Biochemistry (Moscow), Vol. 67, No. 11, 2002, pp. 1262-1270. Translated from Biokhimiya, Vol. 67, No. 11, 2002, pp. 1529-1538.

Lucigenin-Derived Chemiluminescence in Intact Isolated Mitochondria

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Received October 24, 2001
Revision received November 28, 2001

Abstract—There are many data both in favor and against the use of lucigenin as a probe for superoxide anion (SA) in mitochondria, cells, and simple enzymatic systems. In the present work high concentrations (50-400 μM) of lucigenin were used for continuous recording of rapid and reversible changes in the SA level in intact isolated mitochondria. The SA level in the presence of lucigenin rapidly and reversibly changed during the transition of the mitochondria from one functional state to another: under conditions of ATP synthesis from ADP and P_i, of Ca^{2+} accumulation, and of reverse electron transfer. Induction of a Ca^{2+},cyclosporin A-sensitive pore in mitochondria completely suppressed the lucigenin-derived chemiluminescence (LDC). The electron transfer in the Q-cycle of the respiratory chain complex III and high electric potential difference across the inner membrane of mitochondria were obligatory conditions for generation of a SA-dependent chemiluminescent signal. Based on our own and literature data, a scheme of LDC generation is suggested. The origin of superoxide anion detected in intact mitochondria with lucigenin is discussed.

Key words: lucigenin, superoxide anion, mitochondria, Ca^{2+}, mPTP, reverse electron transfer, complex III

The generation of reactive oxygen species (ROS) in mitochondria has been studied since the works of Chance and Boveris [1, 2]. Mitochondria, which have powerful redox-systems, are one of the main cellular producers of superoxide anion (SA) of oxygen and of hydroperoxide. To analyze these species in mitochondria various probes are used, including scopoletin, cytochrome *c*, peroxidase, epinephrine, Tiron, etc. [3-6].

In many works ROS were analyzed in partially damaged mitochondria, e.g., by pretreatment with H_2O_2, long-term storage in the cold, etc., or in submitochondrial particles [7-9]. Under these conditions, the ROS generation was, as a rule, increased in the presence of the respiratory chain inhibitors, in particular, of antimycin A, the effect of which was strengthened with uncouplers [9, 10]. The ROS generation in the respiratory chain of intact mitochondria oxidizing various substrates was found to be inhibited with uncouplers and to be activated with antimycin A [1, 11].

To determine ROS in intact mitochondria some authors used lucigenin (Luc^{2+}) [12] which allowed them to detect SA by its chemiluminescent signal. It is suggested, that lucigenin-derived chemiluminescence (LDC) in mitochondria is a result of interaction of lucigenin cation-radical ($Luc^{+\cdot}$) and SA, both of which are generated in the mitochondrial respiratory chain, followed by degradation of the reaction product dioxetane to two methylacridone molecules with light emission [13]. Due to high sensitivity, LDC measurements are still used for SA determination in mitochondria [14-16] notwithstanding recent data about probable lucigenin-dependent SA generation via autooxidation of cation-radical in several biological systems [17, 18].

Advantages of this probe are high specificity of its interaction with SA (under physiological pH the LDC reflects exclusively the reaction of Luc^{2+} with SA [13]) and presence of two positive charges in a relatively highly hydrophobic molecule that seems to significantly facilitate the transmembrane transfer and accumulation of lucigenin by mitochondria.

In experiments with isolated mitochondria and whole cells relatively low concentrations of Luc^{2+} (5-20

Abbreviations: ROS) reactive oxygen species; LDC) lucigenin-derived chemiluminescence; Luc^{2+}) lucigenin; $Luc^{+\cdot}$) lucigenin cation-radical; SA) superoxide anion; FCCP) carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; mPTP) mitochondrial permeability transition pore (a nonspecific Ca^{2+},cyclosporin A-sensitive mitochondrial pore); CsA) cyclosporin A.

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and 50-250 μM for mitochondria and cells, respectively) were used with a long-term incubation with the probe (30-60 min). Such a prolonged incubation seemed to be necessary for lucigenin penetration and accumulation by mitochondria [12, 19].

To provide the continuous recording of rapid and reversible changes in the LDC and SA production in mitochondria, in the present work we used elevated concentrations of Luc^{2+} (50-400 μM) that allowed us to significantly reduce the time of the incubation of mitochondria with the probe. Under these conditions rapid, pronounced, and reversible changes in the LDC and SA generation were observed during the mitochondrial transition from the fourth to the third metabolic state (phosphorylation of added ADP or transport of Ca^{2+} by the coupled mitochondria. The induction by Ca^{2+} of mitochondrial permeability transition pore (mPTP) opening, as well as uncoupler-caused increase in the proton permeability irreversibly suppressed the LDC and SA generation. The findings suggested that the electron transfer in the respiratory chain complex III and the high electric potential on the mitochondrial inner membrane ($\Delta\varphi$) are prerequisites for the LDC and SA generation.

MATERIALS AND METHODS

Liver mitochondria were isolated from Wistar rats by a routine procedure including differential centrifugation [20]. The isolation medium contained 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris-HCl, 1 mM EDTA, 0.5% BSA (pH 7.4). The resulting mitochondria were washed and suspended in the isolation medium deprived of EDTA and BSA. The protein content was determined according to Lowry [21].

