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EFFECTS OF PHENOPERIDINE ON RAT LIVER MITOCHONDRIAL RESPIRATION

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Abstract—Phenoperidine decreases rat liver mitochondrial oxidation of the following substrates *in state 3*: glutamate, pyruvate/malate mixture, and succinate. The intensity of inhibition is dose-dependent and substrate-dependent. With glutamate which acts at the first phosphorylation site on the respiratory chain, phenoperidine seems to have two effects on mitochondrial oxidative processes: without preincubation, phenoperidine antagonizes mitochondrial oxidation of glutamate presumably by inhibiting enzymes involved in glutamate metabolism such as glutamate dehydrogenase and glutamate oxaloacetate transaminase. Upon a three min preincubation, an additional inhibitory mechanism develops: phenoperidine prevents glutamate from penetrating into the mitochondria. Inhibition without preincubation appears to be non competitive ($K_i = 7.5 \times 10^{-5}$ M) and it is assumed that it takes place at a site before the 2,4-dinitrophenol (DNP)-sensitive site on the energy transfer process. *In state 4*, phenoperidine increases the oxidation of these substrates. It decreases the P/O ratio: thus it acts as an uncoupler, although not as potent as DNP. When compared with phenoperidine, levorphanol and pentazocine act as uncouplers but not as energy-transfer inhibitors. DALA-enkephalinamide increases state 4 respiration on glutamate. As for morphine, etorphine, meperidine and fentanyl, they do not act on mitochondrial processes. The effects of opiates on mitochondrial respiration are irrelevant to their morphinomimetic actions because they are not antagonized by naloxone.

It has been shown that anesthetics such as halothane, methoxyfluorane, trichlorethylene and chloroform act on rat liver mitochondrial oxidative processes [1]. Some authors establish correlations between the lipid solubility of these substances and their *in vitro* and *in vivo* potency [2]. In particular these substances inhibit rat liver mitochondrial respiration in state 3 with NAD-linked substrates (glutamate, NADH); in return the inhibition is low with succinate as substrate [2]. As for barbiturates, they inhibit the electron transfer in the respiratory chain on a NADH-dehydrogenase level [3].

Therefore it would be interesting to study the action of opiate analgesics, particularly those used in anesthesia, on mitochondrial processes. Among opiate analgesics, phenoperidine [1-(3-phenylpropane-3-ol)-4 carbethoxy-4-phenyl piperidine] gives good results in obstetrical anesthesia and its pharmacological effects have been extensively studied [4, 5]. Recently, Nishi *et al.* [6, 7] show that fentanyl inhibits state 3 of rat liver and brain mitochondrial respiration in the fashion of uncoupling effect, especially with NAD-linked substrates (glutamate, ketoglutarate).

On the other hand, Chistyakov *et al.* [8, 9] find that morphine, codeine, dionine and nalorphine at extremely high concentration (5 mM) inhibit state 3 mitochondrial respiration on succinate by acting upon the adenine nucleotide translocase of the mitochondrial membrane and that this effect resembles that of atractyloside. The authors correlate the action of opiates on bioenergetic processes with their pharmacological effects including hypothermia [10, 11], contractibility of smooth muscle [12], modification of ATP concentration in brain [13] and in liver [14] and with their action on ATPase [15] and glycolytic [16] activities in the liver. In addition, it has been shown that opiates at high concentration can accumulate in liver cells by active transport systems [17–19] and thus they could induce the exhaustion of tissue energetic resources as a consequence of their inhibitory effect on oxidative phosphorylation of these tissues.

The purpose of this work was to look for possible modifications caused by phenoperidine on rat liver oxidative phosphorylation, to compare this effect to those obtained with other opiate analgesics and finally to study the mechanism of action of phenoperidine at this level.

MATERIALS AND METHODS

Liver mitochondria from male Wistar rats weighing about 200 g were prepared at 4° according to the method of Hogeboom [20] as modified by Meyer *et al.* [21]. They were kept in ice-cold 0.25 M sucrose, Tris 2 mM at pH = 7.4. The mitochondrial suspension purity and the membranous integrity were checked by electron microscopy. Further, the mitochondrial suspensions which respiratory control index was inferior to six with glutamate, to four with pyruvate/malate mixture, to five with succinate in the presence of rotenone and to 1.7 with ascorbate tetramethylparaphenylene diamine (TMPD) mixture in the presence of rotenone were discarded. Bidistilled water was used.

