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STUDY OF THE MITOCHONDRIAL PHOSPHATE CARRIER IN THE COURSE OF CALCIUM PHOSPHATE ACCUMULATION: A REQUIREMENT FOR Mg^{2+} AND ADP OF ITS SENSITIVITY TO THIOL REAGENTS

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SUMMARY

1. The accumulation of calcium phosphate driven by succinate oxidation is ADP-dependent. In its absence the accumulation stops after a short incubation time and the oxygen uptake is permanently stimulated. This uncoupled oxygen uptake is insensitive to the inhibitors of phosphate transport, like mersalyl and *N*-ethylmaleimide. When ADP plus Mg^{2+} are added to the medium, or when ADP is added in the initial presence of magnesium, the inhibitory action of the thiol reagents on oxygen uptake is re-established. ADP alone or Mg^{2+} alone are without any effect.

2. Phosphate/phosphate exchange has been studied, in the absence of ADP, when calcium phosphate accumulation had stopped and oxygen uptake is uncoupled. Under these conditions the exchange process becomes insensitive to thiol reagents. Sensitivity is recovered solely in the presence of ADP plus Mg^{2+}.

3. When mitochondrial swelling is studied according to the method of Chappell, it also appears that the phosphate carrier loses it sensitivity to mersalyl in the absence of ADP, which confirms the data obtained with phosphate/phosphate exchange experiments. When ADP plus Mg^{2+} are added (or present), together with mersalyl, the action of the thiol inhibitor is recovered. ADP and magnesium are inactive separately. EGTA plus Mg^{2+} (but not EGTA plus ADP) may substitute for ADP plus Mg^{2+} in this process.

4. A possible interaction between the magnesium binding site and the phosphate carrier is considered and discussed.

INTRODUCTION

It has been previously shown that the sustained accumulation of Ca^{2+} by mitochondria in the presence of phosphate and oligomycin, the energy of which is derived from substrate oxidation (for extensive reviews of this aspect of mitochondrial metabolism, [1–3]), depends on the presence of an adenine nucleotide [4], and that the specific nucleotide required is ADP [5, 6]. It has been further demonstrated that

Abbreviation: EGTA, ethyleneglycol-bis-(aminoethyl)-tetraacetic acid.

Mg^{2+} plays an essential role in this accumulation [6], since its presence, together with ADP is stringent for reversing the metabolic changes induced by the accumulation of small amounts of calcium phosphate in the absence of ADP: both this nucleotide and Mg^{2+} have to be present to re-initiate calcium phosphate accumulation and to re-impose respiratory control under these conditions. The present work extends previous findings, demonstrating that in addition to the changes described a complete loss of sensitivity of the phosphate carrier to its classical inhibitors, the thiol reagents is observed [7–10]. Sensitivity is only restored by adding both ADP and Mg^{2+} to the mitochondrial preparation. The model proposed previously [6] to explain the respective roles of ADP and Mg^{2+} on Ca^{2+} accumulation and oxygen uptake is disscussed in the light of the new data obtained. A brief account of this work has been published [11].

MATERIALS AND METHODS

Hog heart mitochondria were prepared as described previously [12]. The study of phosphate accumulation (linked to the accumulation of Ca^{2+}) and of phosphate/phosphate exchange was performed with $H_3{}^{32}PO_4$. After incubation, sarcosomes were separated from the medium by filtration through 0.65 nm Millipore filters under negative pressure, and subsequently washed and counted, as described previously [6] for calcium exchange. Oxygen uptake and its activation during calcium phosphate accumulation were determined with a Gilson oxygraph.

Swelling of mitochondria in ammonium phosphate, according to Chappell and Crofts [13], was measured at 540 nm in an Eppendorf spectrophotometer.

Proteins were measured according to Jacobs et al. [14].

Ruthenium red was purchased from Fluka AG., $Na_2H^{32}PO_4$ was purchased from CEA, Saclay, France.