The incubation medium was as follows: 0.125 M KCl, 2 mM KH_2PO_4, 0.01 M HEPES (pH 7.4). The medium was supplemented with 5 mM succinate with rotenone (2 μg/mg protein), 5 mM pyruvate with 5 mM malate, or 2 mM MgATP as shown in figures legends. In experiments on induction of reverse electron transfer there was no rotenone in the incubation medium. All measurements were carried out at 37°C. The usual concentration of mitochondrial protein was 1 mg/ml.

The $\Delta\varphi$ value in mitochondria was determined by distribution of tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode as described in [22]. The mitochondria were loaded with Ca^{2+} in a standard medium supplemented with oxidation substrates and with cyclosporin A (CsA) in the cases indicated. The threshold concentrations of Ca^{2+} accumulated by the mitochondria without a rapid uncoupling were determined in preliminary experiments. The figures present the data of typical experiments performed in no less than three replicates with different preparations of mitochondria.

Changes in the fluorescence of nicotinamide nucleotides were studied at 360-480 nm (the maxima of the excitation and emission wavelengths, respectively, 5 nm bandwidth) with an MPF 44B spectrofluorimeter (Perkin Elmer, USA).

The LDC was recorded with a Lucifer 02M chemiluminometer (Nika, Russia).

The following reagents were used: Tris-HCl, HEPES, BSA, dimethylsulfoxide (DMSO) (Serva, Germany); EDTA, lucigenin, antimycin A, rotenone, myxothiazol, thenoyltrifluoroacetone (TTFA), succinate, pyruvate, malate, tetraphenylphosphonium (Sigma, USA); carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), ATP (Calbiochem, USA); cyclosporin A (Sandoz, Switzerland). Other reagents were of Russian production, of chemical or special purity. Lucigenin was dissolved in DMSO. Rotenone, antimycin A, myxothiazol, TTFA, and CsA were dissolved in twice-distilled ethanol.

RESULTS

Kinetics and amplitude of lucigenin-derived chemiluminescence in the presence of different concentrations of lucigenin. In preliminary experiments to choose the working concentration of Luc^{2+}, the chemiluminescent response of the mitochondria was titrated with lucigenin. Figure 1 presents concentration dependences of the maximal amplitude of the LDC signal of mitochondria oxidizing succinate (in the presence of rotenone) or malate with pyruvate. The dependence curves flattened at ~300 μM

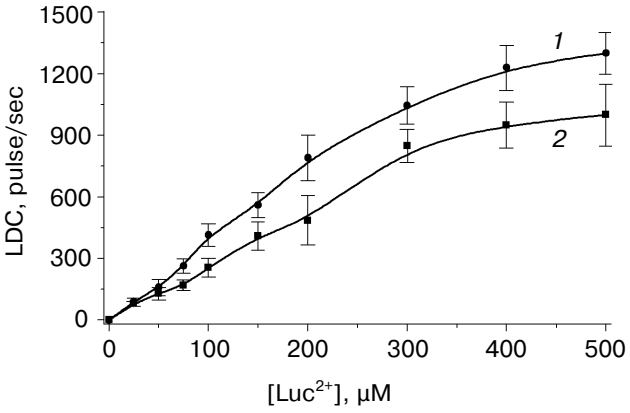

Fig. 1. Dependences of LDC maximal amplitude of mitochondria during the oxidation of succinate or of malate with pyruvate on the Luc^{2+} concentration. The incubation medium described in "Materials and Methods" was supplemented with 5 mM succinate and rotenone (2 μg/mg protein) (*1*) or with 5 mM malate and 5 mM pyruvate (*2*).

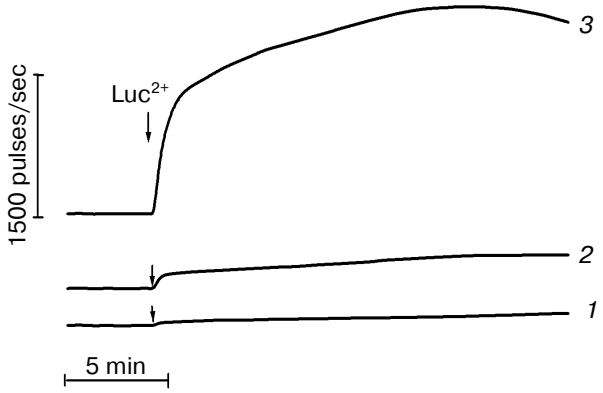

Fig. 2. Chemiluminescent responses of mitochondria during oxidation of pyruvate with malate in the presence of different concentrations of Luc^{2+}. The incubation medium was the same as in Fig. 1. The arrows show the addition of 10 (*1*), 50 (*2*), and 400 μM Luc^{2+} (*3*).

Luc^{2+}. A further increase in the Luc^{2+} concentration to 400-500 μM only slightly increased the LDC amplitude.

Figure 2 presents the LDC responses of mitochondria oxidizing malate with pyruvate in the presence of 10, 50, and 400 μM Luc^{2+}. After an initial leap the LDC increased smoothly with a subsequent flattening (at 400 and 50 μM). At the lower concentration of Luc^{2+} (10 μM) the response was markedly decreased and during the experiment (10-20 min) no flattening was observed. Because the accumulation of lucigenin in the mitochondria could limit the LDC, in the subsequent experiments relatively high concentrations of lucigenin (50-400 μM) were used.

Effects of ADP and Ca^{2+} on LDC. The influence of natural metabolites, such as ADP and Ca^{2+} on mitochondrial SA production were studied.

