. 2005). Accordingly, we studied the effects of CsA, ADP, Mg^{2+}, and RR on the swelling of mitochondria in media containing 50 mM $TlNO_3$ and 100 μM Ca^{2+} (Fig. 2). Swelling of non-energized mitochondria in the nitrate medium decreased in the order of control≥Mg or ADP± Mg≥RR or ADP+CsA±Mg>CsA±Mg. Addition of glutamate and malate stimulated the contraction of mitochondria 3 min after recording of the swelling. The contraction increased in the order of control≤Mg or ADP±Mg≤RR or CsA±Mg<ADP+CsA±Mg (Fig. 2b–e). Both the swelling and the contraction of mitochondria in the sucrose medium illustrated the later order (Fig. 2a).

It is well known that the probability of MPTP opening is lowered by rotenone because electron transport is blocked via complex I and oxidation of matrix pyridine nucleotides is prevented (Rigobello et al. 1995; Fontaine et al. 1998; Waldmeier et al. 2002; Baines 2009). The effect of 0–5 μM rotenone on swelling of mitochondria in the medium containing 50–75 mM $TlNO_3$ and 100 μM Ca^{2+} is shown in Fig. 3. Administration of glutamate and malate (bold trace) or succinate in the medium free of rotenone resulted in marginal contraction of the mitochondria. The swelling of non-energized mitochondria diminished sharply in the presence of rotenone (Fig. 3). Subsequent energization of

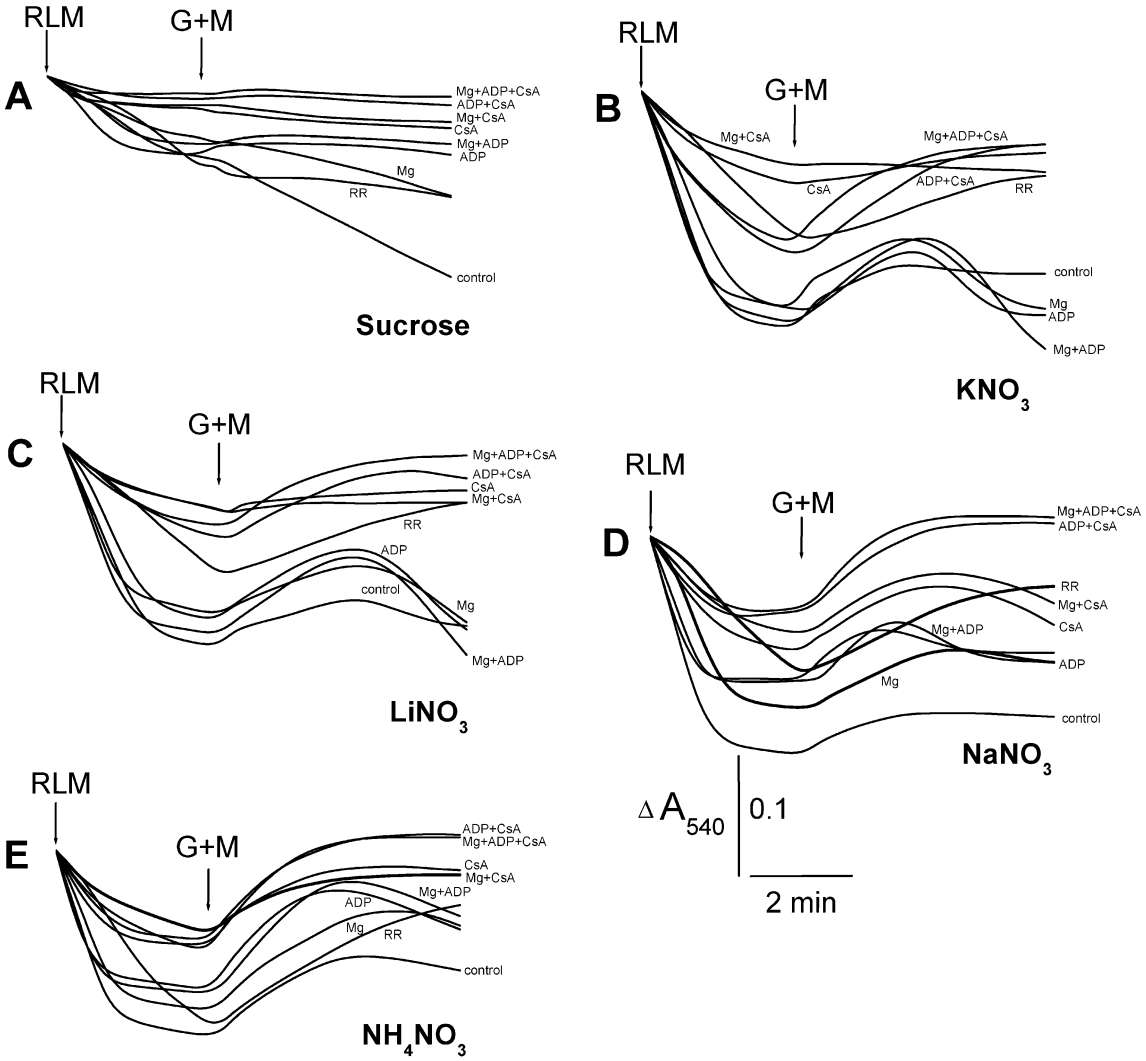

Fig. 2 Effects of CsA, ADP, RR, and Mg^{2+} on Tl^{+}-induced swelling of Ca^{2+}-loaded rat liver mitochondria in the presence of glutamate and malate. Mitochondria (1.5 mg protein/ml) were added to media containing 50 mM $TlNO_3$ and 50 mM sucrose. These media contained additionally 5 mM Tris-NO_3 (pH 7.3), 100 μM Ca^{2+}, and 1 μg/ml of oligomycin, as well as 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $LiNO_3$ (**c**), or $NaNO_3$ (**d**), or NH_4NO_3 (**e**). Additions before mitochondria are indicated on the right of the traces: none (control); 1 μM CsA (CsA); 0.5 mM ADP (ADP); 3 mM Mg^{2+} (Mg); 7 μM RR (RR). Additions of mitochondria (RLM) and of 5 mM of glutamate and malate (G+M) are shown by arrows. Typical traces for three different mitochondrial preparations are presented

the mitochondria by succinate stimulated mitochondrial swelling which increased in the order of sucrose<$LiNO_3$<NH_4NO_3≤$NaNO_3$<KNO_3 (Fig. 3). The swelling slowed down after the rotenone concentration was decreased from 2 to 5 μM. The swelling of non-energized mitochondria in media containing 0–75 mM $TlNO_3$, 2 μM rotenone, and 0–100 μM Ca^{2+}, as well as 250 mM sucrose (Fig. 4a) or 125 mM nitrates (Fig. 4b–e) was not affected by Ca^{2+} and it was gradually enhanced as concentration of $TlNO_3$ increased. At the same $TlNO_3$ concentrations, the swelling increased in the order of sucrose<KNO_3 or $LiNO_3$<$NaNO_3$<NH_4NO_3 (Fig. 4). Further energization of mitochondria by succinate stimulated their contraction in all media containing 0–25 mM $TlNO_3$ and 100 μM Ca^{2+}. The contraction was still detected in the sucrose and $LiNO_3$ media containing 2 μM rotenone, as well as 50 mM $TlNO_3$ and 50–100 μM Ca^{2+} or 75 mM $TlNO_3$ and 25 μM Ca^{2+} (Fig. 4a and c). The contraction of energized mitochondria was replaced by their swelling under increasing the concentrations of both $TlNO_3$ (from 50 to 75 mM) and Ca^{2+} (from 25 to 100 μM) in all media used (Fig. 4). At the same concentrations of Tl^{+} and Ca^{2+}, their combined effect on the swelling increased in the order of sucrose<$LiNO_3$<NH_4NO_3≤$NaNO_3$<KNO_3.