RESULTS

Insensitivity of calcium-stimulated oxygen uptake to thiol reagents (N-ethylmaleimide, mersalyl) in the absence of ADP

It has been shown previously [6] that in the absence of ADP, calcium phosphate accumulation stops, oxygen uptake is uncoupled and becomes insensitive to the specific inhibitor of calcium transport, ruthenium red [19, 20].

Fig. 1 shows that under these very conditions neither mersalyl nor *N*-ethylmaleimide inhibits oxygen uptake; the addition of ADP+Mg^{2+} (Figs 1a, 1b) (when these ions were initially absent) restores the sensitivity of oxygen uptake to thiol reagents. The inhibition of oxygen uptake observed with mersalyl in the absence of ADP or Mg^{2+} (or both) (Fig. 1b) is not a true respiratory control, since it does not respond to uncoupling by 2,4-dinitrophenol. This inhibition is reversed solely by 2-mercaptoethanol. It appears that this phenomenon does not involve the phosphate carrier, but some other parameter of mitochondrial metabolism, as the inhibition of succinate penetration [15], or of succinodehydrogenase [16, 17]. No such inhibition is observed with *N*-ethylmaleimide which supports to some extent the hypothesis of an inhibition at the level of succinate transport [15, 18]. The obvious parallelism between both sets of phenomena suggests analogous explanations: it might at