Figure 3 presents data on LDC (a) and Δφ (b) during the physiological exposure of mitochondria, including ATP synthesis from ADP and P_i in the medium containing malate and pyruvate. An addition of Luc^{2+} into the incubation medium stimulates the LDC that suggested the production of SA in mitochondria, with the curve flattening within about 2-3 min. A subsequent addition of ADP caused a significant and reversible decrease in the LDC (Fig. 3a) accompanied with corresponding changes in the Δφ value (Fig. 3b). After conversion of added ADP into ATP, the restoration of LDC level is observed. Addition to the mitochondria of a protonophore, FCCP, which caused an irreversible dissipation of Δφ resulted in irreversible LDC inhibition.

Figure 4 presents the effect on the mitochondrial LDC of another natural metabolite, Ca^{2+}. A single addition of a relatively small amount of Ca^{2+}, which was rapidly accumulated by the mitochondria, decreased the LDC level and the Δφ value (Figs. 4a and 4b, respectively), and this effect was similar and concurrent with the changes caused by ADP. A subsequent addition of 20 μM Ca^{2+}, which induced the opening of nonselective pores in the mitochondrial internal membrane, resulted in a complete and irreversible dissipation of Δφ, a decrease in the LDC level, and, consequently, in the inhibition of SA production. The presence in the incubation medium of an inhibitor of the mPTP opening, cyclosporin A (Figs. 4c and 4d) significantly increased the amount of Ca^{2+}, which was accumulated by mitochondria without an irreversible decrease in the Δφ and LDC.

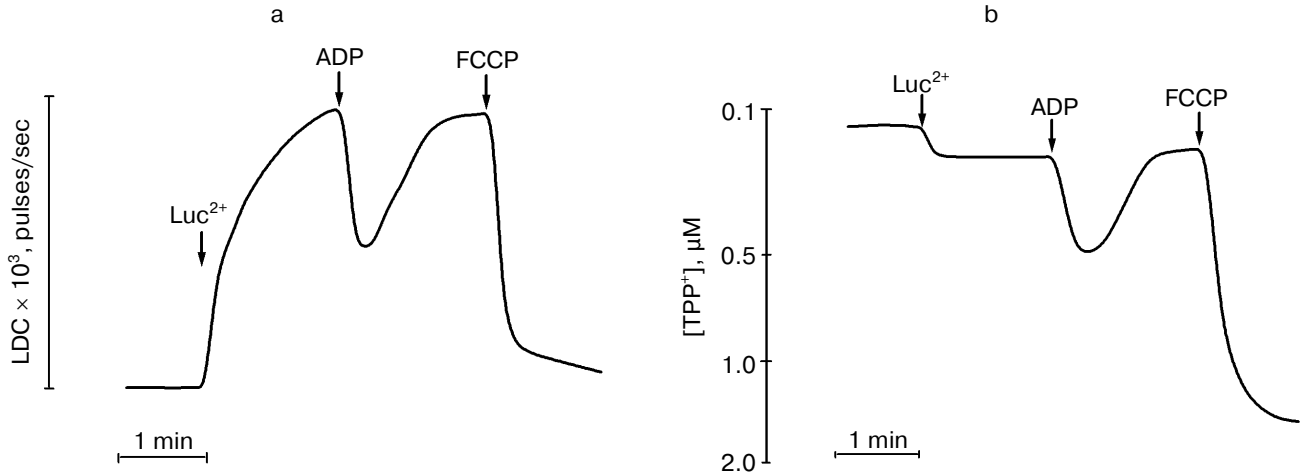

Fig. 3. Changes in LDC (a) and in Δφ value (b) in mitochondria on addition of ADP and FCCP. The incubation medium was the same as in Fig. 2. The arrows show the additions of 400 μM Luc^{2+}, 200 μM ADP, and 0.5 μM FCCP. The TPP^+ concentration was 2 μM.