Effects of the MPTP inhibitors (CsA, ADP, and Mg^{2+}) on the swelling of mitochondria in the media containing 75 mM $TlNO_3$, 100 μM Ca^{2+}, and 2 μM rotenone, are shown in Fig. 5. The swelling of non-energized mitochondria dimin-

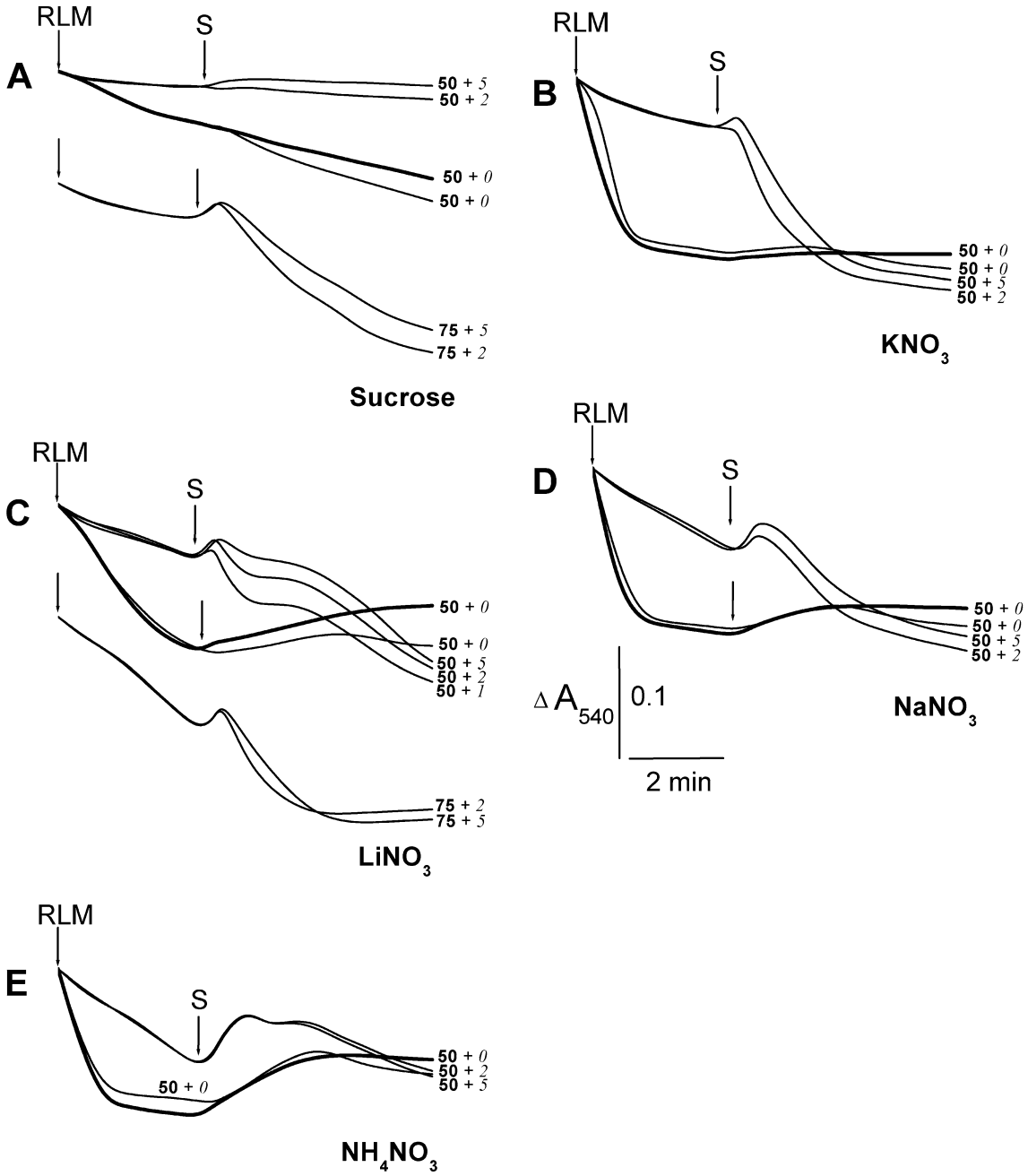

Fig. 3 Effects of rotenone on Tl^+-induced swelling of Ca^{2+}-loaded rat liver mitochondria. Mitochondria (1.5 mg protein/ml) were added to media containing 50–75 mM $TlNO_3$ and 0–50 mM sucrose. These media contained additionally 5 mM Tris-NO_3 (pH 7.3), 100 μM Ca^{2+}, 0–5 μM rotenone, and 1 μg/ml of oligomycin, as well as 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $LiNO_3$ (**c**), or $NaNO_3$ (**d**), or NH_4NO_3 (**e**). The numbers on the right of the traces show concentrations both of $TlNO_3$ (mM) [in bold] and of rotenone (μM) [in italics] in these media. Additions of mitochondria (RLM) and of substrates (S) are shown by arrows. These substrates are: 5 mM glutamate and malate (traces in bold) or 5 mM succinate. Typical traces for three different mitochondrial preparations are presented

ished in the media containing the inhibitors in the order of control, or Mg, or CsA±Mg<ADP±Mg<ADP+CsA±Mg< ADP(2). After being energized by succinate, the mitochondria underwent massive swelling (Fig. 5b–d) which decreased in the nitrate media containing the inhibitors in the order of control>Mg>CsA>Mg+CsA>ADP. Various combinations of the inhibitors eliminated the swelling, after which the succinate-energized mitochondria contracted (Fig. 5b–d). The contraction progressed in the series of no Ca<Mg+ADP or ADP(2)<ADP+CsA<ADP+CsA+Mg. The swelling of energized mitochondria was detected in the sucrose medium containing Ca±Mg only. Other experiments in the medium showed that mitochondrial contraction took place in the presence of different