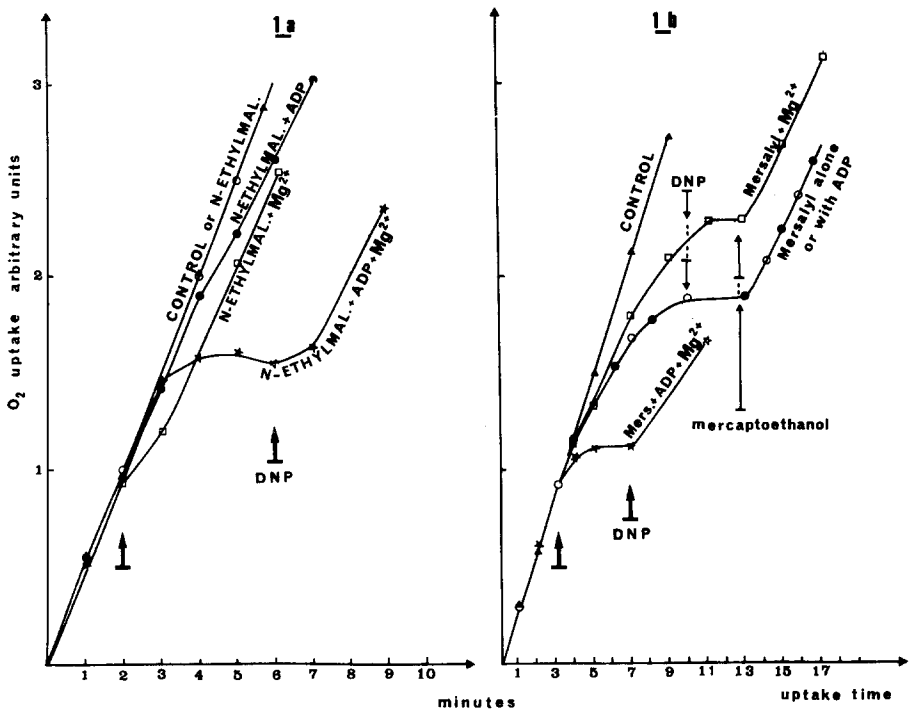

Fig. 1. Loss of *N*-ethylmaleimide and mersalyl effects on the sustained stimulation of oxygen uptake. Standard incubation medium: 200 mM sucrose, 35 mM L-histidine, 12 mM KH_2PO_4, 6 mM KCl, 13 mM succinate, 0.75 mg/ml bovine serum albumin delipidated type F. 2 μg/ml Rotenone, 5 μg/ml oligomycin, plus 1.2 mM ATP, 2.6 mM phosphocreatine, pH 7.0; final volume, 1.5 ml. No Mg^{2+} initially present. The ordinates (arbitrary units) show the increments of oxygen uptakes in the presence of calcium (O_2 uptake with Ca^{2+} present minus O_2 uptake with Ca^{2+} absent). (a) 1 mg/ml proteins. At the time indicated by the first arrow, *N*-ethylmaleimide (130 μM) was added with or without 2 mM ADP and 7 mM Mg^{2+}. ▲–▲, control; ○–○, *N*-ethylmaleimide; ●–●, *N*-ethylmaleimide + ADP; □–□, *N*-ethylmaleimide + Mg^{2+}; ⁎–⁎, *N*-ethylmaleimide + ADP + Mg^{2+}. (b) 0.5 mg/ml proteins. Additions at the first arrow: 20 μM mersalyl with or without 2 mM ADP and 7 mM Mg^{2+}. Other additions are indicated on the figures. ▲–▲, control; ○–○, mersalyl; □–□, mersalyl + Mg^{2+}; ●–●, mersalyl + ADP; ⁎–⁎, mersalyl + ADP + Mg^{2+}.

first sight be concluded that in the absence of ADP, calcium phosphate and oxygen uptake are uncoupled, which explains the insensitivity of the latter parameter to both ruthenium red and the thiol reagents. In both cases ADP plus Mg^{2+} re-instate coupling, whereas they are inactive when added separately. It has, however, been demonstrated [6] that under these conditions the mitochondrial calcium carrier remains sensitive to its inhibitor, ruthenium red; and the following experiments have been designed to investigate if, under the same circumstances, the phosphate carrier remains sensitive to thiol reagents.

Study of the phosphate/[^{32}P]phosphate exchange. The action of thiol reagents on this phenomenon

Fig. 2a shows that in the absence of ADP (7 mM Mg^{2+}) calcium phosphate accumulation stops 2 min after the addition of Ca^{2+}. If [^{32}P]orthophosphate is then