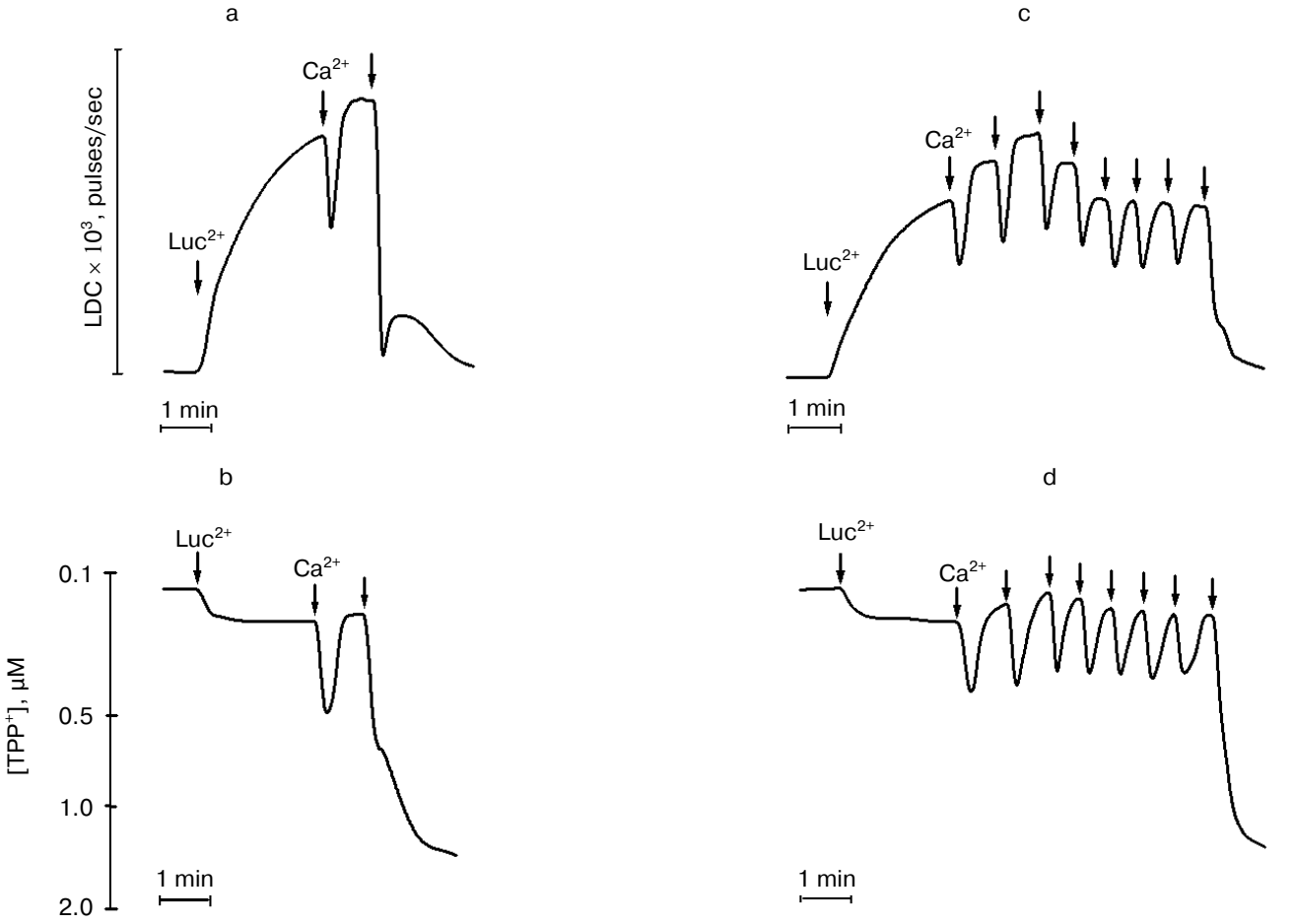

Fig. 4. Reversible changes in the LDC and in the Δφ value in mitochondria in response to addition of Ca^{2+} into the incubation medium in the absence (a, b) and in the presence of cyclosporin A (c, d). The incubation medium was the same as in Fig. 2. The arrows show additions of 400 μM Luc^{2+}, 20 μM Ca^{2+}. The concentration of cyclosporin A was 1 μM, that of TPP^+ was 2 μM.

The data presented show a close correlation between changes in the LDC and in the Δφ value. These changes seemed to be associated with the reduction state of the respiratory chain components, which is known to correlate with the Δφ value of mitochondria. In the next series of experiments (Fig. 5) the effect of the reduction state of nicotinamide nucleotides on the LDC was studied under conditions of reverse electron transfer in the respiratory chain of the mitochondria oxidizing succinate (in rotenone-free medium). Decreasing the reduction state of nicotinamide nucleotides in the mitochondria by successive additions of α-ketoglutarate and ammonium (Fig. 5a, dashed line), one could significantly decrease the LDC level (Fig. 5b, dashed line). While the Δφ value remained virtually unchanged (Fig. 5c, dashed line).

Effect of respiratory chain inhibitors on the LDC level and SA production in mitochondria. To identify the sites of the respiratory chain responsible for the LDC generation and SA production, the Luc^{2+}-dependent chemiluminescence was analyzed with inhibitors. Figure 6 shows the changes in the LDC and Δφ in response on addition to mitochondria of respiratory substrates and of inhibitors of the initial sites of electron transport chain (complexes I and II), rotenone and TTFA, respectively. Thus, the substrates which were utilized with the involvement of complex I, i.e., pyruvate and malate, caused the generation of LDC (Fig. 6a) and Δφ (Fig. 6b). An addition of rotenone, which blocked the electron flow from complex I to complex III, resulted in the dissipation of Δφ and in the suppression of LDC. The Δφ value was recovered and the LDC was even stimulated after addition of the complex II substrate, succinate. In their turn, the Δφ and LDC generated in the presence of succinate could be decreased by addition of the complex II inhibitor—TTFA.

It should be noted that the addition of the respiratory chain inhibitors to mitochondria not only inhibited the

