J Bioenerg Biomembr (2006) 38:137–142
DOI 10.1007/s10863-006-9013-6

ORIGINAL PAPER

Profound effects of the general anesthetic etomidate on oxidative phosphorylation without effects on their yield

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Received: 15 January 2006 / Accepted: 12 April 2006 / Published online: 7 October 2006

Abstract We investigated the effects of the general anesthetic Etomidate on oxidative phosphorylation in isolated rat liver mitochondria. The study of each electron transfer site shows that there is an inhibition: mainly at complex I but also, to a lesser extent, at complex III. Moreover, with succinate as substrate, the increase in non-phosphorylating respiration is accompanied by a decrease in $\Delta\Psi$. However, this effect is not due to classical uncoupling of oxidative phosphorylation, since ADP addition at high Etomidate concentrations restores the transmembrane difference of electrical potential. Also, in the same range of Etomidate concentration, the ATP/O ratio is not significantly affected. In conclusion, the main effect of Etomidate is to decrease the oxidative phosphorylation rate without changing yield. The H^+ leak which appears under non-phosphorylating conditions becomes negligible in physiological conditions.

Keywords Etomidate · Rat liver mitochondria · Respiratory chain · Oxidative phosphorylations

Abbreviations used: DNP, 2,4-dinitrophenol · $\Delta\Psi$, transmembrane difference of electrical potential · Δp, transmembrane difference of proton electrochemical potential.

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Introduction

Even if general anesthetics include a great variety of molecular structures (Fig. 1) and have been reported to act on different mitochondrial functions, most have been characterized as uncouplers of oxidative phosphorylation. For instance, halothane and chloroform (Fig. 1) induce a stimulation of non-phosphorylating respiration (state 4), an inhibition of ATP synthesis and an increase in basal ATPase activity; all effects which define an uncoupling process of oxidative phosphorylation (Rottenberg, 1983; Branca *et al*., 1986; Luvisetto *et al*., 1987). However, in contrast to the wide class of "classical uncouplers" e.g., protonophores or ionophores, both halothane and chloroform only very slightly increase the proton and/or the ion conductance of the inner mitochondrial membrane, and cause only a little Δp depression. The fact that these strong uncouplers can act without a significant effect on bulk to bulk protonmotive force (Δp) has been considered as evidence in favour of the existence of either a parallel (intramembranal) coupling pathway for protons (Rottenberg, 1990) or a mechanism of intrinsic uncoupling at the proton pump level (Pietrobon *et al*., 1983). In a previous study we have shown that the uncoupling effect of the general anesthetic 2,6-diisopropylphenol (see Fig. 1) goes through the ATPsynthase. Indeed, in the absence of phosphorylation, 2,6-diisopropylphenol induces a H^+-leak which becomes negligible when ADP is added. This proton leak can also be inhibited in the presence of high concentrations of oligomycin (Rigoulet *et al*., 1996).

Etomidate is currently used as an intravenous general anesthetic. Whereas most general anesthetics have been studied in regard to their influence on oxidative phosphorylation, no systemic study regarding Etomidate effects on mitochondrial function has ever been described. In order to determine whether all general anesthetics have a comparable influence

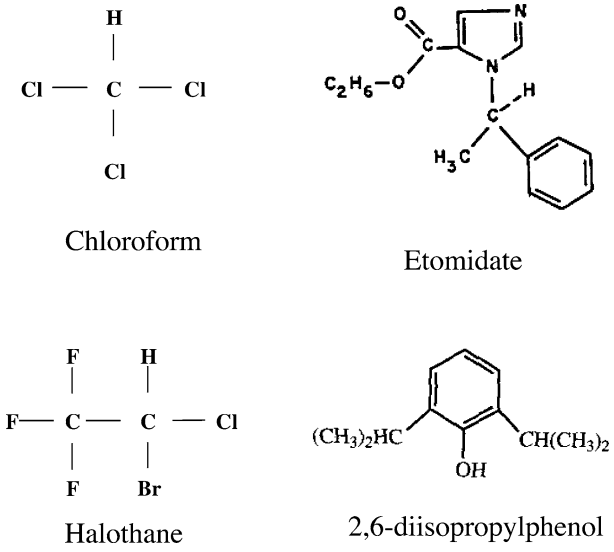

Fig. 1 Chemical structure of some commonly used anesthetics

on oxidative phosphorylation, we investigated the effects of Etomidate on the oxidative phosphorylation of isolated rat liver mitochondria.