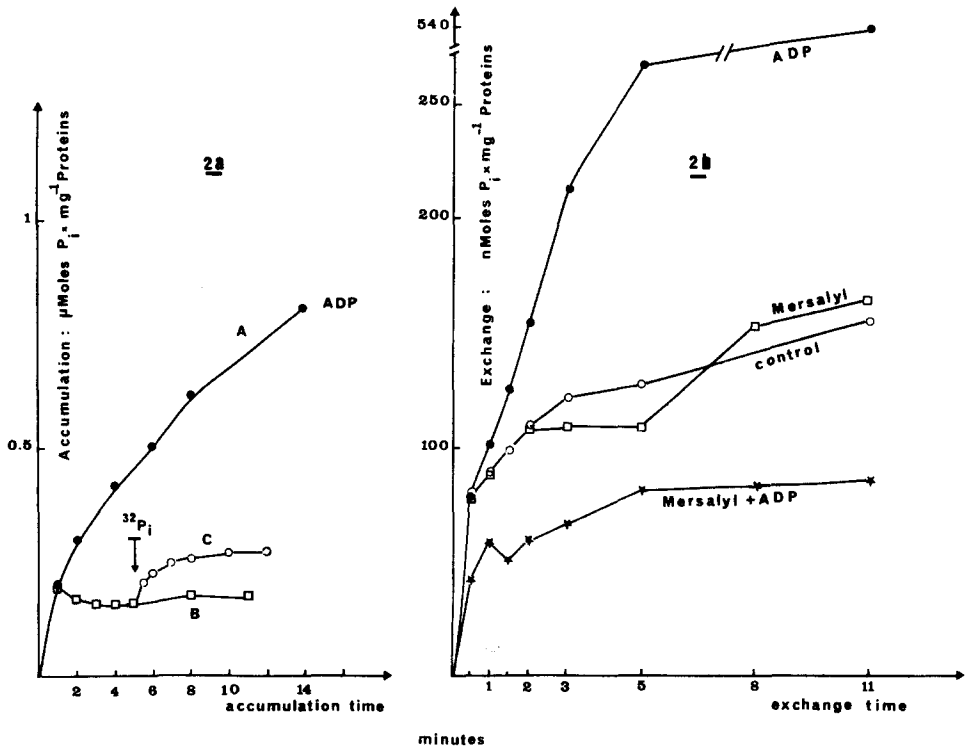

Fig. 2. Study of phosphate/phosphate exchange. Standard incubation medium, plus 1.2 mM ATP, 7 mM $MgCl_2$, 1 mg/ml proteins. Final volume 4.3 ml, pH 7.0, 20 °C. (a) Sarcosomes added 3 min prior to the start of the experiment. $CaCl_2$ (2.2 mM) added at 0 min. (A) ●–●, 2.2 mM ADP, 4.5 μCi [^{32}P]phosphate initially present; (B) □–□, no ADP, 2.6 mM phosphocreatine, [^{32}P]-phosphate initially present; (C) ○–○, no ADP, 2.6 mM phosphocreatine; 4.5 μCi [^{32}P]phosphate added at 5 min (arrow). (b) Exchange experiments as for Curve C of Fig. 2a, plotted on an extended scale. The 0 time for these curves corresponds to the addition of [^{32}P]phosphate (5 min on Fig. 2a). No ADP, 2.6 mM phosphocreatine. 500 μl of the incubate were pipetted off at the times indicated and filtered under negative pressure (see Materials and Methods). □–□, 46 μM mersalyl, added 2 min prior to 4.5 μCi ^{32}P; *–*, mersalyl+2.2 mM ADP, same time; ●–●, 2.2 mM ADP, same time.

added (5 min after calcium) a limited amount of the tracer is taken up (Figs 3a and 3b). This small accumulation proves to be insensitive to ruthenium red (1.6 μM) (results not shown) which inhibits calcium uptake [19, 20] under these conditions [6]; hence the small [^{32}P]phosphate uptake observed cannot be due to a residual accumulation of calcium phosphate but rather represents true phosphate exchange. In addition, the method used for separating the sarcosomes from the incubation medium includes extensive washing of the organelles by a large volume of buffer, which is supposed to minimize any contamination through binding by unspecific sites. Hence, it appears that any major participation of non-accumulated phosphate in the exchange phenomenon may be dismissed.

Fig. 2b shows that mersalyl added 2 min prior to [^{32}P]phosphate does not modify the exchange; an inhibition occurs, however, when ADP is added to the me-