The mitochondrial protein concentration was estimated by the Biuret method [22]. The mitochondrial

suspension contained an average of 100 mg of protein per ml.

Mitochondrial respiratory activity in state 3 and state 4 was measured in a Gilson oxygraph (Gilson Medical Electronics) equipped with a Clark electrode [23]. State 3 is the active state of mitochondrial respiration in the presence of substrate and of ADP and state 4 is the slower rate of respiration after all the ADP has been phosphorylated during state 3. The following measurements were made according to Chance and Williams [24] = QO_2: amount of oxygen consumed expressed as nanoatoms/mg of mitochondrial protein/min in state 3 and state 4; P/O ratio: amount of nmol of ADP esterified per nanoatom of oxygen consumed during state 3; respiratory control index (RCI): ratio between the amount of oxygen consumed in state 3 and the amount of oxygen consumed in state 4.

The experiments were carried out at 25° in 1.8 ml of buffer respiratory medium (BRM) at pH = 7.4 containing: KCl 110 mM, phosphate buffer (pH = 7.4) 16 mM and $MgCl_2$ 6 mM. Under these conditions the BRM contained 240 μM of oxygen [25].

The oxidizable substrates selected in terms of number of phosphorylation sites on the respiratory chain were: 1st site (between NADH and ubiquinone) either potassium glutamate 10 mM or sodium pyruvate 5 mM/potassium malate 5 mM mixture; 2nd site (between cytochromes *b* and c_1) potassium succinate 10 mM in the presence of rotenone 0.8 μM; 3rd site (between cytochrome *c* and cytochrome oxidase) potassium ascorbate 8 mM/TMPD 0.2 mM mixture in the presence of rotenone 4 μM.

Respiration was initiated by the addition of about 3 mg of mitochondrial protein and 400 μM ADP.

Opiates in aqueous solution were added in a total volume that did not exceed 0.05 ml.

The kinetics of inhibition of mitochondrial respiration were followed according to the method described by Wilson and Merz [26], modified. Into the oxygraphic chamber (thermostated at 25°) containing 1.65 ml of BRM at pH 7.4, were added successively: 3 mg of mitochondrial protein, phenoperidine at the desired concentrations in less that 0.01 ml, 400 μM ADP and glutamate to initiate the reaction.

The measurement of mitochondrial swelling in the presence of 0.1 M ammonium glutamate was recorded over a period of 10 min by following the variation of the extinction E at 520 nm [27] with a Beckman Acta III spectrophotometer. The mitochondrial suspension (2 ml in incubation medium) was kept at 25° and contained Tris (pH = 7.4) 20 mM, EDTA 1 mM and rotenone 10 μM. Phenoperidine in aqueous solution (less than 0.015 ml) was preincubated in the above-mentioned medium with 1 mg of mitochondrial protein at different times: 0, 1, 2 and 3 min before inducing the mitochondrial swelling with 0.1 M ammonium glutamate. A control of inhibition of mitochondrial swelling was realized for each assay with NEM at 35 μM [27].

Reagents were purchased from: Lebrun, Paris (Phenoperidine hydrochloride and fentanyl citrate); Francopia, Paris (Morphine hydrochloride); Sterling Winthrop, Surbiton Surrey, England (Pentazocine hydrochloride); Rhône-Poulenc, Paris (Meperidine hydrochloride); Endo, Brussels (Naloxone hydrochloride); Boehringer, Paris (ADP) and Sigma, Saint-Louis, USA (Oligomycin). Other reagents were given by: Reckitt & Colman, Kingston Upon Hull, England (Etorphine hydrochloride); Hoffman-La Roche, Bâle, Switzerland (Levorphanol l-tartrate); and Dr. H. Mazarguil, Toulouse (DALA-enkephalin-amide).

RESULTS

The effects of opiates on oxidative phosphorylation are shown in Figs. 1, 2, 3, Table 1 and Fig. 4.

Figure 1 shows the effect of phenoperidine on the mitochondrial oxidation of four respiratory substrates in states 4 and 3. State 4 respiration on ascorbate/TMPD is not affected whereas that on glutamate, pyruvate/malate and succinate are stimulated. This is particularly evident with succinate, where oxidation was significantly increased with concentration of phenoperidine as low as 25 μM. On the other hand, state 3 respiration is inhibited by phenoperidine especially with the NAD-linked substrates (glutamate and pyruvate/malate); 50 μM of phenoperidine is sufficient to halve the rate of glutamate oxidation.

