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Enalapril Maleate Affects 2-Oxoglutarate Metabolism in Mitochondria from the Rat Kidney Cortex

MA. ELIANE MERLIN, ANNIBAL P. CAMPELLO† AND MA. LÚCIA W. KLÜPPEL

Departamento de Bioquímica da Universidade Federal do Paraná, Caixa Postal 19046, Curitiba, Paraná, Brasil, 8531-970

Enalapril maleate (EM) is the salt of *N*-{(S)-1-ethoxycarbonyl)-3-phenylpropyl}-L-alanyl-L-proline, used therapeutically as an anti-hypertensive agent. The effects of EM on some aspects of the energy metabolism and membrane properties of mitochondria from rat liver and kidney cortex were studied, but only the latter were significantly affected. With 0·8 mM of EM and 2-oxoglutarate as oxidizable substrate for isolated mitochondria from rat kidney cortex, the findings were: (a) inhibition of the respiratory rate in state III (37 per cent) and decrease (45 per cent) in respiratory control ratio (RCR), with only one addition of ADP; (b) reinforcement of the inhibition when a second addition of ADP was made; (c) no significant effect either on the rate of respiration in state IV or on the ADP/O ratio; (d) no effect on the ATPase activity of mitochondria from liver or kidney cortex; (e) inhibition of the transmembrane potential ($\Delta\psi$) after a second addition of ADP; (f) inhibition of the 2-oxoglutarate dehydrogenase complex. It is suggested that in kidney mitochondria, EM interferes in the gluconeogenesis dependence of at least five substrates: 2-oxoglutarate, glutamine, glutamate, lactate, and pyruvate. Also, EM may inhibit Na^+/H^+ exchange causing natriuresis.

KEY WORDS—Enalapril maleate; anti-hypertensive; liver and kidney cortex mitochondria; oxygen uptake; oxidative phosphorylation; transmembrane potential; 2-oxoglutarate dehydrogenase complex.

INTRODUCTION

Enalapril is unusual in being a pro-drug. Serum enalapril concentrations reach a peak at between 0·5 h and 1·5 h after oral administration with little unchanged drug in the circulation after 4·0 h.[1] It was previously reported that peak serum concentrations of the active metabolite, enalaprilat occur at 3–4 h.[2] After 8·0 h, drug excretion is totally in the form of enalaprilat. Some of the changes observed in renal tubular function may be due to unchanged enalapril.[3] The kinetic evaluation of renal elimination in patients with renal dysfunction showed that a progressive accumulation of enalaprilat occurred, and was related to the decrease in renal activity.[4] A number of studies have shown various adverse reactions attributable to the effect of enalapril, as well as the results of those on *in vitro* metabolite, in which the conversion of enalapril to enalaprilat and other biotransformations occurred at the hepatic level.[5–8]

The effect of increasing amounts of enalapril maleate (EM) on substrate utilization (glutamate and succinate) and oxidative phosporylation by rat liver and kidney cortex mitochondria has been reported by Basso *et al.*[8] EM significantly inhibited the activity of purified glutamate-oxalacetic transaminase, and modified the activities of NADH-oxidase and NADH cytochrome c reductase, probably inhibiting electron transfer between complex I and III of rat kidney mitochondria. No effect was observed with rat liver mitochondria. Experiments on biotransformation showed that de-esterification of enalapril to enalaprilat occurred in liver mitochondria.

Studies on the effect of EM on mitochondrial functions are scarce. The promising findings of Basso *et al.*[8] appear to strengthen previous suggestions of the mechanism of action of EM and led to several lines of investigation. The present study, designed to investigate the effects of EM on 2-oxoglutarate utilization by liver and cortex kidney mitochondria, further complements the results obtained by Basso *et al.*[8] which enhances the understanding of the mechanism of action.

†Addressee for correspondence.