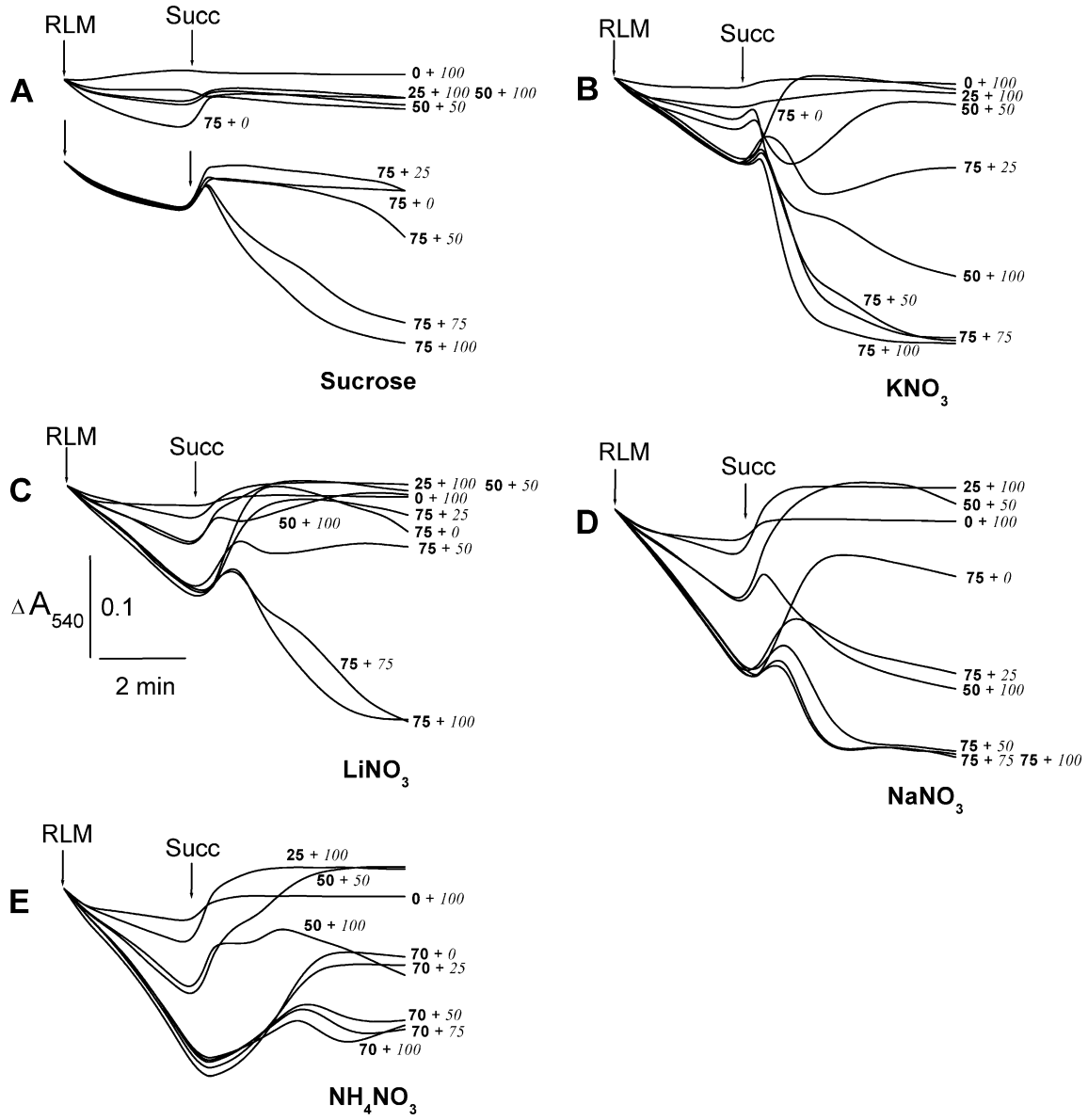

Fig. 4 Effects of Tl^+ and Ca^{2+} on swelling of rat liver mitochondria in the presence of succinate. Mitochondria (1.5 mg protein/ml) were supplemented to media containing 0–75 mM $TlNO_3$ and 0–150 mM sucrose. Additionally these media contained 5 mM Tris-NO_3 (pH 7.3), 0–100 μM Ca^{2+}, 1 μg/ml of oligomycin, and 2 μM rotenone, as well as 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $LiNO_3$ (**c**), or $NaNO_3$ (**d**), or NH_4NO_3 (**e**). Numbers on the right of the traces show concentrations both of $TlNO_3$ (mM) [in bold] and of $CaCl_2$ (μM) [in italics] 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

combinations of the inhibitors (Fig. 5a). Succinate-energized mitochondria in the nitrate medium containing 75 mM $TlNO_3$ showed weak swelling in the Ca^{2+}-free experiments (Fig. 6a–c). The swelling was markedly stimulated only after the addition of Ca^{2+} to the medium, and markedly retarded after administration of ADP, or CsA, or RR, or EGTA (Fig. 6).

Figure 7 illustrates the effects of Ca^{2+} on respiration rates of succinate-energized rat liver mitochondria in the medium containing 75 mM $TlNO_3$ in the presence of P_i or the MPTP inhibitors (ADP, Mg^{2+}, and CsA) and of combinations of the latter. State 4 was stimulated in the medium in contrast to experiments free of $TlNO_3$ (control, figures in braces) (Korotkov 2009). Ca^{2+} in the medium caused a burst of state 4 and following its decrease which occurred also in the presence of the inhibitors in all used media in comparison to Ca^{2+}-free experiments (figures in parentheses). The respiration burst was less visible in the simultaneous presence of Mg^{2+}, ADP, and CsA in the media and state 4 returned to the basic level, as in the