The P/O ratios are diminished by phenoperidine at concentrations in the range 15–150 μM (Fig 2). This inhibitory effect is more evident with glutamate, pyruvate/malate and succinate than with ascorbate/TMPD.

RCI values (Fig. 3) were much decreased in the presence of phenoperidine with the three first

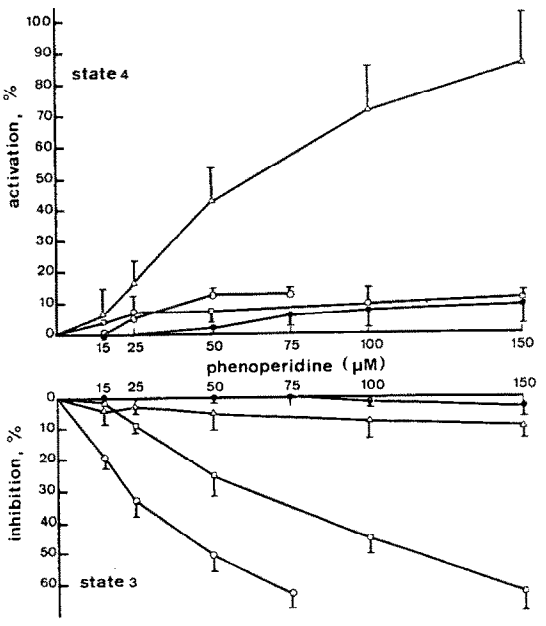

Fig. 1. The effect of phenoperidine at different concentrations on state 4 or 3 respiration by rat liver mitochondria. Controls values of QO_2, expressed as nanoatoms oxygen × min^{-1} × mg^{-1} of mitochondrial protein, are the means ± S.D. of five or six mitochondrial preparations with glutamate (○): 7.75 ± 0.37 (state 4), 52.82 ± 1.28 (state 3); pyruvate/malate mixture (□): 9.46 ± 0.72 (state 4), 42.34 ± 1.37 (state 3); succinate (△): 14.38 ± 0.90 (state 4), 85.62 ± 5.06 (state 3); or ascorbate/TMPD mixture (●); 48.74 ± 1.62 (state 4), 91.53 ± 2.29 (state 3).

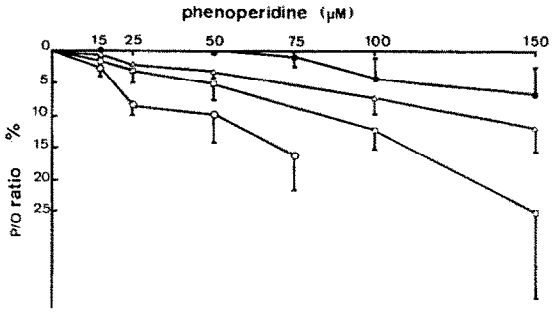

Fig. 2. The effect of phenoperidine at different concentrations on P/O ratio of rat liver mitochondrial respiration. Control values are the means ± S.D. of five or six mitochondrial preparations with glutamate (○): 2.74 ± 0.11; pyruvate/malate (□): 3.04 ± 0.05; succinate (△): 1.78 ± 0.04; or ascorbate/TMPD mixture (●): 1.02 ± 0.04.

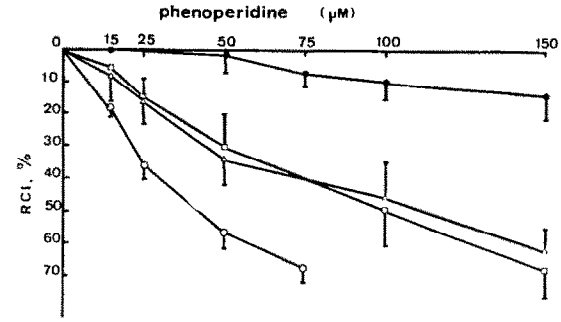

Fig. 3. The effect of phenoperidine at different concentrations on RCI of rat liver mitochondrial respiration. Control values are the means ± S.D. of five or six mitochondrial preparations with glutamate (○): 6.85 ± 0.38; pyruvate/malate mixture(□): 4.50 ± 0.33; succinate (△): 5.98 ± 0.38; or ascorbate/TMPD mixture (●): 1.88 ± 0.08.