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MATERIALS AND METHODS

Preparation of Mitochondria

Rat liver or rat kidney cortex mitochondria, were prepared according to the procedure of Voss *et al.*[9] using a mannitol extraction medium with modifications (mannitol 260·0 mM, EGTA 1·0 mM, Tris–HCl 10·0 mM (pH 7·2), and bovine serum albumin (BSA) 0·1 per cent). In all cases the mitochondrial pellet was washed once, suspended in a mannitol medium, and used with minimum delay for the determination of oxygen uptake and assays of oxidative phosphorylation.

Polarographic Determinations

Polarographic determinations of oxygen uptake and oxidative phosphorylation were made with an oxygen electrode.[10] The reaction system and procedure were essentially as described earlier.[9] The respiratory rate is expressed as nanomoles of oxygen consumed $min^{-1}\,mg^{-1}$ of mitochondrial protein, according to Estabrook.[11]

Determination of Activity of the 2-Oxoglutarate Dehydrogenase Complex

The determination of the activity of the 2-oxoglutarate dehydrogenase complex was done according to Schnaitman and Greenawalt[12] by treating liver or kidney cortex mitochondria with digitonin, followed by differential centrifugation.

Isolation of Mitochondria. Mitochondria were isolated using a medium containing 220·0 mM mannitol, 60·0 mM sucrose, 2·0 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer and 0·5 mg ml^{-1} of BSA.

Digitonin Fractionation of Mitochondria. A mitochondrial suspension was gently stirred for 5 min in 2·0 ml of 20·0 mg of digitonin dissolved in the isolation medium containing 0·5 mg ml^{-1} of BSA. The resulting suspension was diluted with three volumes of the isolation medium, gently homogenized, and centrifuged at 8000 *g* for 10 min. The supernatant was carefully drawn off, and the pellet resuspended in the isolation medium and centrifuged at 12 000 *g* for 10 min. The pellet from the second centrifugation was suspended in the isolation medium to give 37·0 mg ml^{-1} of mitochondrial protein.

Enzymatic Assays. 2-Oxoglutarate dehydrogenase activity was assayed spectrophotometrically by following the reduction of NAD^+ at 25°C. Samples were incubated for 3 min in a medium containing in 1·5 ml of water: 2·5 mg of BSA, 0·6 mM $MgCl_2$, 0·15 mM EDTA, 0·6 mM KCN, 1·4 mM thiamine pyrophosphate, 66·0 mM HEPES buffer (pH 7·4), 4·0 mM NAD^+, and 0·3 mg Lubrol PX mg^{-1} of mitochondrial protein. To this was added 1·5 ml of water containing 6·0 mM cysteine, 0·86 mM coenzyme A, 1·0 mM $MgCl_2$ and 133·0 mM HEPES buffer (pH 7·4). The reaction was initiated by the addition of 1·6 mM of 2-oxoglutarate. This enzyme is quite labile and was assayed immediately after fractionation of the mitochondria. The system was supplemented with 0·8 mM of EM and 0·42 mM ATP where stated. The activity is expressed as μmoles of NAD^+ reduced min^{-1} mg^{-1} of mitochondrial protein, considering the extinction coefficient to be 6220 $M^{-1}\,cm^{-1}$ at 340 nm.[13]

Determination of ATPase (ATP Phosphohydrolase, E.C.3.6.1.3)

The ATPase in broken or FCCP-treated mitochondria was assayed as described by Pullman *et al.*[14] Mitochondria were isolated according to Voss *et al.*[9] The results are expressed as nmoles of phosphate released $min^{-1}\,mg^{-1}$ of mitochondrial protein. The orthophosphate content was determined according to Sumner.[15]

Determination of the Transmembrane Potential ($\Delta\psi$)

A sensitive tetraphenylphosphonium (TPP^+) electrode, prepared according to Kamo *et al.*[16] and Cohen *et al.*[17] was used to determine $\Delta\psi$, monitoring the distribution of TPP^+ across the mitochondrial membrane. The standard respiratory medium was: 250·0 mM mannitol, 10·0 mM Tris–HCl (pH 7·4), 10·0 mM KCl, 0·5 mM phosphate buffer, 0·1 mM EGTA, and 0·05 per cent BSA. The reaction was initiated by adding 2·0 mg of mitochondrial protein and 2·0 mM of 2-oxoglutarate or succinate as substrates. The electrode signals were amplified by a Metrohn pH-meter and the output recorded on a linear chart.