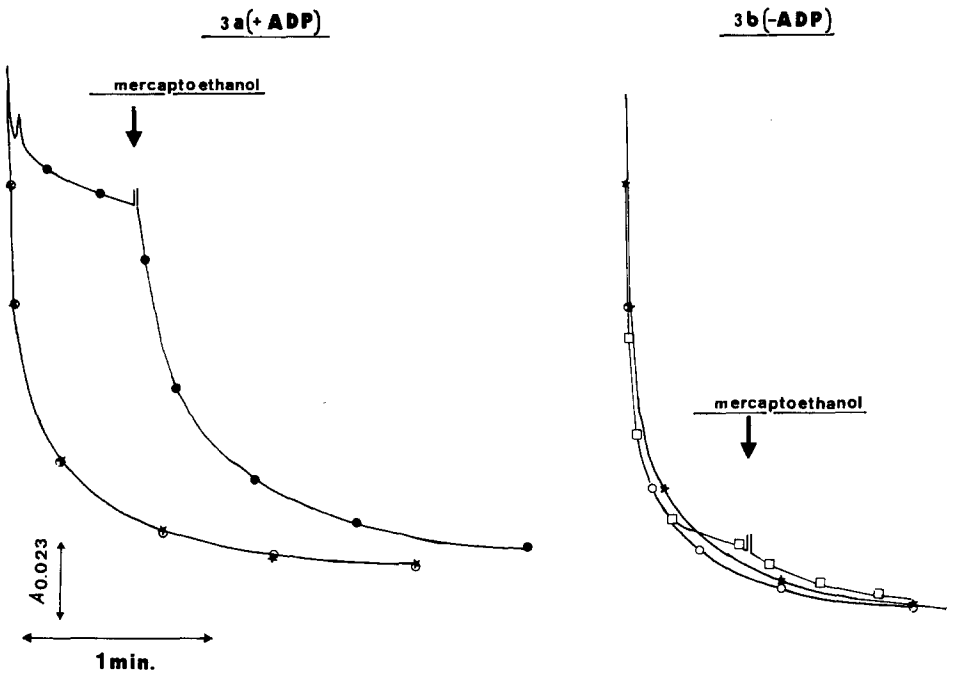

Fig. 3. The swelling of heart mitochondria in 100 mM ammonium phosphate. Standard incubation medium; no $MgCl_2$, 1.2 mM ATP, 1 mg/ml proteins, pH 7.0, 20 °C, final volume 4.3 ml. At the time indicated 200 μl of the incubate are pipetted off with an Eppendorf micropipette and added to 1.5 ml 100 mM ammonium phosphate, pH 7.0, in a 1 cm thick cuvette located in an Eppendorf Spectrophotometer. The variation of absorbance is recorded at 540 nm (recording speed 5 cm/min). (a) 2.2 mM ADP. Heart mitochondria added at 0 time; 2.2 mM $CaCl_2$ at 3 min. ○–○, control swelling at 2 min (no $CaCl_2$ added); ✶–✶, 1.2 μM ruthenium red at 7 min, swelling recorded immediately; ●–●, 1.2 μM ruthenium red at 7 min, 70 nmoles mersalyl/mg protein at 8 min. The swelling is recorded at 9 min. At 9 min 45 s 2-mercaptoethanol (5 mM) was added to the cuvette. (b) No ADP; 2.6 mM phosphocreatine. Heart mitochondria added at 0 time, $CaCl_2$ (2.2 mM) at 3 min. ○–○, control swelling at 2 min (no $CaCl_2$ added); ✶–✶, 1.2 μM ruthenium red at 6 min, swelling recorded immediately; □–□, 1.2 μM ruthenium red at 6 min, 70 nmoles mersalyl/mg protein at 7 min, swelling is recorded at 8 min.

dium simultaneously with the thiol reagents. Identical results (not shown) are observed with *N*-ethylmaleimide. In both cases ADP restores alone calcium phosphate accumulation, as previously shown [6].

It seems, however, difficult to postulate a loss of sensitivity of the phosphate carrier to mersalyl and *N*-ethylmaleimide solely on the basis of a sustained phosphate exchange in the presence of these inhibitors: it has been demonstrated that the mitochondrial dicarboxylic acid carrier [21] may promote such an exchange [18]. *N*-Butylmalonate, the inhibitor of this carrier [22] proves to be deleterious to the inner mitochondrial membrane under these very conditions (experiments not shown in the present manuscript). Hence, it seemed necessary to analyze the sensitivity of the phosphate carrier to its inhibitors under conditions where an unidirectional flow of phosphate precludes any participation of the dicarboxylic acid carrier in this phenomenon.