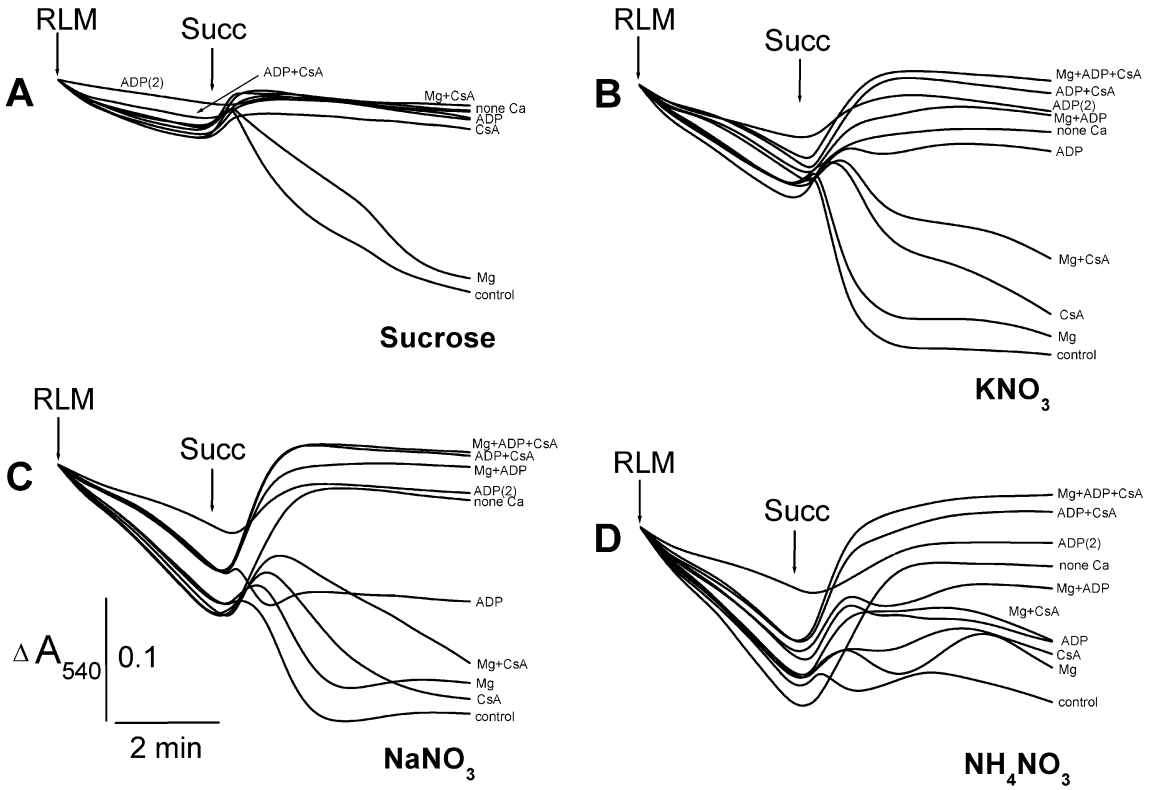

Fig. 5 Effects of CsA, ADP, and Mg^{2+} on Tl^{+}-induced swelling of Ca^{2+}-loaded rat liver mitochondria in the presence of succinate. Mitochondria (1.5 mg protein/ml) were added to media containing 75 mM $TlNO_3$ and 100 μM Ca^{2+}. Additionally these media contained 5 mM Tris-NO_3 (pH 7.3), 2 μM rotenone, and 1 μg/ml of oligomycin, as well as 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**). Additions before mitochondria are indicated on the right of the traces: none (control); free of Ca^{2+} (none Ca); 1 μM CsA (CsA); 0.5 mM ADP (ADP); 2 mM ADP (ADP(2)); 3 mM Mg^{2+} (Mg). Additions of mitochondria (RLM) and of 5 mM succinate (Succ) are shown by arrows. Typical traces for three different mitochondrial preparations are presented

experiments without Ca^{2+} (figures in parentheses). The Ca^{2+}-induced increase of state 4 respiration was maximal in the media with P_i (Fig. 7). DNP-stimulated respiration in the media containing $TlNO_3$ and Ca^{2+} showed a visible decrease after addition of Ca^{2+} (control) in comparison to the minimal decrease observed in the Ca^{2+}-free experiments (control, figures in parentheses). The decrease was preserved in the presence of the MPTP inhibitors alone and leveled off in their simultaneous presence in the media (Fig. 7). A maximal decrease in DNP-stimulated respiration was observed in the nitrate media containing Ca^{2+} and P_i (Fig. 7). Regardless of the presence of Ca^{2+}, state 4 (Fig. 8) was stimulated in the media containing 75 mM $TlNO_3$, Mg^{2+} and P_i. State 3 or DNP-stimulated respiration was less visible in the nitrate medium than in the sucrose medium (Fig. 8) (Korotkov 2009). This respiration increased in the nitrate media in the order of $TlNO_3$ + Ca^{2+} < $TlNO_3$ (figures in parentheses)<media free of both $TlNO_3$ and Ca^{2+} (Korotkov 2009). The dissipation of $\Delta\Psi_{mito}$ was negligible within 4–5 min after addition of succinate to the $TlNO_3$ medium (Fig. 9, trace 1). The dissipation was strongly accelerated after administration of Ca^{2+} to the medium (Fig. 9, trace 2). At the same time, the dissipation in the sucrose medium expressed a biphasic mode (Fig. 9a, trace 2). The MPTP inhibitors (ADP, Mg^{2+}, and CsA) markedly retarded the Ca^{2+}-induced dissipation of $\Delta\Psi_{mito}$ in the nitrate medium (Fig. 9b–d, traces 3–5). The effects of the inhibitors were maximal in the sucrose medium (Fig. 9a, traces 3–5).

Discussion

This study showed that the swelling of non-energized mitochondria was stimulated by both Tl^{+} and Ca^{2+} in all of the used rotenone-free media (Figs. 1 and 3). However, the swelling was increased by Tl^{+} but not Ca^{2+} in the presence of 2 μM (Fig. 4) or 4 μM rotenone (Korotkov and Lapin 2003). Earlier we found that the Cd^{2+}-stimulated swelling of non-energized rat liver mitochondria in rotenone-free media was more intense than the swelling in the presence of rotenone (Belyaeva and Korotkov 2003). Rat liver mitochondria created visible $\Delta\Psi_{mito}$ before addition of glutamate and malate to the sucrose medium free of rotenone