Protein Determination

Mitochondrial protein was assayed by the method of Lowry *et al.*[18] using bovine serum albumin as standard.

Statistical Analysis

Statistical analysis was by linear regression using the method of least squares of the means, and the Student's *t*-test for analysis of significance. Mean values ± SD were used. Results were considered significant as a $p < 0.05$ level.

RESULTS AND DISCUSSION

McNabb *et al.*[3] observed that enalapril and enalaprilat contribute separately to acute renal effects, independently of haemodynamic renal changes or reduction of the angiotension converting enzyme (ACE) or aldosterone activity. Our results agree with the findings of McNabb *et al.*[3] and Basso *et al.*[8] and further support the clinical results of Lau[19] who described a patient who had ingested an overdose of EM and in whom there were greater changes in renal function. No effect on liver function was observed, and the hydrolysis of enalapril to the active form was not impeded.

The concentration of EM used in the present study was based on the therapeutic serum and tissue concentrations calculated according to Johnston *et al.*,[20] Pang *et al.*[21] and Basso *et al.*[8]

The polarographic results (Figure 1) showed that EM significantly inhibited the respiratory rate of state III by about 37 per cent ($p < 0.05$) of the control and the respiratory control ratio (RCR) by about 47 per cent ($p < 0.05$) of the control when 2-oxoglutarate was used as the respiratory substrate in renal mitochondria, and with one addition of ADP. No significant effect was observed either on the rate of respiration in state IV or on the ADP/O ratio. When liver mitochondria were used the small variations observed were not significant.

Interesting results were obtained when a second addition of ADP was made. As shown in Figure 2B, EM (0·4 mM) inhibited state III by about 30 per cent and RCR by about 33 per cent of the control after the first addition of ADP (0·2 mM). These results were similar when 0·8 mM of EM was used, namely state III was inhibited by about 37 per cent and RCR by about 45 per cent of the control, after the first addition of ADP (Figure 2C). However after the second addition of ADP and with 0·4 mM EM the inhibition was 60 per cent for state III, and RCR was decreased from 3·7 to 1·3 (Figure 3B); when 0·8 mM of EM was used the inhibition was about 72 per cent for state III and RCR decreased to unity (Figure 2C). These results are quite different from those in the control (Figure 2A).

It was possible that these effects were due to the time gap between the addition of EM and the second addition of ADP (approximately 4 min). However, if kidney cortex mitochondria were pre-incubated for 4 min with 0·8 mM of EM, the results obtained were similar to those in Figure 2C.

These observations excluded the possibility that the results obtained were related only to the time of contact of the mitochondrial preparation with the drug, firstly, because pre-incubation did not alter the effect, and secondly, the phenomenon only occurred with kidney cortex mitochondria. Furthermore, the effect appeared only with 2-oxoglutarate as the oxidizable substrate (see Basso *et al.*)[8] These results suggest a combination of the presence of ADP or ATP at high concentration, together with an effect of EM, since this did not occur with the control.

The results of Basso *et al.*[8] and our own, eliminated the first and second possibilities. Furthermore, the finding that EM did not inhibit ATPase activity (results not shown) either of FCCP-treated or broken mitochondria from liver or kidney cortex mitochondria excluded an hypothesis that the effects were due to inhibition of this activity.

To examine this issue further, the effect of EM on the 2-oxoglutarate dehydrogenase complex was studied. It is known[22] that ATP (0·05 mM) inhibits 50 per cent of the 2-oxoglutarate dehydrogenase activity. As shown in Table 1, these results were confirmed by us with the enzyme isolated from kidney cortex mitochondria, using 0·42 mM of ATP. EM alone had no effect on the activity of the isolated enzyme, either from liver or kidney cortex mitochondria. However, when 0·8 mM of EM was added to the system before the 0·42 mM of ATP, only the enzyme isolated from the renal tissue was inhibited (83 per cent). EM added after the ATP inhibited the enzymatic activity by 46 per cent, similar to that observed with ATP alone (Table 1). It is important to remember that the inhibition observed on the polarographic parameters (state III and RCR) with renal mitochondria occurred after the second addition of ADP, in those experiments in which EM was previously added. Apparently EM promotes a partial restriction of the respiratory chain electron transfer through the inhibition of 2-oxoglutarate dehydrogenase complex conditioned to the presence of ATP.