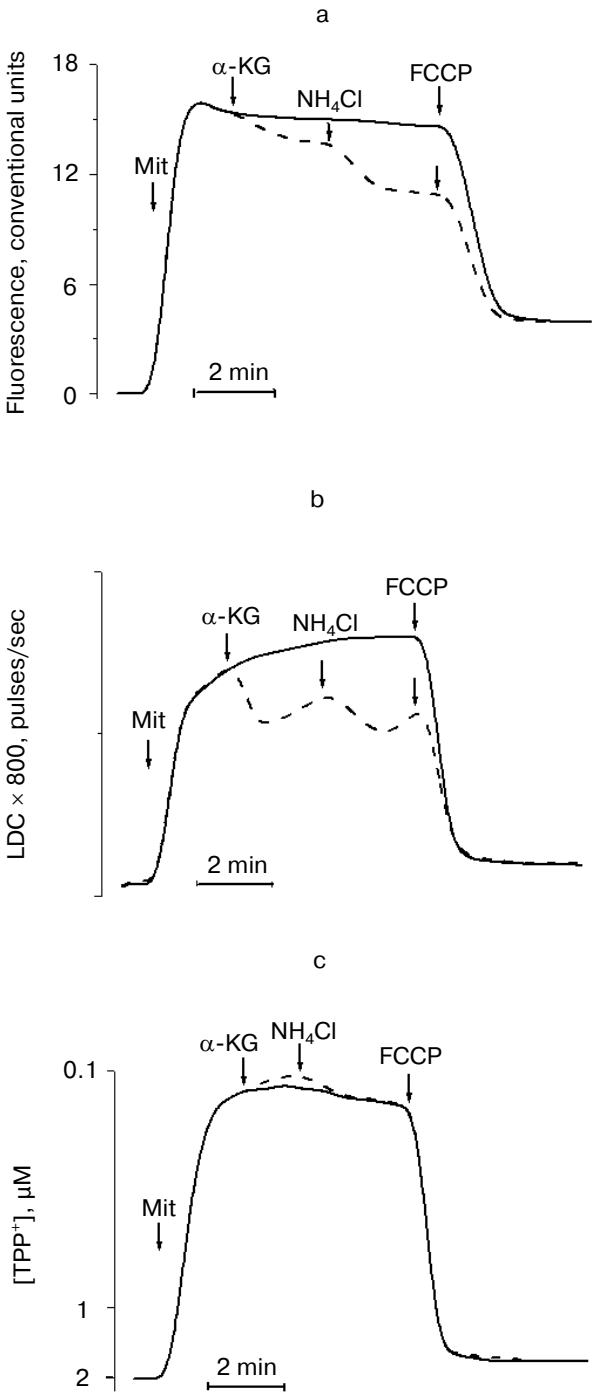

Fig. 5. Changes in the fluorescence of reduced nicotinamide nucleotides (a), LDC (b), and Δφ value (c) of mitochondria which oxidized succinate on addition of α-ketoglutarate and NH_4^+. The incubation medium was the same as in Fig. 1 but supplemented with 5 mM succinate (without rotenone) and 2 mM sodium arsenite. The arrows show the addition of 3 mM α-ketoglutarate (α-KG), 2 mM NH_4Cl, and 1 μM FCCP. The initial concentrations of Luc^{2+} and TPP^+ in the incubation medium were 50 μM (b) and 2 μM (c), respectively. The protein contents in the mitochondria (Mit) were 0.5 (a) and 1 mg/ml (b, c). Changes in the parameters recorded in the absence and in the presence of α-ketoglutarate and NH_4^+ are shown by solid and dashed lines, respectively.

electron transfer at one or another point of the respiratory chain but also decreased the Δφ value that alone could decrease the LDC level and the SA production. To assess an immediate effect of the electron flow inhibition on the SA production, we used the approach of Δφ recovery at the cost of F_0F_1-ATPase-induced hydrolysis of the substrate added, Mg-ATP. Figure 6 shows that the addition of Mg-ATP after rotenone (Fig. 6b, dashed arrow) resulted in a partial but significant recovery of the Δφ. The LDC was also increased in this case (Fig. 6a, dashed line).

The addition of Mg-ATP to mitochondria (in the presence of rotenone and TTFA) increased the Δφ value, whereas the LDC stimulation was insignificant (it was no more than 10% of the LDC in the medium with pyruvate and malate for different preparations of mitochondria), and this suggested that the electron transfer in complex III of the respiratory chain should play a predominant role in the LDC generation.

Figure 7 presents the effect of inhibitors of *i*- and *o*-centers on the LDC and Δφ of mitochondria oxidizing malate with pyruvate in a rotenone-free medium. Myxothiazol and antimycin A both separately and combined decreased the Δφ value (Fig. 7b) and suppressed the LDC (Fig. 7a). A partial recovery of the Δφ at the cost of exogenous Mg-ATP in the presence of antimycin A markedly increased the mitochondrial LDC, and this increase was significantly higher than in the presence of myxothiazol. The inhibition of both centers of the Q-cycle by a combined effect of myxothiazol and antimycin A prevented LDC generation (Fig. 7a), notwithstanding the Δφ recovery in response to the addition of Mg-ATP (Fig. 7b).

DISCUSSION

In the present work the changes in LDC, resulting from interaction of lucigenin cation-radical with SA, in different functional states of mitochondria were studied. The LDC generation was preceded by reduction of Luc^{2+} to $Luc^{+\cdot}$ in the respiratory chain [13]. Therefore, the LDC level when using Luc^{2+} depends on the stationary concentration of both SA and lucigenin cation-radical. To minimize the limiting effect of the reduction stage of Luc^{2+} and correspondingly of the stationary concentration of $Luc^{+\cdot}$ in mitochondria on reactions which determine the LDC generation, we have used relatively high concentrations of Luc^{2+} (50-400 μM).

This allowed us to register rapid and in some cases reversible changes in the LDC and SA production at various functional states of mitochondria. In other works lower concentrations of Luc^{2+} were used. In this case, intensity of measured LDC in mitochondria may be limited by slow lucigenin penetration and one electron reduction, i.e. steady state $Luc^{+\cdot}$ concentration [12, 14, 19].