Study of sarcosomal swelling in 0.1 M ammonium phosphate

Fig. 3b demonstrates that after a 3 min accumulation of calcium phosphate (ADP and Mg^{2+} absent), swelling is nc longer sensitive to the thiol reagents, whereas in the presence of ADP added prior to the addition of Ca^{2+} swelling proves to be sensitive to these reagents (Fig. 3a). In Fig. 4a this phenomenon is studied under various conditions when ADP or magnesium are added separately to the heart mitochondria incubate after the uptake of a limited amount of calcium phosphate: the swelling phenomenon does not recover its sensitivity to mersalyl. Conversely, if both Mg^{2+} and ADP are added simultaneously to the incubate, the swelling sensitivity of heart mitochondria to thiol reagents is recovered. In addition, it must be emphasized that in Figs 3a and 3b control swelling experiments have been performed both with heart mitochondria which have been incubated in the absence of Ca^{2+} and with heart

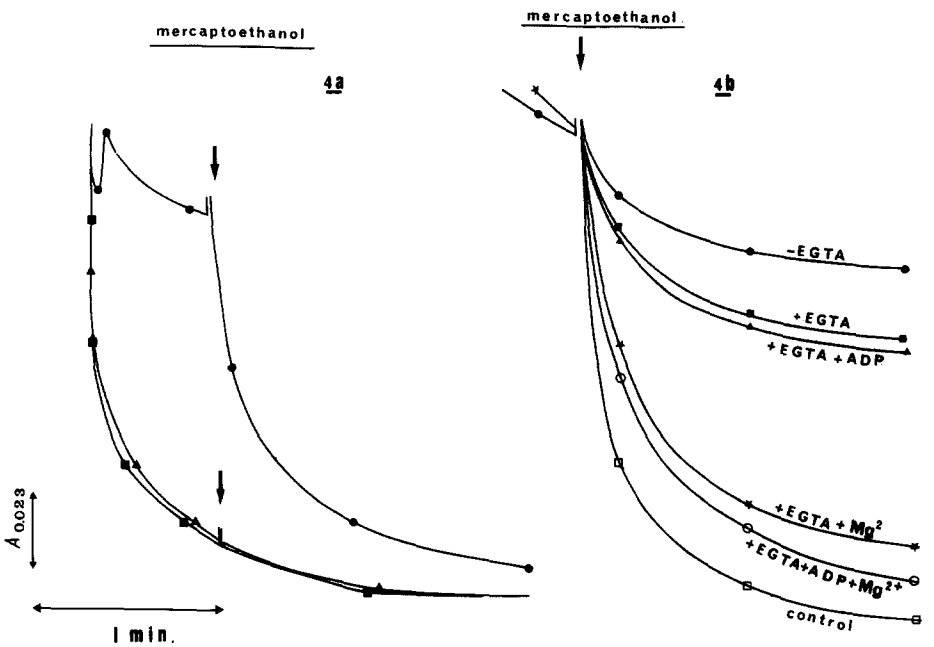

Fig. 4. The action of ADP+Mg^{2+} and EGTA+Mg^{2+} on the recovery of the mersalyl effect on swelling. Conditions as in Fig. 3. (a) No ADP, no Mg^{2+}; 2.6 mM phosphocreatine, 2.2 mM $CaCl_2$ added at 3 min. ■–■, 1.2 μM ruthenium red+2.2 mM ADP at 6 min; 70 nmoles mersalyl/mg protein at 7 min; ▲–▲, 1.2 μM ruthenium red+12 mM $MgCl_2$ at 6 min; 70 nmoles mersalyl/mg protein at 7 min; ●–●, 1.2 μM ruthenium red+2.2 mM ADP+12 mM $MgCl_2$ at 6 min; 70 nmoles mersalyl/mg protein at 7 min. (b) Heart mitochondria added to the medium at 0 min; 2.2 mM $CaCl_2$ added at 3 min; EGTA (4 mM) added at 6 min; 70 nmoles mersalyl/mg protein added at 8 min. $MgCl_2$ (10 mM) or/and 2.2 mM ADP (when added) at 8 min, simultaneously with mersalyl. ●–●, no EGTA; ■–■, EGTA present; ▲–▲, EGTA present, ADP as indicated; ○–○, EGTA present, ADP and Mg^{2+} as indicated; ⁎–⁎, EGTA present, Mg^{2+} as indicated; □–□, control swelling; obtained with 2-mercaptoethanol, after maximal inhibition of the phosphate carrier by mersalyl added simultaneously with Ca^{2+} at 3 min. The swellings recorded on this figure are the swellings recovered after the addition of 2-mercaptoethanol to heart mitochondria previously treated with mersalyl. Hence the extent of the swelling recorded is proportional to the inhibitory effect of mersalyl on the phosphate carrier.