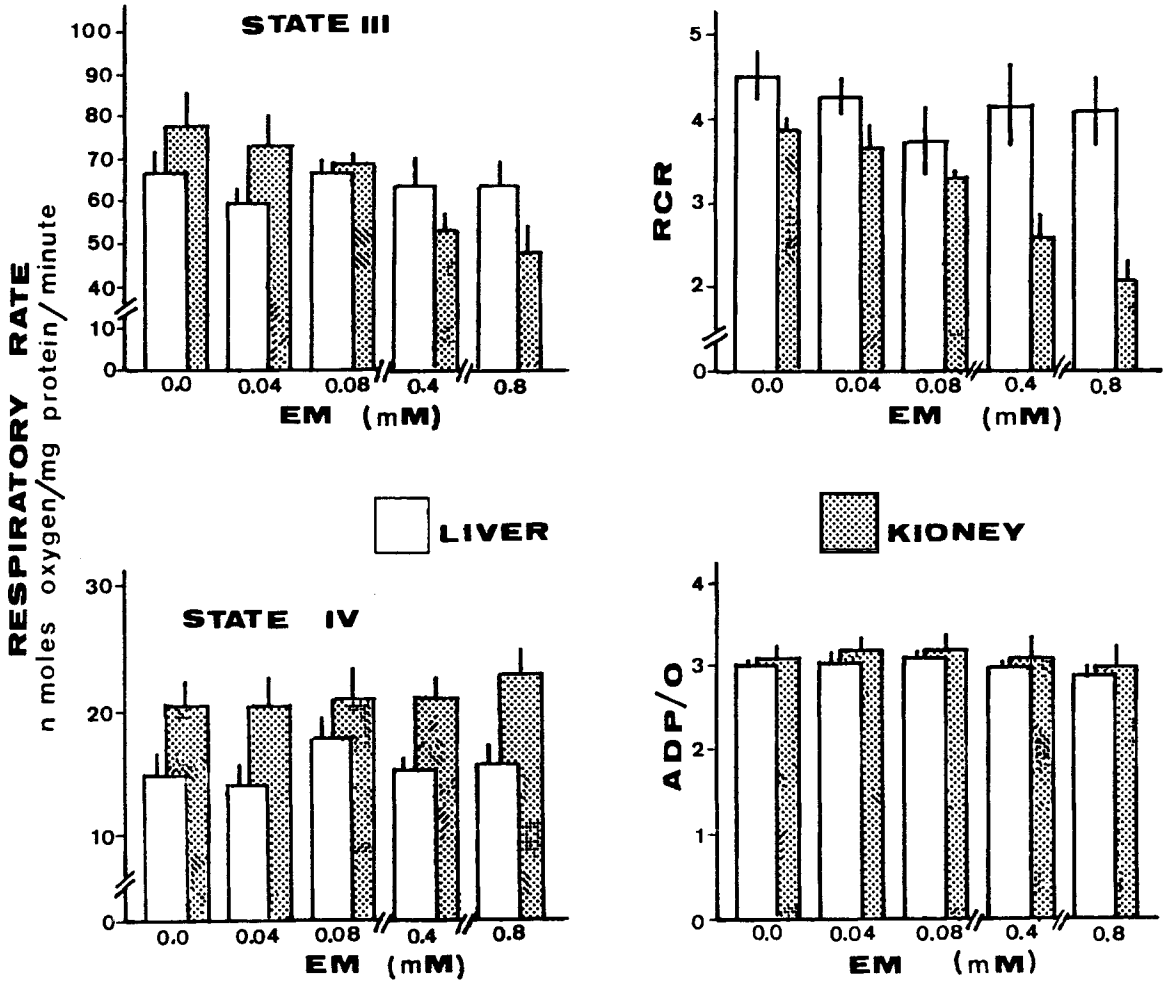

Figure 1. Effect of EM on the oxygen uptake and oxidative phosphorylation of rat liver and kidney cortex mitochondria. ADP (0·2 mM) was added to the system after 4 min of pre-incubation with the drug. Each value represents the mean ± SD of six different experiments. The values for state III and RCR with kidney cortex mitochondria are significantly different from control values (0·00 mM) at the $p < 0·05$ level. State III = mitochondrial oxygen consumption in the presence of substrate and ADP. State IV = mitochondrial oxygen consumption in the presence of substrate only. RCR = respiratory control ratio. ADP/O = ADP concentration/oxygen consumption ratio.

Together with the results shown in Figures 1 and 2, and as is apparent from Figure 3 and the results summarized in Table 2, there is an indication that EM at increasing concentrations promotes alterations in kidney cortex mitochondrial membrane, whose consequence would be a modification in the electrochemical gradient generation. The effects of EM on the mitochondrial transmembrane electrical potential ($\Delta\psi$) are shown in Figure 3 and Table 2.