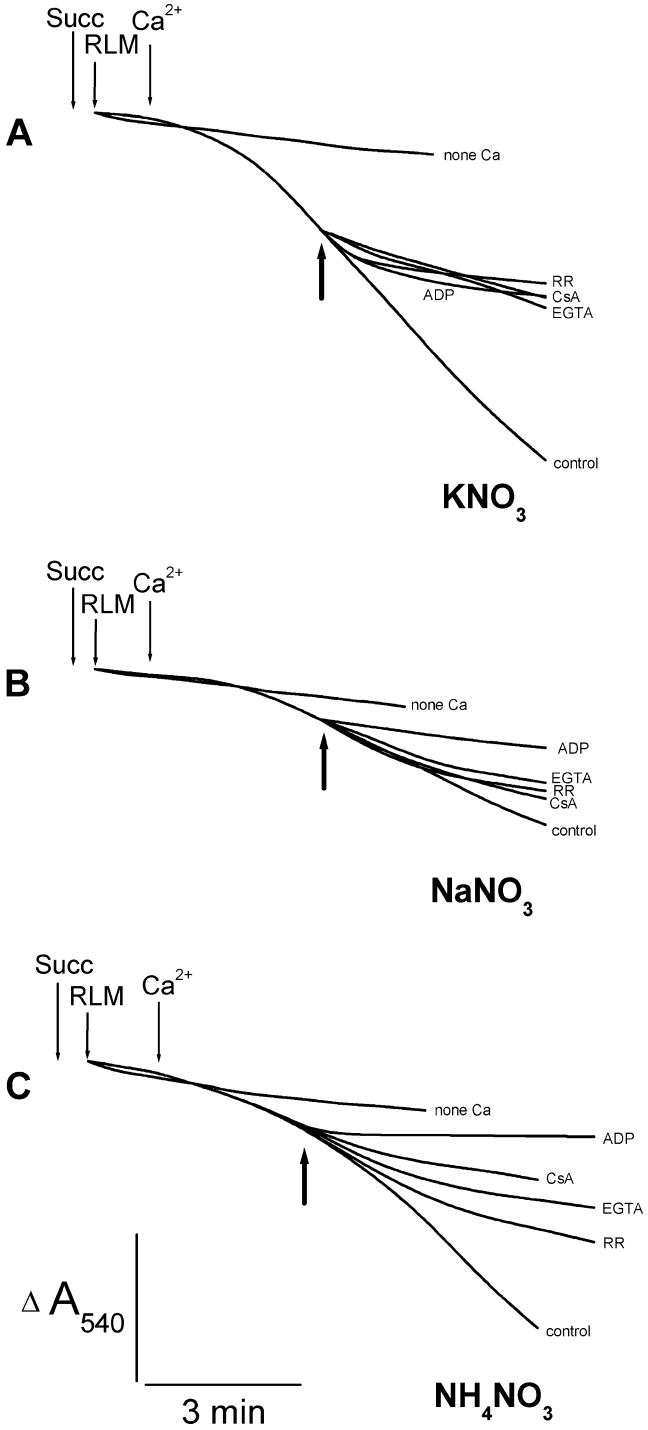

Fig. 6 Effects of ADP, CsA, EGTA, and RR on Tl^+-induced swelling of Ca^{2+}-loaded rat liver mitochondria. Mitochondria (1.5 mg protein/ml) were added to media containing 75 mM $TlNO_3$. Additionally these media contained 5 mM succinate, 5 mM Tris-NO_3 (pH 7.3), 4 μM rotenone, and 1 μg/ml of oligomycin, as well as 125 mM of KNO_3 (**a**), or $NaNO_3$ (**b**), or NH_4NO_3 (**c**). Additions of mitochondria (RLM), 5 mM succinate (Succ), and 100 μM Ca^{2+} (Ca^{2+}) are shown by arrows. Additions after mitochondria are shown by an arrow (in bold) and indicated on the right of the traces: none (control); free of Ca^{2+} (none Ca); 1 μM CsA (CsA), 0.5 mM ADP (ADP), 7 μM RR (RR), and 1 mM EGTA (EGTA). Typical traces for three different mitochondrial preparations are presented

(unpublished data). Thus, it is possible that the more intense swelling of mitochondria in rotenone-free media may be due to the induction of energy-dependent uptake of Ca^{2+} (Fig. 1) or Cd^{2+} (Belyaeva and Korotkov 2003) together with the participation of mitochondrial endogenous substrates under conditions in which pyridine nucleotides are in a more oxidized state. Cd^{2+} therefore demonstrated the substrate specificity which manifested as more potent action of Cd^{2+} on both swelling and state 4 or DNP-stimulated respiration of rat liver mitochondria, energized by glutamate plus malate or succinate alone, versus the Cd^{2+} action on mitochondria energized by succinate in the presence of rotenone (Belyaeva and Korotkov 2003). Thus, like Cd^{2+} (Belyaeva and Korotkov 2003) and Ca^{2+} (Fontaine et al. 1998; Belyaeva et al. 2004a; Baines 2009), the substrate specificity in the combined effects of Tl^+ and Ca^{2+} is corroborated by the finding that the total concentrations of Ca^{2+} stimulated the stronger swelling of mitochondria energized by glutamate *plus* malate (Fig. 1), but not by succinate *plus* rotenone (Fig. 4). On the other hand, this effect of rotenone on the Tl^+-induced swelling suggests that the Ca^{2+}-binding sites of respiratory complex I (Fontaine et al. 1998; Fontaine and Bernardi 1999; Baines 2009) play a part in opening the Tl-induced MPTP in CaRLM. Our earlier discussion of experiments with Cd^{2+}-induced swelling of rat liver mitochondria dealt with the role of these sites (Belyaeva et al. 2004a).

It is known that CsA and ADP, respectively, prevent the binding of CyP-D and Ca^{2+} with ANT that underlies their ability to inhibit opening of the MPTP (Halestrap and Brenner 2003; Halestrap 2009; Zorov et al. 2009). The swelling due to MPTP opening, induced by both Cd^{2+} and Ca^{2+}, was significantly retarded by ADP, CsA, and Mg^{2+}, individually and especially in combination (Novgorodov et al. 1994; Bravo et al. 1997; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Dorta et al. 2003; Belyaeva et al. 2004a). CsA (in contrast to ADP or Mg^{2+}) inhibited more strongly the swelling of energized mitochondria in the rotenone-free media containing Ca^{2+} and KNO_3, or $LiNO_3$, or NH_4NO_3 (Fig. 2). This inhibition may be due to the participation of the Ca^{2+}-binding sites near the CyP-D domain of ANT in opening of the Tl^+-induced MPTP opening in CaRLM (Halestrap and Brenner 2003; Halestrap 2009). On the other hand, the swelling was more strongly inhibited by ADP than by CsA in the media containing sucrose or $NaNO_3$ (Figs. 2a and d) or in all media containing succinate and rotenone (Fig. 5). This one points to the primary participation of the Ca^{2+}-binding sites close to the ADP domain of ANT in inducing opening of the MPTP (Halestrap and Brenner 2003). Diminishing both the Tl^+-induced swelling (Figs. 2 and 5) and the dissipation of