Table 1. Comparative effects of some opiate narcotics (50 μM) on rat liver mitochondrial respiration oxidizing glutamate

Drugs	Parameter measured			
	QO_2		P/O	RCI
	state 4	state 3		
Morphine	−4.7	−1.8	0	+2.9
Etorphine	0	+0.7	−1	+0.7
Levorphanol	+21.6	+3.6	−10.7	−14.5
Pentazocine	+26.3	+1.7	−14.9	−19.4
Meperidine	0	−0.4	−0.3	−0.3
Fentanyl	0	−2.9	−3.6	−2.9
DALA-enkephalinamide	+16.6	−5.7	0	−0.9

Values are the mean of effect per cent of five mitochondrial preparations; +: activation −: inhibition.

substrates, especially with glutamate: 40 μM phenoperidine is found to reduce the RCI by 50 per cent with this substrate. In contrast to this phenoperidine has no inhibitory effect with ascorbate/TMPD.

Table 1 shows the comparative effects of other opiate analgesics at 50 μM (concentration corresponding to phenoperidine's ID_{50} in state 3) on mitochondrial respiration oxidizing glutamate. Both morphine and etorphine do not affect mitochondrial processes. In return, levorphanol (morphinan) stimulates state 4 and has practically no effect in state 3; but it decreases P/O ratio and RCI values. Pentazocine (benzomorphan) alters the above-mentioned parameter of mitochondrial respiration as levorphanol does. In the 4-phenyl-piperidines series, neither meperidine nor fentanyl alter mitochondrial oxidative processes. As for DALA-enkephalinamide, it stimulates state 4, but it does not act in state 3, P/O ratio and RCI values.

The opiates antagonist naloxone, at 50 or 100 μM, added up to 5 min before or after 50 μM of phenoperidine and other opiates, was not found to prevent the above-mentioned effects of these drugs.

In Figure 4, the inhibition of mitochondrial oxidation of glutamate by phenoperidine was compared with the effects of oligomycin and DNP. Oligomycin inhibited state 3 respiration down to the level of state 4 and the inhibition was reversed by DNP. The inhibition of state 3 respiration by phenoperidine followed a time course to the by oligomycin and it was not reversed by DNP (Fig 4a). This result suggests that phenoperidine has a property somewhat different from a typical energy-transfer inhibitor and its blocking site is located before the DNP-sensitive site. On the other hand, oligomycin had no effect on DNP-stimulated respiration, whereas phenoperidine had an inhibitory effect that is similar to that on state 3 respiration (Fig. 4b). This observation also supports the assumption concerning the blocking site of phenoperidine described above.

The Lineweaver–Burk plot (Fig. 5) shows that the inhibition of mitochondrial respiration in state 3 by increasing concentrations of phenoperidine is non competitive with glutamate as substrate. The values found are: $K_i = 7.5 \times 10^{-5}$ M and $K_m = 6.6 \times 10^{-4}$ M.

Figure 6 shows that mitochondrial swelling induced by 0.1 M ammonium glutamate is not inhibited by phenoperidine when the preincubation time is inferior to 3 min. In return, NEM (35 μM) inhibits swelling without preincubation (Fig. 6a). But when the preincubation time is 3 min, phenoperidine inhibits swelling in a dose dependent manner (Fig. 6b).

DISCUSSION

Our results indicate that the various effects of phenoperidine on mitochondrial metabolism are reminiscent of those reported by Cohen and McIntyre [2] with general anesthetics that strongly inhibit the oxidation of NAD-linked substrates, but only mildly that of succinate in state 3, and diminish the RCI for all the substrates.

Among a number of possible mechanisms, phenoperidine might: (1) inhibit the transport of glutamate across the mitochondrial membrane by acting upon