On the addition of 2-oxoglutarate, mitochondria developed a $\Delta\psi$ of about 210 mV (negative inside). The potential suddenly decreased to −172 mV, on addition of ADP. After a short lag phase, the mitochondrial membrane repolarized close to its state IV value. After a second addition of ADP, $\Delta\psi$ decayed to −177 mV, and another repolarization of the mitochondrial membrane occurred (Figure 3A). When EM (0·08, 0·4, or 0·8 mM) (Figures 3B, C and D) was mixed together with the kidney cortex mitochondrial suspension, the $\Delta\psi$ induced by the oxidation of 2-oxoglutarate was unaffected, confirming that no inhibition of 2-oxoglutarate dehydrogenase

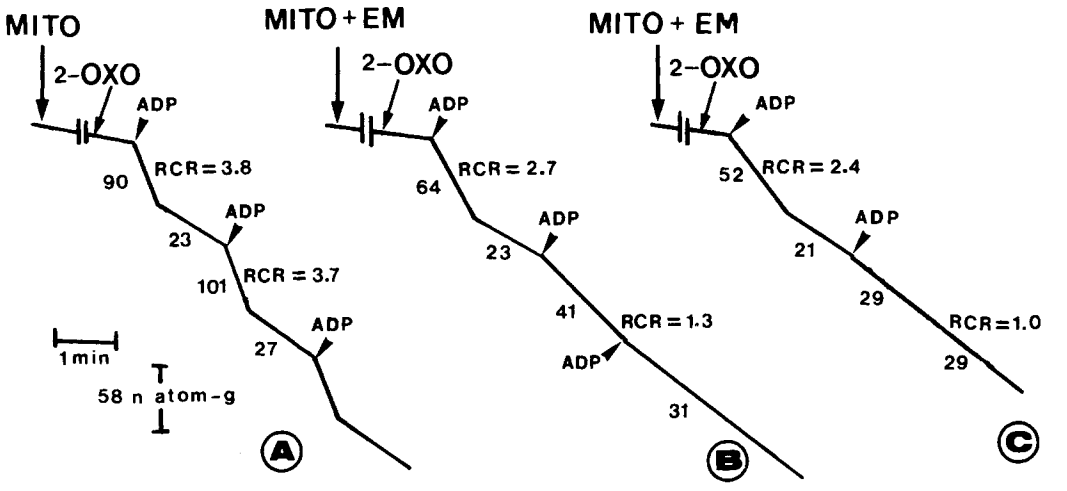

Figure 2. Effect of EM on the polarographic parameters of kidney cortex mitochondria. 2-Oxoglutarate (2·0 mM) and ADP (0·2 mM) were added as indicated in the tracings. A — control (0·00 mM EM), B — 0·4 mM EM, and C — 0·8 mM EM.