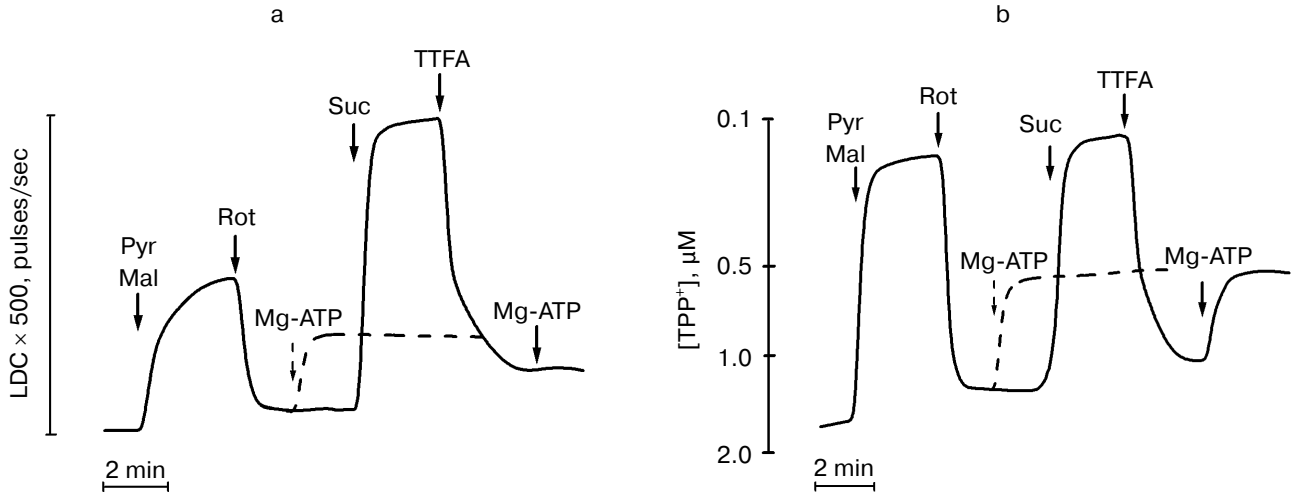

Fig. 6. Changes in LDC and Δφ of mitochondria in response to addition of respiratory substrates, inhibitors of electron transport chain, and Mg-ATP. The incubation medium was the same as in Fig. 1. The arrows show additions of 5 mM pyruvate (Pyr) with 5 mM malate (Mal), 2 μM rotenone (Rot), 5 mM succinate (Suc), 50 μM TTFA, and 2 mM Mg-ATP. Changes in the LDC and Δφ on addition of Mg-ATP after rotenone without the subsequent additions are shown by the dashed arrow and the dashed line. The Luc^{2+} and TPP^+ concentrations were 50 and 2 μM, respectively.

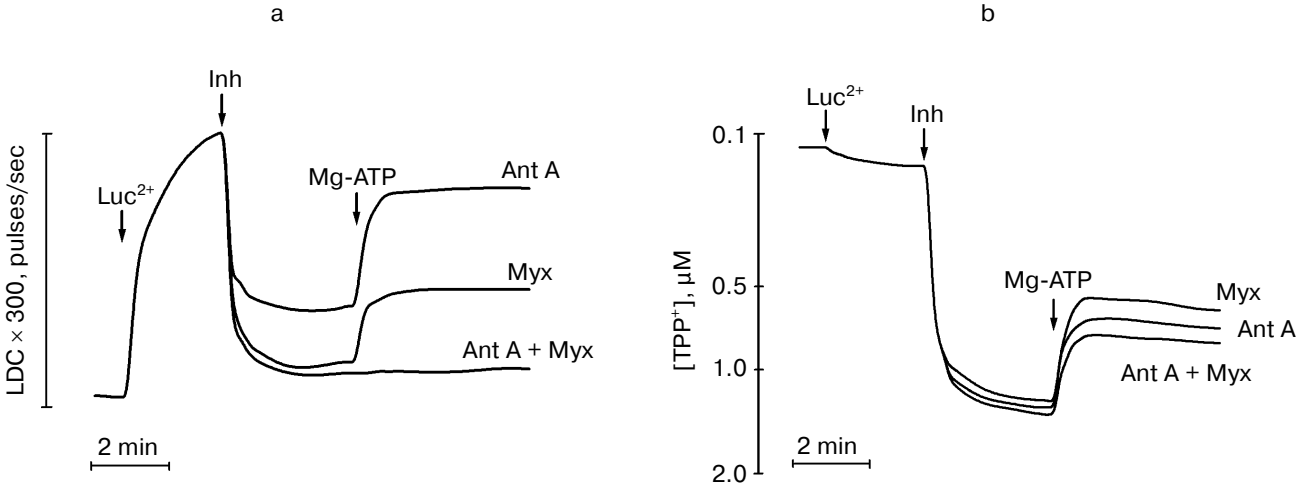

Fig. 7. Effect of electron transfer inhibitors in respiratory chain complex III on LDC and Δφ generation by mitochondria. The incubation medium was the same as in Fig. 2. The arrows show the addition of 50 μM Luc^{2+}, the respiratory chain inhibitors (Inh), antimycin A (Ant A) (0.5 μg/mg protein), 1 μM myxothiazol (Myx), or antimycin A + myxothiazol, 2 mM Mg-ATP. The TPP^+ concentration was 2 μM.

In the present work rapid and reversible changes in the LDC were observed when mitochondria was exposed to various metabolites: ADP (Fig. 3a) and of relatively small amounts of Ca^{2+} (Fig. 4a). However, after mPTP induction or Δφ dissipation by uncoupling protonophores the LDC was virtually completely inhibited.