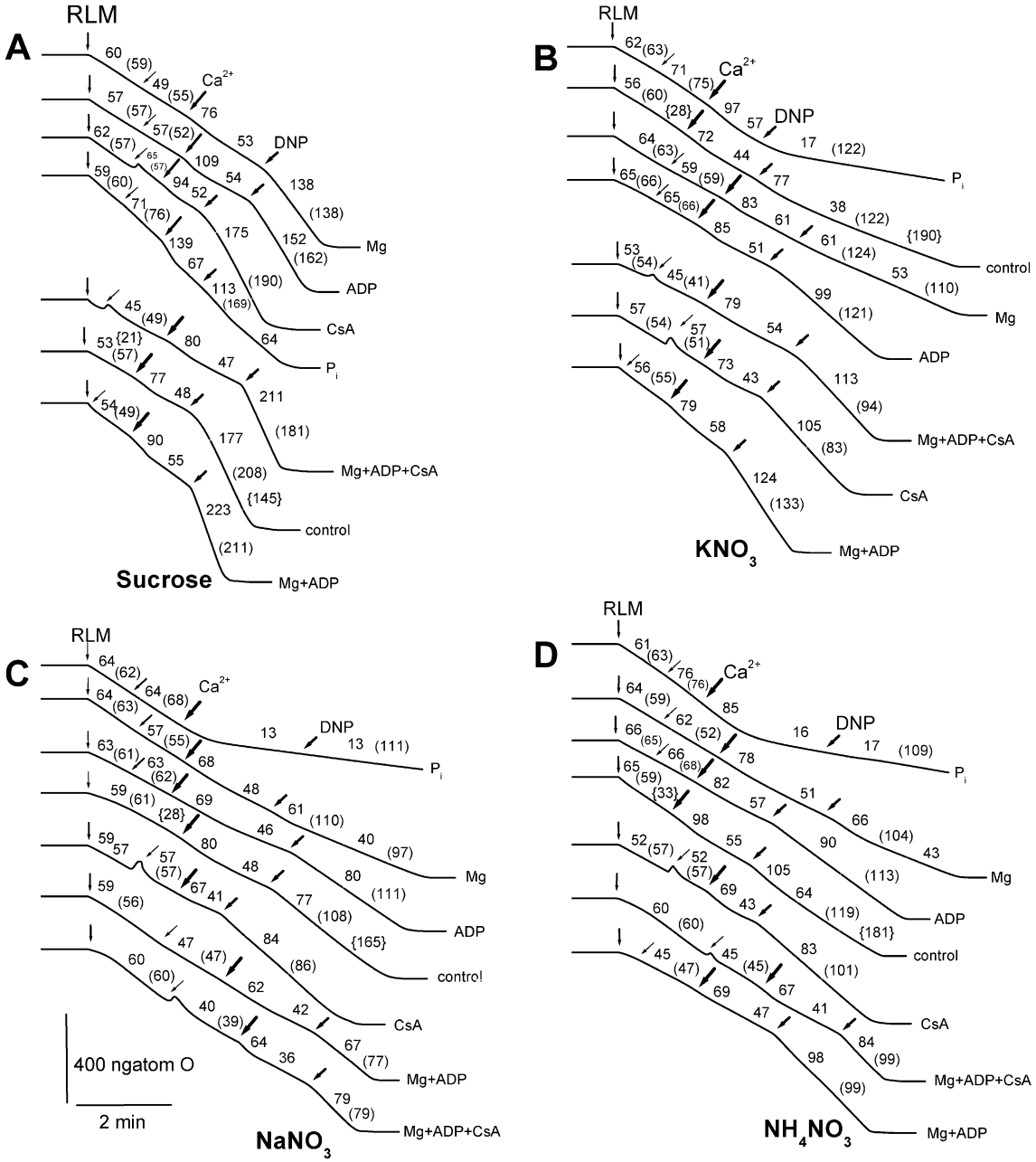

Fig. 7 Effects of $TlNO_3$ and Ca^{2+} on oxygen consumption rates (ng atom O min/mg of protein) in energized rat liver mitochondria in the presence of ADP, CsA, Mg^{2+}, and P_i. Mitochondria (1.5 mg protein/ml) were suspended in media containing 75 mM $TlNO_3$, 5 mM Tris-NO_3 (pH 7.3), 5 mM succinate, 4 μM rotenone, and 3 μg/ml of oligomycin, as well as 250 mM sucrose (**a**), or 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**). Additions of mitochondria (RLM), 100 μM Ca^{2+}, and 30 μM DNP (DNP) are shown accordingly by vertical arrows, inclined long bold arrows, and inclined bold arrows. Other additions are shown by inclined arrows and indicated on the right of the traces: none (control); 5 mM $Mg(NO_3)_2$ (Mg); 0.5 mM ADP (ADP); 1 μM CsA (CsA); 3 mM Tris-P_i.(P_i.). Oxygen consumption rates (ng atom O min/mg of protein) are presented as numbers placed above experimental traces. Numbers in parentheses were obtained from experiments with Ca^{2+}-free of media. The numbers in braces were calculated from experiments with the Ca^{2+}-free media, where 75 mM $TlNO_3$ was substituted by 150 mM sucrose (Korotkov 2009). Typical traces for three different mitochondrial preparations are presented

$\Delta\Psi_{mito}$ (Fig. 9) in energized CaRLM was therefore maximal in the simultaneous presence of Mg^{2+}, CsA, and ADP in the nitrate media. A more potent decrease of the swelling in the simultaneous presence of ADP and CsA (Figs. 2 and 5) allows us to speculate about the existence of co-operative interactions between these inhibitors and the Ca^{2+}-binding sites of ANT (Novgorodov et al. 1992; Halestrap and Brenner 2003). It should be stressed that

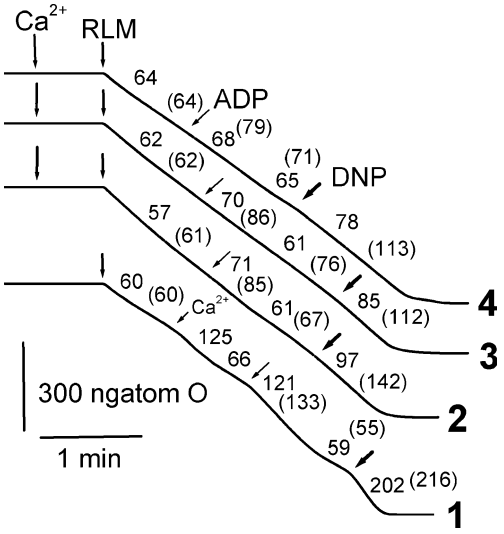

Fig. 8 Effects of $TlNO_3$ and Ca^{2+} on oxygen consumption rates (ng atom O min/mg of protein) in the energized rat liver mitochondria in different energy states. Mitochondria (1.5 mg protein/ml) were suspended in media with 75 mM $TlNO_3$, 5 mM Tris-NO_3 (pH 7.3), 250 mM sucrose (trace 1) or 125 mM 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), 30 μM DNP (DNP), and 100 μM Ca^{2+} (Ca^{2+}) are shown by arrows. Oxygen consumption rates (ng atom O min/mg of protein) are shown as numbers above the experimental traces. The numbers in parentheses were obtained from experiments with medium containing 75 mM $TlNO_3$ and free of Ca^{2+} (Korotkov 2009). Typical traces for three different mitochondrial preparations are presented

the swelling of rat liver mitochondria, stimulated by Tl^+ (Fig. 5) or Cd^{2+} (Belyaeva and Korotkov, unpublished data), decreased substantially in nitrate media containing 2 mM ADP. These findings may be explained by the ability of ADP to reduce the affinity of Ca^{2+} for the binding sites of ANT (Halestrap et al. 1997; Halestrap and Brenner 2003). Thus, we propose that swelling of energized mitochondria in the nitrate media containing Ca^{2+} and Tl^+ may be caused by opening of CsA-inhibited and ADP-dependent pores in the MIM, which we observed already earlier in electrolyte media with Cd^{2+} (Belyaeva et al. 2002; Belyaeva et al. 2004a).

The mitochondria swollen in the nitrate media that contained $TlNO_3$, RR and Ca^{2+} contracted markedly after their energization by glutamate *plus* malate (Fig. 2). At the same time, RR and EGTA completely inhibited the Tl^+-induced swelling (Fig. 6) and eliminated the Tl^+-induced reduction of state 4 and DNP-stimulated respiration rates of CaRLM (not shown there) in the nitrate media containing succinate and rotenone. We observed similar effects of these inhibitors on the Cd^{2+}-induced swelling and the Cd^{2+}-induced reduction of the respiration rate in media containing KNO_3, or NH_4NO_3, or KCl (Korotkov and Skulskii 1996; Belyaeva et al. 2001; Belyaeva et al. 2002). Thus, our findings (Figs. 2 and 6) show feasible reversibility in the capacity of Ca^{2+} to associate with the Ca^{2+}-binding sites in the media with $TlNO_3$. It is possible that in this case mitochondria remove Ca^{2+} from the matrix by the Ca^{2+}/H^+ exchanger (Ichas and Mazat 1998; Bernardi 1999; Gunter and Sheu 2009). The Tl^+-induced swelling of CaRLM regardless of the presence of rotenone was slightly inhibited by Mg^{2+} and in this case the effects of ADP or CsA on the swelling in the presence of Mg^{2+} were stronger (Figs. 2 and 5). This is in agreement with the capacity of Mg^{2+} to compete with Ca^{2+} in the Ca^{2+}-binding sites and to modulate the effects of ADP and CsA (Novgorodov et al. 1994; Zoratti and Szabó 1995; Ichas and Mazat 1998). Previously we observed much less Cd^{2+}-induced swelling of non-energized rat liver mitochondria in $LiNO_3$ medium than in media containing NH_4NO_3, or KNO_3, or $NaNO_3$ (Korotkov et al. 1998). It has been shown recently that the association of Li^+ with the outer surface of the MIM inhibitited the Ca^{2+}-induced MPTP opening in rat brain mitochondria (Shalbuyeva et al. 2007). We found that the swelling of energized CaRLM in $LiNO_3$ medium was considerably lower than in KNO_3 or $NaNO_3$ media (Figs. 1, 3, and 4). This indicates that the ability of Li^+ to prevent the effects of Tl^+ *plus* Ca^{2+} (the present study), Ca^{2+} (Shalbuyeva et al. 2007), and Cd^{2+} (Korotkov et al. 1998) is possible related to the involvement of Ca^{2+} sites on the outer surface of the MIM, in opening of MPTP.