Materials and methods

Mitochondria preparation

Liver mitochondria were prepared from male Wistar rats (250–300 g body weight) fed ad libitum. Rats were killed by cervical dislocation and liver was rapidly removed and put into an ice-cold isolation medium containing 225 mM sucrose, 20 mM Tris-HCl (pH 7.2) and 1 mM EGTA. Mitochondria were isolated according to (Cooper and Lehninger, 1956) in the same medium. The mitochondrial pellet was finally resuspended in the isolation medium. Protein concentration was estimated by the biuret method using bovin serum albumin as standard (Gornall *et al.*, 1949).

Mitochondrial respiration and ATP/O measurements

The rate of oxygen consumption was measured polarographically at 37°C using a Clark-type oxygen electrode connected to a microcomputer giving an online display of rate values. Since it has been shown that ionic medium is more physiological for studies performed on isolated mitochondria (Devin *et al.*, 1997), respiration medium contained 150 mM KCl, 20 mM Tris-HCl (pH 7.2), 1 mM EGTA, 5 mM Tris-Pi and either 6 mM glutamate +6 mM malate or 6 mM succinate (in the presence of 5 μM rotenone and 0.5 mM malate) in a final volume of 2 ml. Oxygen concentration in the medium was determined with spectrophotometrically quantitated NADH and yeast mitochondria as being equal to 480 natom O·ml^{-1}.

ATP synthesis rate was determined (after addition of 1 mM ADP) by ATP formation measured by a bioluminescence technique (Lundin, 1976) using an ATP monitoring kit. The ATP/O ratio stoichiometries were determined from the yield of oligomycin-sensitive phosphorylation rates versus respiratory rates.

Measurement of $\Delta\Psi$

The $\Delta\Psi$ was assessed by two different techniques: by using either a fluorescent probe, rhodamine 123 or a radioactive lipophilic cation, $[^3H][P(Ph)_3Me]^+$.

Using rhodamine 123, we determined the transmembrane potential under different steady states as described following and according to (Emaus *et al.*, 1986): mitochondria were incubated in 2 ml of respiratory buffer containing 5 mM Pi as well as 6 mM succinate/0.5 mM malate/5 μM rotenone. State 3 $\Delta\Psi$ is measured in the presence of 1 mM ADP. The amount of rhodamine bound to the membranes is evaluated in the presence of 0.2 μM CCCP and 2 μg/ml of antimycin.

Using $[^3H][P(Ph)_3Me]^+$, the $\Delta\Psi$ under different steady states was determined as follows and according to (Rottenberg, 1979): matrix space was estimated using $[^3H]$ water and $[^{14}C]$ mannitol, an impermeable sugar, in the respiratory buffer (see above); in a parallel experiment, $\Delta\Psi$ was measured by $[^3H][P(Ph)_3Me]^+$ distribution between intra and extramitochondrial space. Since $[P(Ph)_3Me]^+$ is a lipophilic cation which is mainly bound to membranes, an apparent activity coefficient of 0.38 was applied to our measurements of matricial $[P(Ph)_3Me]^+$ accumulation (Espié *et al.*, 1995).

Rate of electron transfer at complex I and II+III level

Complex I (NADH-decylubiquinone oxidoreductase) (EC 1.6.5.3)

The oxidation of NADH by complex I was recorded using decylubiquinone as electron acceptor in the basic assay medium: 10 mM Tris-maleate pH 7.2, 2 μg antimycin, 0.3 mM decylubiquinone (Sigma Co) and 0.1 mM NADH in a final volume of 1 ml. The reaction was started by the addition of 2 mg of mitochondrial protein previously sonicated twice for 30s. The decrease in absorbance due to NADH oxidation was measured at 340 nm both in the absence and in the presence of 5 μM rotenone. The reported activity was sensitive to rotenone.