The data presented in Figs. 3 and 4 show a good correlation of changes in the LDC with changes in the Δφ. The ADP- and Ca^{2+}-induced changes in Δφ are known to be also accompanied by changes in the reduction degree of the respiratory chain carriers that can strongly affect the LDC level and Δφ generation. The role of the respiratory chain reduction state was confirmed by experiments on induction of reverse electron transfer in the mitochondria when changes in the LDC (Fig. 5b) were recorded at virtually constant values of Δφ (Fig. 5c).

The high level of LDC recorded in state 4 and its reversible decrease in state 3 after addition of ADP confirmed the hypothesis of V. P. Skulachev about the regulation of mitochondrial functions and on their defense against oxidative stress via a decrease in the reduction state of the respiratory chain and in the rate of ROS

generation along with the reversible decrease in Δφ [8, 23].

Changes in Δφ could influence the redistribution of the positively charged lucigenin between the mitochondrial matrix and the incubation medium or in the inner membrane of mitochondria. However, the use of elevated concentrations of Luc^{2+} in our experiments suggested that even in the absence of Δφ the content of lipophilic lucigenin in mitochondria should be rather high. We have additionally determined the binding of Luc^{2+} to mitochondria by spectral analysis of supernatants resulting after incubation of mitochondria with Luc^{2+} and their rapid sedimentation. At the lucigenin concentration in the incubation medium of 250 μM about 5-10 nmol lucigenin per mg mitochondrial protein was bound to the mitochondria. This suggested a high concentration of lucigenin in the mitochondria and was in agreement with the data presented in work [14]. Moreover, the Luc^{2+} accumulation in mitochondria was a rather slow process (Fig. 2), whose rate is incommensurable to rapid changes in the LDC, e.g., recorded on addition of Ca^{2+} to mitochondria (Fig. 4). That is why an influence of Δφ on the Luc^{2+} distribution between the mitochondria and environment is unlikely. However, changes in the Δφ value were likely to influence the intramembrane redistribution of the positively charged Luc^{2+} and $Luc^{+\cdot}$ and also the rate of their interaction with redox centers of the respiratory chain or SA.

By the inhibitory analysis with rotenone, TTFA, antimycin A, and myxothiazol the LDC was shown to be