complex occurred in the absence of ADP. On the first addition of ADP, the mitochondria developed a $\Delta\psi$ similar to that shown in Figure 3A. It is noteworthy that EM depressed the depolarization induced by ADP and lengthened the lag phase preceding repolarization. On the second addition of ADP, EM depressed the total developed $\Delta\psi$, the depolarization induced by ADP, and repolarization resumes at a reduced rate and level. This effect was not observed with succinate as substrate, showing that there is a dependence for 2-oxoglutarate. With succinate the repolarization rate was only partially affected (Table 2).

Table 2 summarizes the effect of EM on the re-energization rate of kidney cortex mitochondrial membrane after addition of ADP with the substrates 2-oxoglutarate or succinate. The maximum inhibition (0·8 mM of EM) obtained with succinate was 43 per cent and with 2-oxoglutarate it was 99 per cent, i.e. the inhibition was 56 per cent higher with the latter substrate, after the second addition of ADP. All values are statistically significant $p < 0{\cdot}05$. The inhibition was related to the concentration of EM. EM did not affect the trans-membrane potential of liver mitochondria, either with succinate or with 2-oxoglutarate.

Table 1. Effect of EM on the activity of rat kidney cortex mitochondrial 2-oxoglutarate dehydrogenase complex.

Additions	Activity μmoles min^{-1} × mg of protein	% Inhibition
Control	$7{\cdot}12 \times 10^{-3}$	0
EM (0·8 mM)	$6{\cdot}41 \times 10^{-3}$	10
ATP (0·42 mM)	$4{\cdot}06 \times 10^{-3}$	43
ATP (0·42 mM) + EM (0·8 mM)	$3{\cdot}84 \times 10^{-3}$	46
EM (0·8 mM) + ATP (0·42 mM)	$1{\cdot}21 \times 10^{-3}$	83

The values represent the results of at least six independent experiments.

These results lead us to suggest that EM has two kinds of effect on the transmembrane potential. Firstly it is responsible for 43 per cent of the $\Delta\psi$ inhibition, common to both substrates, which is a consequence of the action of the drug on the mitochondrial membrane; secondly, it produces 56 per cent of $\Delta\psi$ inhibition, which is apparently only related to the oxidation of 2-oxoglutarate. This effect occurs because of the effect of EM on complex I of the respiratory chain[8] and on 2-oxoglutarate dehydrogenase (Table 1).

According to Gordon and Hartog,[23] succinate and 2-oxoglutarate are the most effective precursors for gluconeogenesis in the kidney cortex, followed by malate, glutamate, glutamine, lactate, and pyruvate. 2-Oxoglutarate is the common product of transaminase and glutamate dehydrogenase, and that formed by the transaminase reaction is a potent inhibitor of glutamate

EM (mM)	ΔΨ (mV) 2-OXO	ADP 1st	ADP 2nd	REPOLARIZATION 1st	REPOLARIZATION 2nd
0.00	210	172	177	204	204
0.08	206	170	169	200	197
0.40	206	166	170	190	175
0.80	204	167	169	181	169