Complex II+III (Succinate-Ferricyanide Oxidoreductase) (EC 1.3.99.1+EC 1.10.2.2)

This activity was determined in the respiration medium (see above) supplemented with 5 mM succinate by observing the

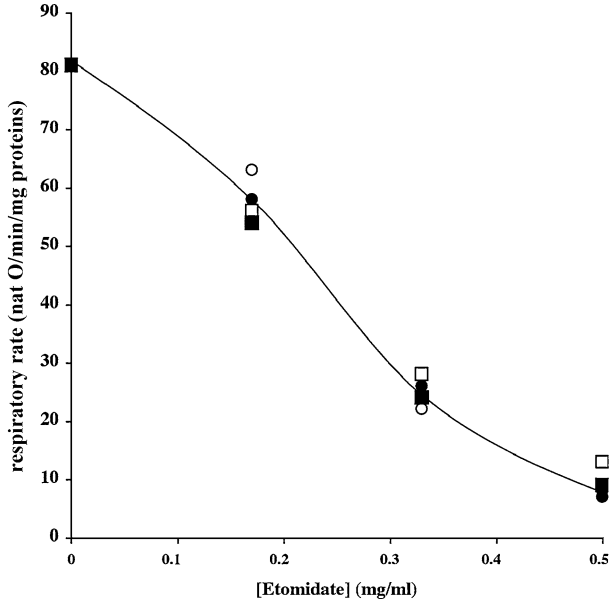

Fig. 2 Effect of Etomidate on respiratory rate as a function of mitochondria amount. Mitochondria (● 0.5 mg protein; ○ 1 mg protein; ■ 1.5 mg protein; or □ 2 mg protein) were suspended in 1.5 ml of respiration medium (see Materials and Methods section) containing 6 mM glutamate +6 mM malate and 1 mM ADP. Respiratory rate was measured after 2 min of incubation in the presence of the indicated Etomidate concentration

reduction of 1 mM ferricyanide in the presence of 5 μM rotenone and 1 mM KCN. The reaction was started by addition of 2 mg mitochondrial protein and the decrease in absorbance was monitored at 436 nm. The activity reported was that sensitive to 2 μg antimycin.

Results

Effects of etomidate on respiratory chain

It is well known that numerous substituted phenols acting as protonophores strongly adsorb to the membrane-solution interface (Mc Laughlin and Dilger, 1980). Since Etomidate is an alkyl-substituted phenol, we first considered the effect of various quantities of mitochondria on the magnitude of the modification induced by this drug. Figure 2 shows that Etomidate addition from 0.175 to 0.5 mg/ml inhibited the state 3 of oxygen consumption rate of rat liver mitochondria when glutamate plus malate were the substrates. The extent of this inhibition was independent of the mitochondria content.

We then compared the dose-dependent effect of Etomidate on various respiratory states supported by glutamate + malate (Fig. 3). Etomidate slightly increases the non-phosphorylating respiratory rate. In contrast, uncoupled and phosphorylating respiratory rates were drastically inhibited.

Figure 4 shows that Etomidate drastically inhibited electron flow at site 1 level (Fig. 4A) whereas, at site 2, the