It was earlier hypothesized that heavy metals (Cd^{2+}, Hg^{2+}, Cu^{2+}, and Zn^{2+}) can induce MPTP opening in the MIM (Zoratti and Szabó 1995; Korotkov and Skulskii 1996; Korotkov et al. 1998; Bernardi 1999; Belyaeva et al. 2001). Experiments with isolated mitochondria showed that Cd^{2+} (Zazueta et al. 2000; Belyaeva et al. 2001; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Dorta et al. 2003; Belyaeva et al. 2004a; Lee et al. 2005), Zn^{2+} (Wudarczyk et al. 1999), Pb^{2+} (Miyahara and Utsumi 1975), Hg^{2+} and Cu^{2+} (Chavez and Holguin 1988; Belyaeva et al. 2004b) stimulated opening of MPTP in the MIM. This opening could cause mitochondrial uncoupling, influx of K^+ into the matrix, extensive mitochondrial swelling, dissipation of $\Delta\Psi_{mito}$, and lowered matrix concentrations of pyridine nucleotides, ATP, and Ca^{2+}. We showed earlier that the Cd^{2+}-induced MPTP opening led to interaction of Cd^{2+} both with the Ca^{2+}-binding sites, located on the matrix side of the MIM, and with the SH groups in the mitochondrial respiratory complexes I and III (Belyaeva et al. 2004a). The participation of vicinal SH groups both of some mitochondrial respiratory complexes as well as of ANT was suggested to induce MPTP opening (Leung et al. 2008; Baines 2009; Halestrap 2009; Zorov et al. 2009). The presence of Ca^{2+} was not essential in experiments with the bivalent heavy metals which were able to induce MPTP opening in the Ca^{2+}-free buffers

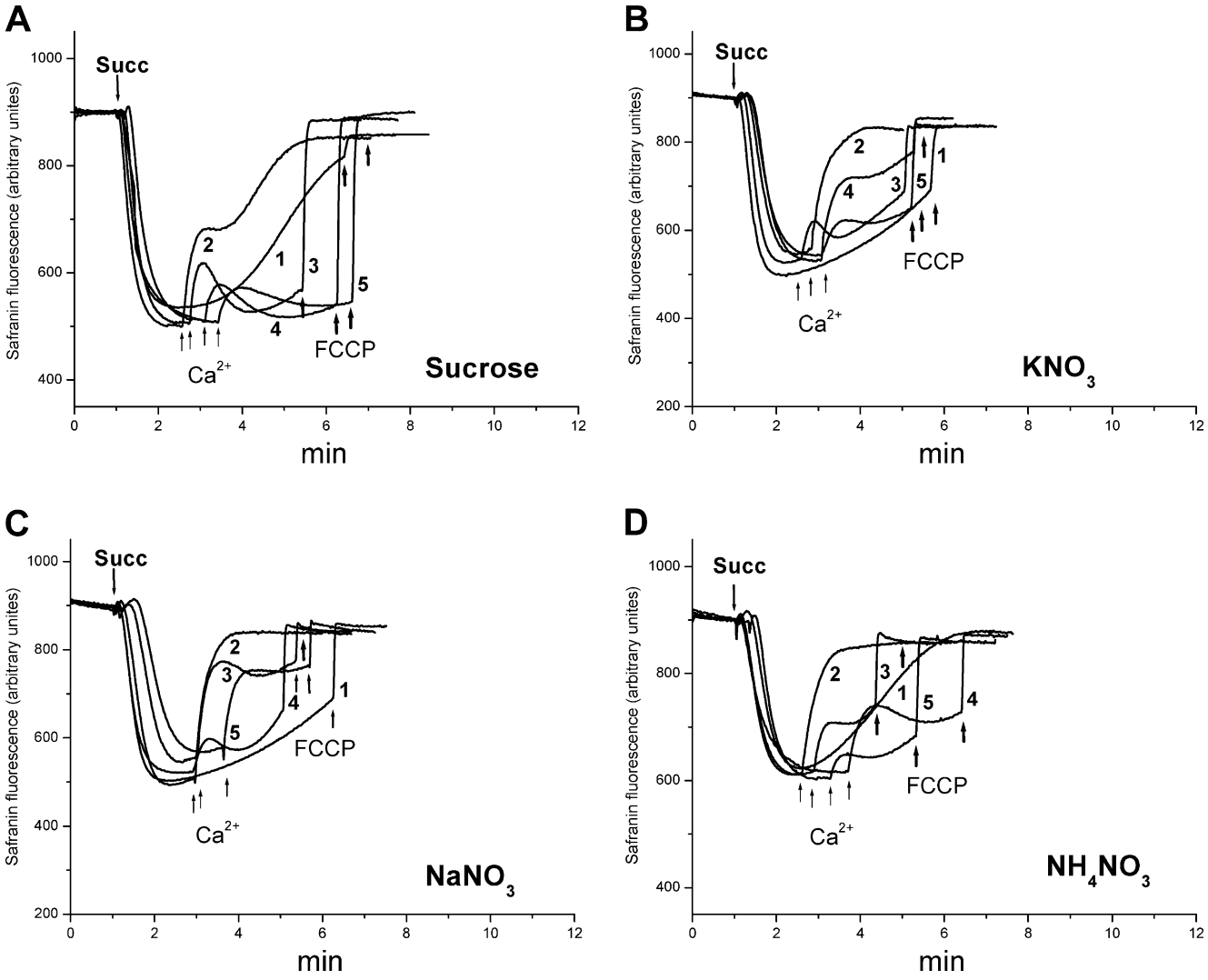

Fig. 9 Effects of Tl^+ and Ca^{2+} on the succinate-induced potential in rat liver mitochondria. Mitochondria (0.5 mg protein/ml) were added to medium containing 30 mM $TlNO_3$, 5 mM Tris-NO_3 (pH 7.3), 1 mM Tris-P_i, 3 μM safranin, 5 μM rotenone, and 3 μg/ml of oligomycin, as well as 340 mM sucrose (**a**), or 90 mM sucrose and 125 mM of KNO_3 (**b**), or $NaNO_3$ (**c**), or NH_4NO_3 (**d**). Additions of 5 mM succinate (Succ), 30 μM (b–d) or 50 μM (a) of Ca^{2+} (Ca^{2+}) [traces 2–5], and 1 μM FCCP (FCCP) are shown by ordinary and bold short arrows. The additions before mitochondria were: 1–2, none; 3, 1 μM CsA; 4, 3 mM $Mg(NO_3)_2$ and 0.5 mM ADP; 5, 3 mM $Mg(NO_3)_2$, 0.5 mM ADP, and 1 μM CsA. Typical traces for three different mitochondrial preparations are presented

(Wudarczyk et al. 1999; Zazueta et al. 2000; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Belyaeva et al. 2004a; Belyaeva et al. 2004b; Lee et al. 2005). Although Tl^+ uncouples mitochondria (stimulates state 4 and decreases $\Delta\Psi_{mito}$) (Melnick et al. 1976; Saris et al. 1981; Korotkov et al. 2007; Korotkov et al. 2008; Korotkov 2009), unlike Ag^+ (Inoue et al. 2009) and Cd^{2+} (Belyaeva et al. 2001; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Belyaeva et al. 2004a) it can induce MPTP opening only in the presence of Ca^{2+}, regardless of which respiratory complex substrate was used (Figs. 1, 3, and 4). Possibly, Tl^+ does not bind to the Ca^{2+}-binding sites due to its single-charge and low affinity to molecular SH groups (Perrin 1979).