mitochondria which have accumulated a limited amount of calcium phosphate in the absence of ADP. No significant differences have been found between these controls, which demonstrates that the uptake of calcium phosphate does not, by itself, modify the kinetics of the swelling phenomenon.

Action of EGTA

4 mM EGTA [23] added 3 min after Ca^{2+} and 2 min prior to mersalyl (Fig. 4b) proves inactive in restoring the effect of this inhibitor. As expected, ADP plus Mg^{2+} completely reverse the loss of sensitivity to mersalyl. However, Mg^{2+} alone is active in the presence of EGTA, which is not the case for ADP. Hence the requirement of the mersalyl effect for ADP, Mg^{2+} and EGTA (or combinations of these agents) is identical to the requirement reported for the coupling between calcium and oxygen uptakes [6].

DISCUSSION

Reversible modification of the mitochondrial phosphate carrier in the absence of ADP and Mg^{2+}

The results obtained when analyzing mitochondrial swelling support the data of the phosphate/phosphate exchange experiments and their interpretation: the decreased action of mersalyl and *N*-ethylmaleimide on this exchange may indeed be attributed to a decreased capacity of inhibiting phosphate transport, rather than to an increase of phosphate exchange mediated through the dicarboxylic acid carrier, which in turn could mask the actual inhibition of the phosphate carrier.

However an increase in the passive permeability of the mitochondrial membrane to phosphate cannot be completely dismissed. This permeability would have to be rather selective, since under the same conditions [6], Ca^{2+} transport and exchange remain sensitive to their inhibitor, ruthenium red [20, 21]. Also a passive phenomenon should result in an outflow of accumulated phosphate from the heart mitochondria, which as has been shown (Fig. 2a, Curve B), is not the case; any phosphate outflow could hardly escape attention since the small phosphate exchange mediated through the carrier (Fig. 2a, Curve C) is easily demonstrated under the same experimental conditions.

Correlation with the uncoupling of oxygen uptake

The requirements of mersalyl sensitivity for ADP and Mg^{2+} are strikingly similar to the conditions which have been established in a previous work [6] for the coupling between sustained calcium phosphate accumulation and oxygen uptake.

Hence the correlation between this loss of sensitivity of the phosphate carrier to its inhibitors, the uncoupling of oxygen uptake and the discontinuance of calcium phosphate accumulation seems well established. It remains to be explained how this parameter could correlate with the energy transducing role of ADP and Mg^{2+}, which has been postulated previously [6]. With reference to previous findings published by E. Kun and co-workers [24–27], several hypotheses are to be envisioned: one consists in a possible conformational change of the entire inner mitochondrial membrane, which may mask the sensitive thiol groups of the phosphate carrier to the access of its inhibitors. According to a more sophisticated hypothesis, one would

assume a direct interaction between the phosphate carrier and the Mg^{2+} binding site: recent data of Guérin and Guérin [28] suggest that inhibition of the phosphate carrier through mersalyl impairs the phosphorylation of ADP by intramitochondrial inorganic phosphate which leads these authors to postulate a direct role for this carrier in energy transduction and ATP synthesis. In this case, an uncoupling through displacement of Mg^{2+} by Ca^{2+} might result in a conformational change of the phosphate carrier itself and give way to a loss of the mersalyl effect.

In both cases it would appear that the action of Mg^{2+}+ADP or of Mg^{2+}+ EGTA in restoring the action of mersalyl on the phosphate carrier, and in re-instating respiratory control, even when energy transfer is inhibited, implies a structural change in one or several elements of the energy transducing system as such.

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