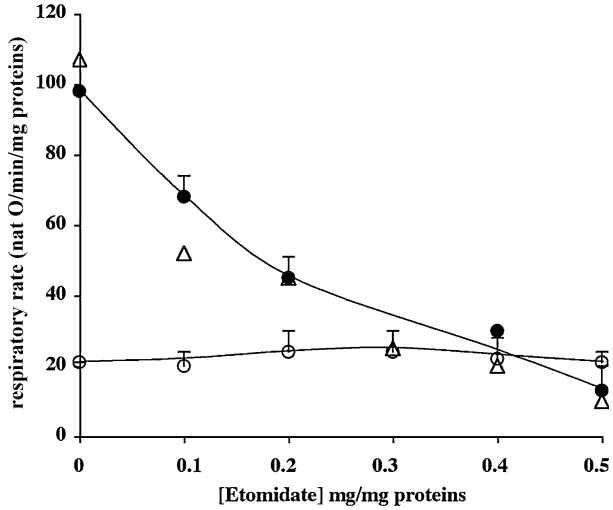

Fig. 3 Dependence of respiratory rates on Etomidate concentration with glutamate + malate as substrates. Mitochondria (1 mg protein·ml^{-1}) were suspended in the respiration medium containing 6 mM glutamate +6 mM malate. Respiratory rate was measured in state 4 (○); after addition of either 1 mM ADP (●) or 0.15 mM DNP (△). The values presented are from at least three different experiments carried out with three different mitochondria preparations and expressed as mean ± SEM

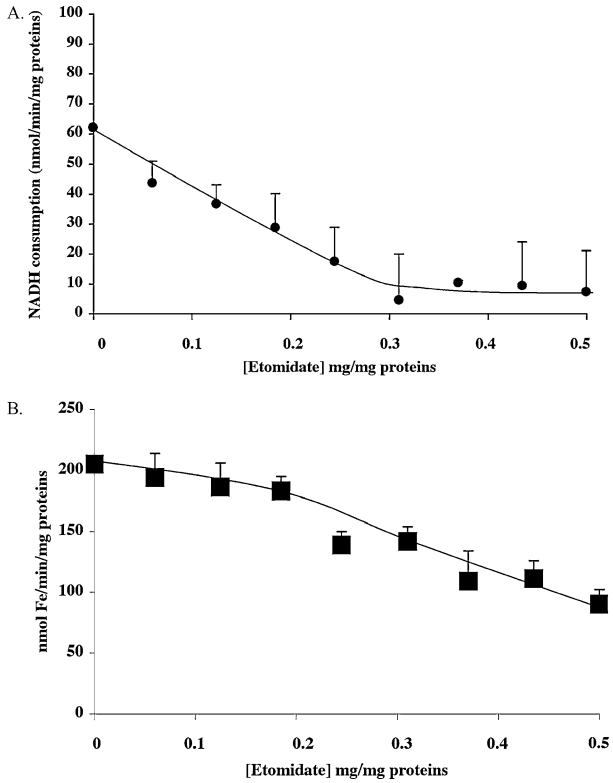

Fig. 4 Effect of Etomidate on electron transport rate at complex I and II+III level. The rate of electron transfer at either complex I (●, A) or complex II+III (■ B) level was measured as described in Materials and Methods section. The values presented are from at least three different experiments carried out with three different mitochondria preparations and expressed as mean ± SEM

electron transfer rate was almost constant up to 0.2 mg/ml Etomidate and, for higher concentrations, decreased up to about 50% (Fig. 4B). The electron transport rate at coupling site 3 with $Ph(NMe_2)_2$ + ascorbate as substrates was insensitive to Etomidate, in the same range of concentration and whatever the respiratory state considered (not shown). Thus, the main effect of Etomidate with glutamate + malate as substrates is an inhibition of complex I.

Effects of etomidate on energetic parameters with succinate as substrate

We then developed this study using succinate as substrate since the inhibitory effect of Etomidate on the respiratory chain is less important with this substrate, allowing a thorough study of oxidative phosphorylation.

Figure 5A shows that with succinate as substrate, mitochondria have a respiratory control ratio of approximately 3.4. Figure 5B shows that adding Etomidate (0.4 mg/mg protein) prior to substrate addition leads to an increase in state 4 respiratory rate, and that ADP no longer stimulates state 4 respiratory rate. When Etomidate is added after ADP, the respiratory rate decreases to a value comparable to the one obtained when Etomidate is added before (Fig. 5C).