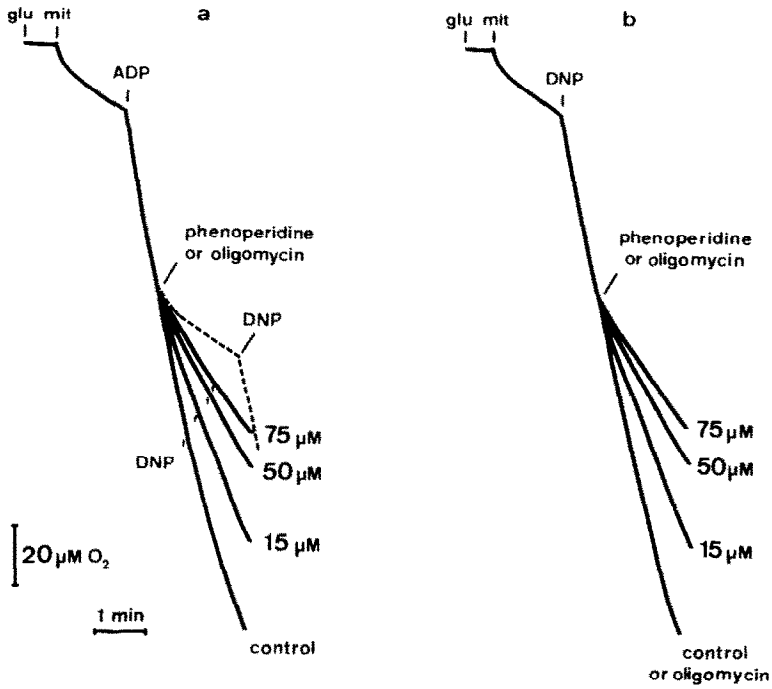

Fig. 4. Polarographic traces showing oxygen consumption by rat liver mitochondria. Each composite curve consists of a series of superimposed traces of individual experiments in which the amount of inhibitor added at the points indicated was varied. Trace a is a comparison between the inhibitory effects of phenoperidine and oligomycin. Trace showing the effect of oligomycin is indicated by the dotted line. At the indicated point, DNP was added to test its effect in releasing the respiratory inhibition. Trace b shows the effect of phenoperidine on the DNP-stimulated respiration. Concentration: DNP 70 μM; oligomycin 3 μg in 1,8 ml of final volume of BRM.

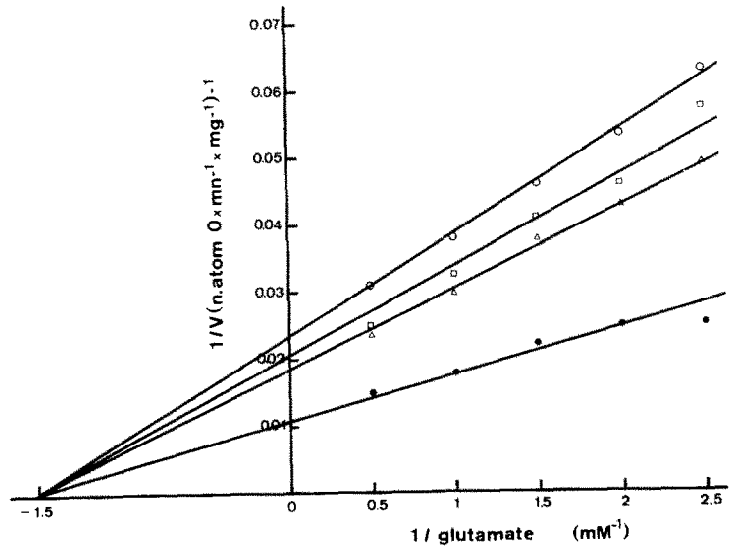

Fig. 5. Lineweaver–Burk plot state 3 glutamate respiration at various phenoperidine concentrations (μM): control = none (●), 15 (△), 50 (□), 75 (⊖).