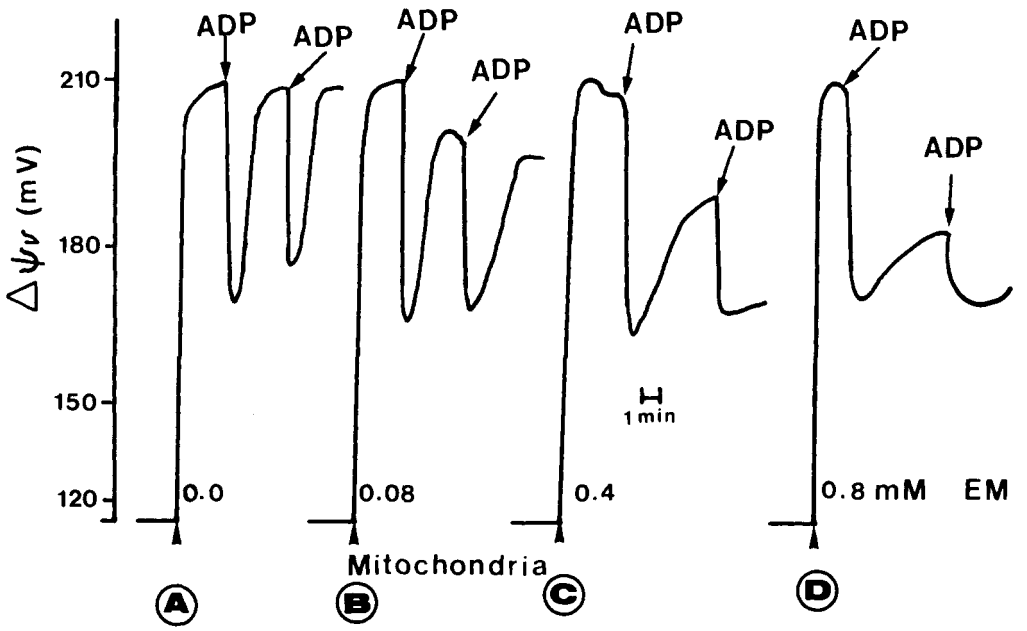

Figure 3. Effect of EM on the mitochondrial transmembrane potential ($\Delta\psi$). The traces represent typical recordings from several experiments with different mitochondrial preparations. Lag phase is the period between the end of depolarization and the onset of repolarization.

dehydrogenase.[24–26] It has since become apparent that what inhibits transamination or the production of matrix 2-oxoglutarate[27] will stimulate the glutamate dehydrogenase flux.

With the above considerations and the results already obtained, several aspects should be stressed: (1) EM inhibits glutamate-oxalacetic transaminase (GOT),[3] giving rise to glutamate dehydrogenase activity, interfering not only in gluconeogenesis, but also in the sodium transport by mammalian kidney.[28] (2) The gluconeogenesis from lactate as substrate, under conditions of

Table 2. Effect of EM on the re-energization rate of rat kidney cortex mitochondrial membrane after addition of ADP.

EM (mM)	After first addition of ADP		After second addition of ADP	
	2-oxoglutarate	succinate	2-oxoglutarate	succinate
	% Inhibition		% Inhibition	
0·00	0	0	0	0
0·04	10 ± 2·9	0	23 ± 5·3	10 ± 2·8
0·08	35 ± 5·2	3 ± 1·8	47 ± 5·3	13 ± 1·4
0·40	69 ± 7·4	13 ± 3·9	95 ± 2·5	27 ± 8·4
0·80	91 ± 3·3	40 ± 4·6	99 ± 0·7	43 ± 3·7

The values are expressed as percentage of inhibition and are the means ± SD of at least six experiments.

severe exercise, might be compromised since cytosolic and mitochondrial GOT would be involved. (3) Regulation by Ca^{2+} is not the only means whereby the flux through the 2-oxoglutarate dehydrogenase complex may be controlled; it may also be controlled by increases in the ADP/ATP and $NAD^+/NADH$ concentration ratios.[22] Gluconeogenesis from 2-oxoglutarate may be controlled in a concerted manner, and presumably the properties that the 2-oxoglutarate dehydrogenase complex exhibits is important in kidney to ensure that the rate through this dehydrogenase is matched closely to the ATP requirements of the cells. (4) Our results (Figure 3 and Table 2) showed that the transmembrane electric potential was depressed by EM. This effect could interfere directly with the cellular energetic gain, transamination, gluconeogenesis, and indirectly with sodium transport in kidneys.[28]

Finally, it can be suggested that EM interferes with gluconeogenesis that is dependent on at least five substrates: 2-oxoglutarate, glutamate, glutamine, lactate, and pyruvate. Also EM may inhibit Na^+/H^+ exchange thus increasing the urinary pH while causing significant natriuresis. Other studies are currently in progress in our laboratory in order to define more accurately the relative contributions of enalapril maleate to observed changes in renal mitochondria.

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