Figure 6A shows that Etomidate increases the state 4 respiratory rate and decreases state 3 and uncoupled state respiratory rates in such a way that for 0.4 mg/ml Etomidate the state 4 respiratory rate is no longer stimulated by ADP addition (see also Fig. 5B). The increase in state 4 respiration with up to 0.2 mg/ml Etomidate was not linked to any change in $\Delta\Psi$ (Fig. 6B). For higher concentrations of Etomidate, the increase in this respiratory rate was linked to a large decrease in transmembranal electric potential, indicating an enhancement of membranal proton permeability (Fig. 6B). The large decrease in membrane electrical potential is almost totally reversed by ADP addition (Fig. 6B). This relationship between non-phosphorylating respiratory rate and $\Delta\Psi$ obtained by Etomidate titration was different from the one obtained with a classical protonophore like dinitrophenol. Indeed, with Etomidate a larger decrease in $\Delta\Psi$ led to a lower increase in respiratory rate (not shown, but see Rigoulet *et al*., 1996). This fact may be a consequence of a double effect of Etomidate: an increase in proton permeability and an inhibition of respiratory chain activity which could modify its kinetic response to the $\Delta\Psi$ change. In state 3, Etomidate addition greatly decreased the respiratory rate with a weak change in $\Delta\Psi$ (Fig. 6B). Only for a high concentration of Etomidate and a strong inhibition of the respiratory rate (about 60%) did the $\Delta\Psi$ decrease significantly. It is worth noting that at high concentration of Etomidate (0.4 mg/mg prot to 0.5 mg/mg prot) the addition of ADP does not stimulate respiratory rate, but significantly increases the $\Delta\Psi$,