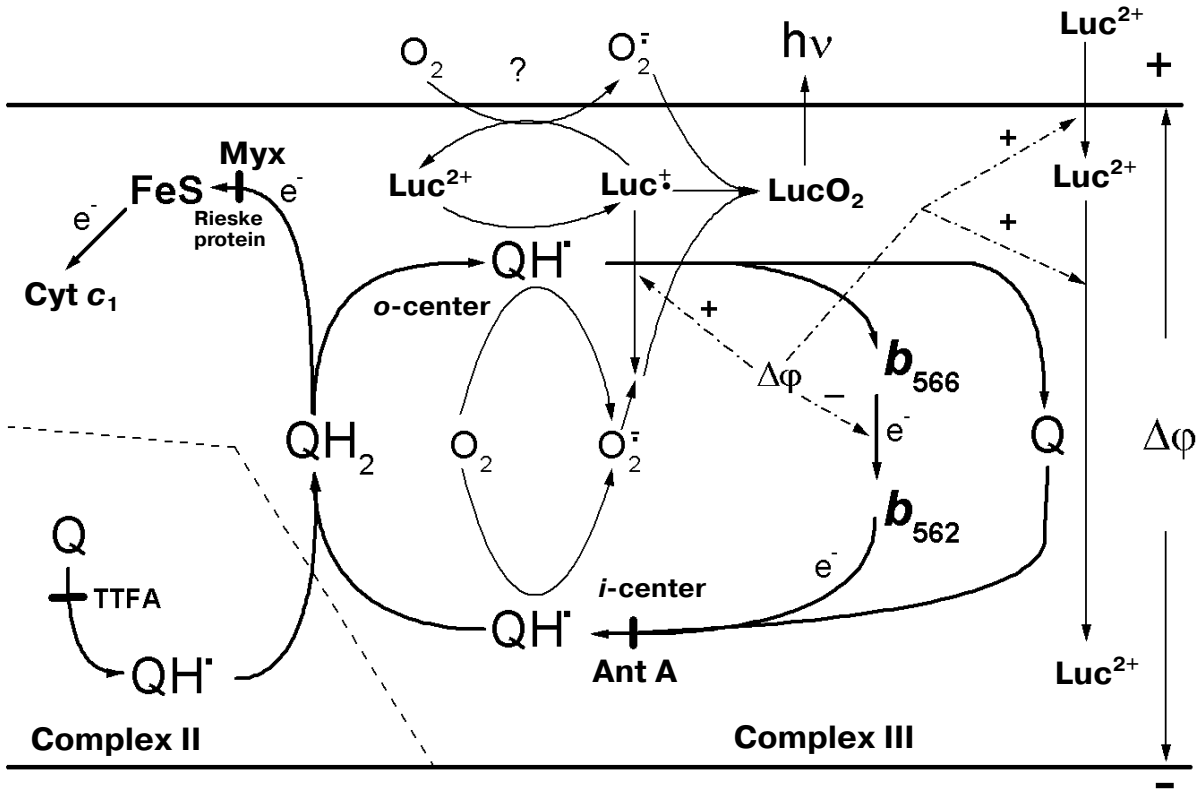

Fig. 8. Hypothetical mechanism of LDC generation in the framework of the Q-cycle scheme. The thick arrows show the pathways of ubiquinone transformation and electron transfer in the Q-cycle of complexes II and III (in the scheme they are arbitrarily delimited with dashed lines) of the mitochondrial respiratory chain with indication of the points of action of the main inhibitors and of supposed oxygen reduction to superoxide (the *i*- and *o*-centers). The thin arrows show the reactions of one-electron reduction of oxygen and of oxidized lucigenin which are located in the *o*- or *i*-centers of the Q-cycle and also interactions of endogenous superoxide-anion with the cation-radical of lucigenin and subsequent conversion of lucigenin which occur in the membrane hydrophobic phase and are accompanied by emission of chemiluminescence quanta. In the absence of inhibitors lucigenin is suggested to be reduced in both redox centers of complex III. For the sake of simplicity, in the scheme only the reactions in the *o*-center are presented. The scheme also presents a hypothetical (?) reaction of autooxidation of the lucigenin cation-radical with oxygen which can induce an additional production of superoxide and LDC generation. The dashed arrows indicate the reactions which are strongly dependent on Δφ (the signs "plus" and "minus" denote an activation and inhibition, respectively) and can influence the generation of endogenous superoxide and/or of LDC. In particular, these are the reactions of transmembrane and intramembrane distribution of the oxidized and semiquinone forms of lucigenin.

mainly generated during the electron transfer in the Q-cycle of respiratory chain complex III. It should be mentioned that the respiratory chain inhibitors not only inhibited the electron transfer but sharply decreased $\Delta\varphi$ as a result of a passive H^+ flow through the internal mitochondrial membrane.

To prevent the effect of the $\Delta\varphi$ decrease, we introduced the inhibitors combined with Mg-ATP. Under certain conditions Mg-ATP significantly increased $\Delta\varphi$ and LDC in the presence of the respiratory chain inhibitors.

The presence of a significant LDC in the mitochondria oxidizing malate with pyruvate on addition of Mg-ATP in the presence of rotenone (Fig. 6a) seemed to be first of all associated with the oxidation of endogenous substrates through complex II of the respiratory chain. A high rate of the LDC reaction in complex I was unlikely because complete inhibition with rotenone and TTFA of the electron entrance into complex III resulted in a very low (or absent) LDC at the ATP-induced generation of potential, notwithstanding the presence of pyruvate and malate, which were substrates of respiratory chain complex I. However, a low residual LDC in mitochondria energized with Mg-ATP combined with the respiratory chain inhibitors could also reflect SA generation in complex I.

Our results of inhibitory analysis of the lucigenin-derived chemiluminescence are in agreement with the data of other authors on the predominant generation of ROS in the complex III and on a probable role of semiubiquinone $QH^{\cdot}$ bound to *o*- (under conditions of antimycin inhibition) or *i*-centers of complex III (in the absence of inhibitors) as of a component responsible for a direct reduction of oxygen to superoxide [24, 25]. These data and also the findings of Li et al. [12, 14] suggested that lucigenin could be used for detection of endogenous SA in complex III but not in complex I.

On the other hand, the strong inhibition of LDC found by us during $\Delta\varphi$ dissipation (even in the presence of such an inhibitor as antimycin A), although confirmed by some works [26], contradicts the bulk of the data by other authors on a high rate of ROS production in the presence of antimycin A and uncouplers of oxidative phosphorylation [9, 24]. These discrepancies seem to be associated with a specificity of the interaction of Luc^{2+} with the respiratory chain components.

A scheme of the hypothetical mechanism of the lucigenin-dependent chemiluminescence in complex III is shown in Fig. 8. Because redox potentials of the one-electron reduced lucigenin and ubiquinone are close [27, 28], Luc^{2+} seemed to be reduced to $Luc^{+\cdot}$, in particular on the level of the respiratory chain components which oxidized QH_2 or reduced Q to $QH^{\cdot}$, i.e., in the places of probable natural generation of SA. The close values of redox potentials of Q and Luc^{2+} also suggested that Luc^{2+} could be reduced by a direct interaction with QH_2 and/or $QH^{\cdot}$ bound to the *i*- or *o*-centers of complex III.

Under certain conditions, if the ratio of generation of $Luc^{+\cdot}$ and of endogenous SA in the mitochondrial respiratory chain was high ($v_{Luc^{+\cdot}} >> v_{SA}$) lucigenin-induced generation of SA could appear, and the LDC could reflect not only the natural SA generation in mitochondria but also the additional lucigenin-induced production of SA. The autooxidation of the lucigenin cation-radical with oxygen is also shown in Fig. 8.

Figure 8 shows how changes in the $\Delta\varphi$ value can influence the LDC and SA generation. High values of $\Delta\varphi$ promote an increase in the local concentrations of positively charged Luc^{2+} and $Luc^{+\cdot}$ in the places of endogenous generation of SA: *i*- and *o*-centers of the Q-cycle. Another general prerequisite of activation of the LDC and SA generation in the mitochondrial respiratory chain at high $\Delta\varphi$ values is the inhibition of electron transfer between cytochromes b_{562} and b_{566}, which is known to be accompanied by $QH^{\cdot}$ stabilization in the *o*-center [29].

This work was supported by the program "Integration" (grant No. A0108) and by a Russian–Dutch NWO grant (No. 047-006-005).

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