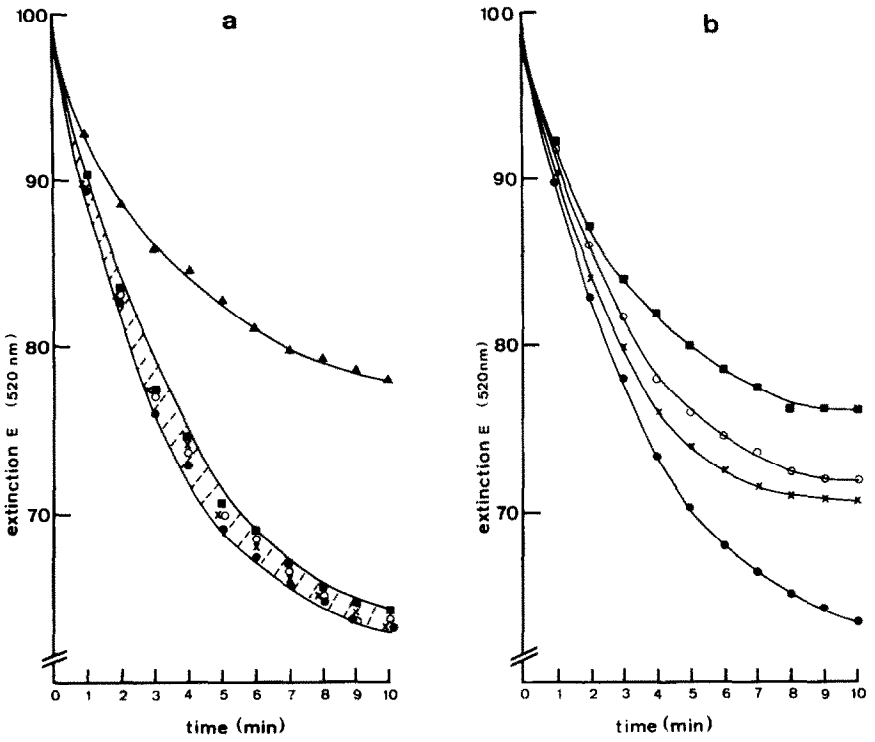

Fig. 6. Effect of phenoperidine on mitochondrial swelling induced by 0.1 M ammonium glutamate. Ordinate: the extinction E normalized to 100 and abscissa: time in min. (a) without preincubation (b) preincubation 3 min. Controls: without phenoperidine (●) and with NEM 35 μM (▲). With phenoperidine (μM): 25 (×), 50 (○), 75 (■).

the glutamate–hydroxyl carrier, (2) enter the mitochondria where it would inhibit enzymes involved in glutamate metabolism such as glutamate dehydrogenase and glutamate oxaloacetate transaminase.

Indeed, phenoperidine does inhibit the mitochondrial swelling induced by 0.1 M ammonium glutamate, suggesting that the former would prevent the latter from penetrating into the organelle. This effect mimics that of NEM which inhibits mitochondrial swelling, most likely by alkylating the glutamate–hydroxyl carrier [28], a mechanism that can hardly be retained for phenoperidine. Also, in contrast with NEM, phenoperidine must be present in the medium for at least 3 min before its effect is observed.

Without preincubation, phenoperidine does not inhibit the mitochondrial swelling, but does inhibit mitochrondrial respiration of glutamate. Inside this organelle, phenoperidine appears to exert its action at the first phosphorylation site in the respiratory chain. With glutamate as substrate, the inhibition of state 3 respiration is non competitive, explaining the non competition on the enzymes involved in glutamate metabolism. The inhibitory effect of phenoperidine on glutamate oxidation is observed mainly on state 3 respiration and P/O ratio, suggesting that phenoperidine acts as an energy-transfer inhibitor. When this drug is compared with oligomycin, a typical energy-transfer inhibitor, their inhibition kinetics are somewhat different, especially on DNP-stimulated respiration. Phenoperidine shows properties reminiscent of those of uncoupling agents, but is not as potent as DNP, a typical uncoupler. When compared with oligomycin and DNP, it may be assumed that the blocking site of phenoperidine lies before the DNP-sensitive site in the energy transfer process.

Among other opiates used, only levorphanol, pentazocine and DALA-enkephalinamide at 50 μM alter mitochondrial respiration on glutamate. Levorphanol acts on mitochondrial processes in the same fashion as pentazocine. These two drugs stimulate state 4 more than phenoperidine does, but they do not influence state 3. In addition, they decrease P/O. These results suggest that levorphanol and pentazocine act as uncouplers but not as energy-transfer inhibitors. Among pentapeptides, DALA-enkephalinamide (Tyr-Dala-Gly-Phe-Metamide) was selected because it seems the most resistant to hydrolysis. It stimulates electron transfer down the respiratory chain only in the absence of ADP. Other drugs such as morphine, etorphine, meperidine and fentanyl at 50 μM do not practically act on mitochondrial oxidative phosphorylation. Additional work is required to determine the effects of these drugs at higher concentration on mitochondrial processes as those obtained by Nishi *et al.* [6, 7] with fentanyl and pentazocine and by Chistyakov *et al.* [8, 9] with morphine.

Finally, the effects of phenoperidine and other opiates reported here depend on their chemical

structure and they are not relevant to its pharmacological action as narcotic analgesics since they are not prevented by naloxone, a well characterized opiate antagonist.

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