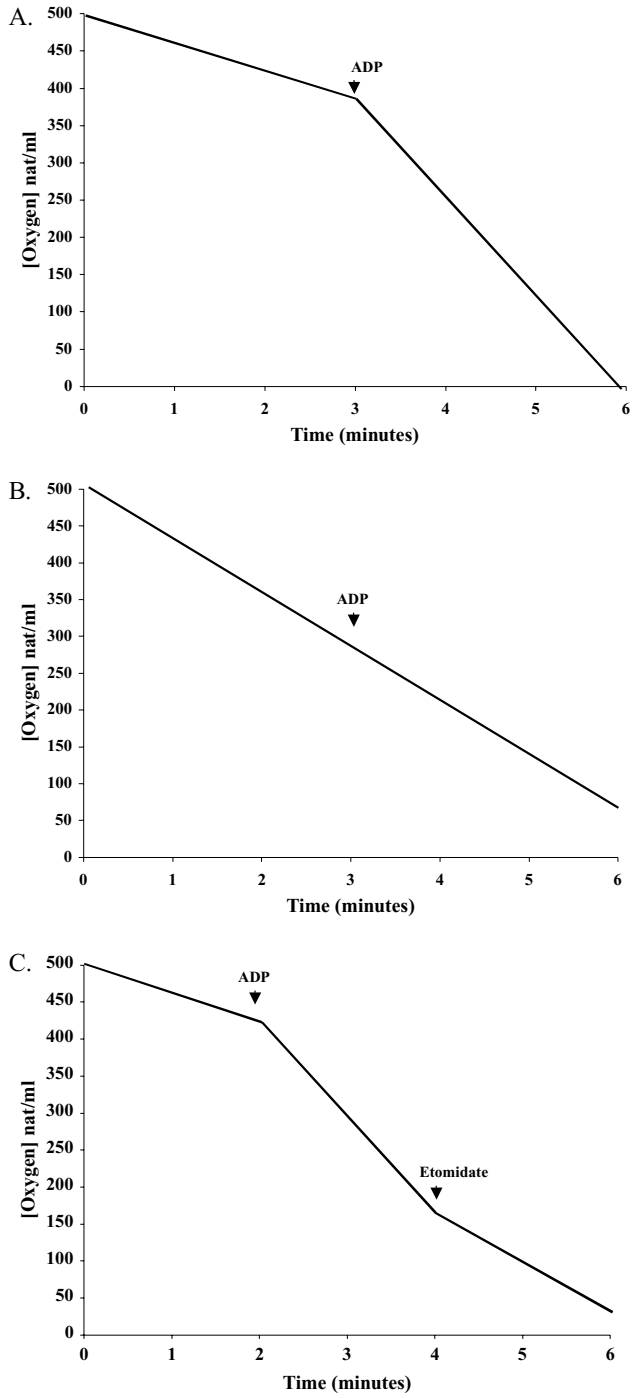

Fig. 5 Original traces of oxygraphic measures. Mitochondria were incubated in respiration medium containing 6 mM succinate in the presence of 5 μM rotenone and 0.5 mM malate. (A) Respiratory rate was measured in state 4 and state 3 was assessed in the presence of 1 mM ADP. (B) 0.4 mg/mg protein Etomidate was added preliminary to respiratory rate assessment in state 4 and state 3. (C) Respiratory rate was measured in state 4 and state 3 was assessed in the presence of 1 mM ADP, Etomidate was then added. The data are representative of four separate experiments performed with four different mitochondria preparations

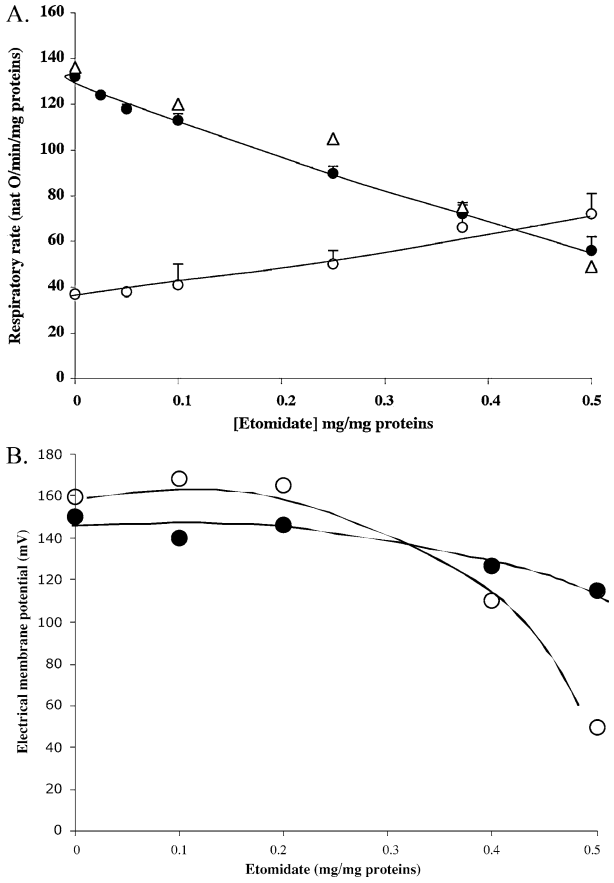

Fig. 6 Dependence of respiratory rates and membrane electrical potential on Etomidate concentration with succinate as substrate. Mitochondria were incubated in respiration medium containing 6 mM succinate in the presence of 5 μM rotenone and 0.5 mM malate. Respiratory rate was measured in state 4 (○); after addition of either 1 mM ADP (●) or 0.15 mM DNP (△). Respiratory rates and $\Delta\Psi$ were measured as described in Materials and Methods section : state 3 (●) and state 4 (○) in the presence of various concentrations (from 0.1 to 0.5 mg/ml) of Etomidate. The data represent the mean ± SEM of four separate experiments performed with four different mitochondria preparations

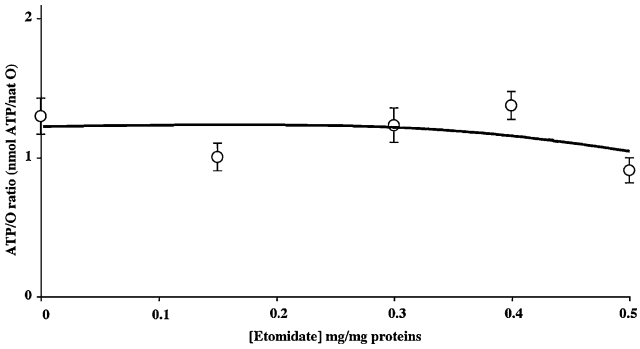

Fig. 7 Dependence of ATP/O on Etomidate concentration with succinate as substrate. Experimental conditions were those of Figure 6A and oligomycin-sensitive ATP synthesis rate was determined as described in Materials and Methods section. The data represent the mean ± SEM of three different experiments performed with three different mitochondria preparations

which raises the question of oxidative phosphorylation efficiency in the presence of Etomidate.