It was demonstrated earlier that both an increase in the swelling and a decrease in state 4, state 3 or DNP-stimulated respiration were even more pronounced in electrolyte media containing Cd^{2+} and Ca^{2+} (Korotkov and Skulskii 1996; Belyaeva et al. 2001; Belyaeva et al. 2004a). These combined effects of Cd^{2+} and Ca^{2+} were eliminated markedly by addition of dithiothreitol or a mixture of ADP, CsA, and Mg^{2+} (Belyaeva and Korotkov 2003; Belyaeva et al. 2004a). It has been shown earlier that Tl^+ does not affect state 3 and DNP-stimulated respiration of rat liver mitochondria in iso-osmotic sucrose media (Herman and Bensch 1967; Barrera and Gomez-Puyou 1975; Melnick et al. 1976; Bragadin et al. 2003; Korotkov et al. 2007; Korotkov et al. 2008; Korotkov 2009), due to

the lack of marked inhibition of mitochondrial respiratory enzymes (Melnick et al. 1976; Perrin 1979; Woods and Fowler 1986). At the same time, we have not observed a decrease in state 3 or DNP-stimulated respiration in Ca^{2+}-free sucrose medium (Korotkov 2009) (Fig. 7a). However, the decrease was seen in the Ca^{2+}-free nitrate media containing $TlNO_3$ (Fig. 7b–d). We postulated that this decrease might be due to the pronounced swelling of mitochondria (Korotkov 2009). Our experiments with the nitrate media containing $TlNO_3$ and Ca^{2+} revealed that the decrease in state 4, state 3 or DNP-stimulated respiration (Figs. 7 and 8) and also the increased swelling of energized mitochondria (Figs. 4) (Korotkov et al. 2008; Korotkov 2009) increased in the order of non-Ca^{2+} < Ca^{2+} < Ca^{2+} + P_i. The decrease of state 4 or DNP-stimulated respiration (Fig. 7) and the increase in the swelling (Fig. 5) were less pronounced in the presence of ADP + Mg^{2+} ± CsA. On the other hand, the swelling (Korotkov et al. 2008) and the decrease in state 4 and DNP-stimulated respiration (Fig. 7) of energized mitochondria were maximal in the sucrose and nitrate media containing Ca^{2+} and P_i. It was earlier shown that state 3 in hyperosmotic media containing sucrose or electrolytes could be restricted accordingly by the functional activity of ANT or mitochondrial respiratory enzymes together with the strong correlation between state 4 respiration and mitochondrial volume (Devin et al. 1997). Hence, we can speculate that the decrease in state 4, state 3 and DNP-stimulated respiration in the nitrate media with Ca^{2+} (Figs. 7 and 8) may be associated with the reduced activity of the respiratory enzymes, because the mitochondrial structure is disturbed by the more massive swelling of CaRLM (Figs. 4 and 6). However, the mitochondrial structure is not affected by the reaction of Tl^+ with SH groups of mitochondrial respiratory enzymes (Verstraeten 2006; Hanzel and Verstraeten 2006), as opposed to the results obtained in experiments with Cd^{2+} and other heavy metals where this reaction did take place (Miccadei and Floridi 1993; Korotkov and Skulskii 1996; Korotkov et al. 1998; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Belyaeva et al. 2004a; Belyaeva et al. 2004b).

Experiments with isolated mitochondria showed that Tl^+ inhibited both influx and release of K^+ (Barrera and Gomez-Puyou 1975; Diwan and Lehrer 1977). However, the Tl^+-induced swelling of energized CaRLM was maximal in the KNO_3 medium (Figs. 1b and 4b). It is possible that this was due to increased MIM leakage under these experimental conditions. Another reason could be stimulation of a Ca^{2+}-activated K^+-channel of the MIM (Cheng et al. 2008). Further studies are nevertheless needed to settle this question. We have also found that the effects of Tl^+ (Figs. 1, 3, and 4), Cd^{2+} (Korotkov et al. 1998; Belyaeva et al. 2002; Belyaeva and Korotkov 2003; Belyaeva et al. 2004a), and Ca^{2+} (Belyaeva et al. 2004a) on the swelling of energized rat liver mitochondria in sucrose media were always less pronounced than the effects occurring in electrolyte media. The effects increased in the order of Ca^{2+} + P_i<Tl^+ + Ca^{2+} < Cd^{2+} in parallel with the chemical affinity of the cations to the SH-groups (Perrin 1979). Thus, comparing our experiments with the sucrose and electrolyte media (Figs. 1, 3, and 4), it is possible that Tl^+, similar to Cd^{2+} and Ca^{2+}, induces opening of the MPTP less actively in the high conduction states (Ichas and Mazat 1998; Bernardi 1999) and furthermore, that this effect of Tl^+ occurs in the presence of the substrates of respiratory complex I (glutamate plus malate) and II (succinate).

It is known that a massive mitochondrial swelling and disruption of the membranes of endoplasmic reticulum and mitochondria is induced by long-term exposure of rats to Tl salts (Herman and Bensch 1967; Woods and Fowler 1986). On the other hand, Tl^+ has triggered apoptosis in Jurkat and PC12 cells (Bragadin et al. 2003; Verstraeten 2006; Hanzel and Verstraeten 2009). It was postulated earlier that Tl^+ can stimulate release of Ca^{2+} from intracellular compartments (Herman and Bensch 1967). Experiments with isolated rat hepatocytes in medium containing TlCl showed that Tl^+ increased the Ca^{2+}concentration in their cytoplasm (Zierold 2000). Quite recently it was discovered that Tl^+ could injure isolated rat hepatocytes. The cytotoxic effect of Tl^+ was manifested as $\Delta\Psi_{mito}$ decline, hepatocyte proteolysis, ROS formation, lipid peroxidation, and glutathione depletion (Pourahmad et al. 2010). These deleterious effects of Tl^+ were considerably reduced by the MPTP inhibitors (CsA and carnitine). The Tl^+-induced MPTP opening in the MIM of Ca^{2+}-loaded rat liver mitochondria, regardless of which respiratory substrate was used (glutamate *plus* malate or succinate), is in line with these latter studies. The participation of the active Ca^{2+}-sites, located near ANT and Complex I, as well as the sites on the external side of MIM, is quite possible in the view of the above-mentioned data and our observations. Taking into consideration recent findings on the involvement of the mitochondrial phosphate carrier in the MPTP complex (Leung et al. 2008; Baines 2009; Halestrap 2009; Zorov et al. 2009), we will in future focus our efforts on the carrier participation in the Tl-induced MPTP in experiments with CaRLM.

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