Yield of oxidative phosphorylation

The yield of oxidative phosphorylation, estimated by the ATP/O ratio, was only slightly affected at the highest Etomidate concentration with succinate as substrate (Fig. 7). It is noteworthy that at 0.4 mg/mg prot Etomidate, the ATP/O ratio remained constant, even if ADP addition did not significantly increase the respiratory rate.

We have previously shown that the uncoupling effect of the general anesthetic 2–6 diisopropylphenol goes through the ATPase (Rigoulet *et al.*, 1996). Indeed, the H^+-leak induced by this anesthetic becomes negligible when oxidative phosphorylation is functioning and 2–6 diisopropylphenol decreases the sensitivity of the ATPase towards oligomycin. Since the paradoxical uncoupling effect of Etomidate is very similar to what was previously shown with 2–6 diisopropylphenol, we investigated the inhibitory action of oligomycin. Oligomycin in low amounts (as little as 0.2 μg·mg protein^{-1}) inhibited state 3 respiration either in the absence or in the presence of Etomidate (not shown). Moreover, the ATP synthesis rate was abolished for 0.2 μg oligomycin.mg protein^{-1} under both experimental conditions (not shown). However, neither state 4 stimulated by Etomidate nor the $\Delta\Psi$ were affected by increasing oligomycin addition up to 1 μg·mg protein^{-1} (not shown). This shows that, contrary to what was shown with 2–6 diisopropylphenol, the uncoupling effect of Etomidate on non-phosphorylating mitochondria is insensitive to oligomycin.

Discussion

The results reported here show that Etomidate may impair the bioenergetic metabolism of rat liver mitochondria by two processes. First, Etomidate inhibits the electron flow in the respiratory chain mainly at the coupling site 1 level even if, at high concentrations, it also inhibits the second site. Second, it acts as an uncoupler in non-phosphorylating mitochondria, leading to an increase in respiratory rate and to a large decrease in $\Delta\Psi$. However, this effect cannot be due to a classical protonophoric property of this drug. Indeed, under phosphorylating conditions, Etomidate does not seem to induce a significant increase in proton passive permeability: (i) with succinate as substrate and up to 0.3 mg/mg prot, it does not decrease the $\Delta\Psi$ or the ATP/O ratio even if the respiratory rate is largely inhibited (see Figs. 6 and 7); with glutamate and malate, when the state 3 respiratory rate inhibition is not higher than 80% the same results were obtained (not shown). Such effects were observed when oxidative

phosphorylation was titrated by a respiratory chain inhibitor on well-coupled mitochondria; (ii) with succinate as substrate and at very high Etomidate concentrations (0.4 mg/mg prot), the transition state 4-state 3 was not linked to a significant change in respiratory rate (see Fig. 5B) although the ATP/O ratio was unchanged (see Fig. 7). Taken together, these facts indicate that in the absence of an ATP synthesis, Etomidate induced a proton leak which was almost completely suppressed when the ATP synthase was active. We propose that Etomidate causes a proton leak only under non-phosphorylating conditions. However, at the onset of ATP synthase functioning, the flux through this proton channel became an effective proton movement supporting ATP formation without significant leakage. Indeed, for an amount of uncoupler which induces the same state 4 stimulation (50%), the ATP/O ratio decreases by 60% (Nogueira *et al.*, 2001).

In conclusion, we show that Etomidate has two main effects on rat liver mitochondria: an inhibition of the respiratory chain at the complex I level and in the absence of phosphorylation, it catalyses a H^+-leak which becomes negligible when oxidative phosphorylation is functioning. Thus, under physiological conditions (phosphorylating) the only effect of Etomidate would be complex I inhibition.

Acknowledgements This work was supported by the Conseil régional d'Aquitaine. We are grateful to Odile Bunoust for her technical assistance. We would like to thank A. Tillet and E. Bernard for their technical skills. The authors would like to thank Dr. William Clark for his contribution to the editing the manuscript.

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