STUDIES ON THE MECHANISM OF ACTION OF UNCOUPLERS ON THE TRANSPORT OF INORGANIC PHOSPHATE IN RAT LIVER MITOCHONDRIA

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1. Introduction

Evidence has recently been obtained [1] that the translocation across the membrane of rat liver mitichondria of inorganic phosphate (P_i), but not that of a dicarboxylate anion (contrast refs. [2, 3]) can be directly coupled to an OH^- counterflux (or H^+ symport [3]). The occurrence in rat liver mitochondria of an exchange-diffusion reaction between P_i and dicarboxylate [2] has also been directly demonstrated [1]. Both translocation reactions of P_i are inhibited by mersalyl [4, 5], but only the P_i/dicarboxylate antiport is inhibited by butylmalonate [5], thus suggesting that they are mediated by separate translocators [2, 6]. It has been found that uncouplers of oxidative phosphorylation promote the efflux of P_i from rat liver mitochondria, but not directly that of dicarboxylate or tricarboxylate [7, 8]. In this paper, further aspects of the effect of uncouplers on the transport of P_i in rat liver mitochondria have been studied.

2. Experimental

Mitochondria were incubated with $^{32}P_i$ and ^{14}C-sucrose in the presence of rotenone, antimycin and oligomycin. $^{32}P_i$-loaded mitochondria were layered on the top of a second incubation layer and centrifuged through this into $HClO_4$. A discontinuous density gradient increasing towards the bottom of the centrifuge tube was made by the addition of dextran to the second layer. This was separated from the $HClO_4$, at the bottom of the tube, by a layer of silicone oil. The exposure time of mitochondria to the second incubation layer (0.35 ml) was estimated to be about 15 sec by measuring the oxidation of β-hydroxybutyrate to acetoacetate. The rate of this reaction was determined in separate controls [9]. The amount of $^{32}P_i$ remaining in the matrix space was calculated from the radioactivity in the $HClO_4$ extract. This was corrected for the $^{32}P_i$ present in the sucrose-permeable space *plus* adherent medium.

3. Results

The $^{32}P_i$-loaded mitochondria were transferred to a second layer free of P_i. During the exposure to the second layer P_i moved out of the mitochondria. The presence of 1 μM FCCP* in this layer caused at pH 6.5 a marked stimulation of the efflux of P_i. As the pH was increased, the efflux of P_i became progressively higher [1] and the *extra* efflux of P_i induced by FCCP progressively smaller. At pH 8.0 or 8.5, the efflux of P_i was practically the same as in the absence of FCCP. Butylmalonate did not inhibit the efflux of P_i either in the absence or presence of FCCP. It should be noted that at pH 6.5, 1 nmole of FCCP promoted the efflux of about 30 nmoles of P_i. This ratio varied with the pH of the medium (fig. 1).

In other experiments the effects of FCCP,

* Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

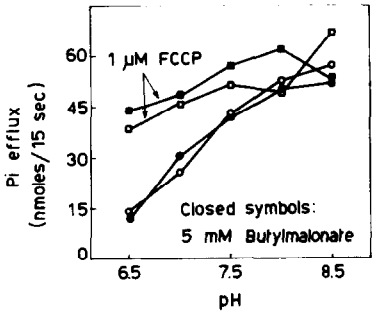

Fig. 1. Effect of pH and FCCP on the efflux of P_i from rat liver mitochondria. Mitochondria (6.9 mg protein) were preincubated for 1 min in a reaction mixture containing 200 mM sucrose, 20 mM tris HCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 μg rotenone, 0.5 μg antimycin and 10 μg oligomycin. 2 mM $^{32}P_i$ (10^6 counts/min) was added. Final volume, 1 ml; final pH, 7.5. The uptake of $^{32}P_i$ was complete in 1 min with the accumulation of 110 nmoles. 2 min after the addition of $^{32}P_i$, mitochondria were centrifuged through a second incubation layer containing the same components as the preincubation mixture (except P_i). The pH of this layer was (in separate incubations) adjusted to the values shown in the figure. Where indicated, 5 mM butylmalonate was added in layer II.

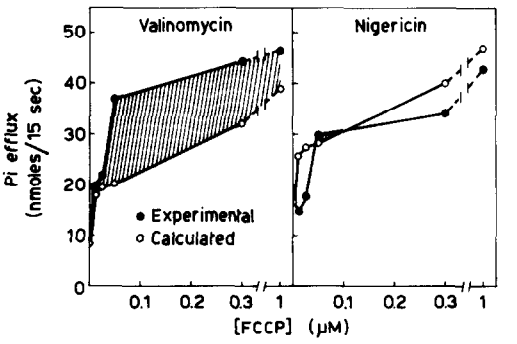

Fig. 2. Effect of FCCP, valinomycin and nigericin on the efflux of P_i from rat liver mitochondria. Mitochondria (6.7 mg protein) loaded with $^{32}P_i$ as described in the legend to fig. 1, were centrifuged through a second incubation layer. This layer (pH 6.5) contained (where indicated) FCCP, 0.2 μg/ml valinomycin and 0.2 μg/ml nigericin. The values in fig. 2 represent the extra efflux of P_i, namely the difference between the amount of $^{32}P_i$ left in the matrix in the absence of any addition *minus* that left in the matrix in the presence of the various additions. The calculated values for P_i efflux represents the sum of the efflux in the presence of FCCP alone *plus* that in the presence of valinomycin alone or of nigericin alone. Note that under conditions of maximal efflux about 30% of the $^{32}P_i$ taken up by mitochondria was still left in the matrix.

nigericin and valinomycin on the efflux of P_i from mitochondria were examined. In fig. 2, the extra efflux of P_i induced by the combined addition of FCCP and valinomycin or of FCCP and nigericin is compared with the sum of the extra efflux of P_i observed in the presence of FCCP alone and that caused by valinomycin and nigericin alone, respectively. It can be seen that the extra efflux of P_i induced by the combination of FCCP and valinomycin is greater than the sum of the extra effluxes caused by the two substances added separately. This was not the case with FCCP and nigericin. It could be calculated from this and similar experiments that the concentration of FCCP required for 50% stimulation of the efflux of P_i was about 0.3 μM in the absence of valinomycin and 0.03 μM in the presence of valinomycin. The latter value is practically equal to that found in titrating the stimulation of respiration by FCCP [10, 11]. The experiment of fig. 2 was carried out at pH 6.5 (second layer). The data of table 1 show that also at higher pH (7.0 and 7.5) the stimulation of the P_i efflux by FCCP and that by valinomycin potentiated each other. The experiment of table 1 shows also that under all the conditions tested the efflux of P_i was severely inhibited by mersalyl.

4. Discussion

P_i can move across the membrane of rat liver mitochondria either by exchange-diffusion with OH^- (or by H^+ symport; see [1] and fig. 1) or by exchange-diffusion with dicarboxylate [1, 2]. Uncouplers promote the efflux of P_i from mitochondria, in the absence of a counteranion, but inhibit the P_i/dicarboxylate antiport [7, 8]. The uncoupler-induced efflux of P_i is inhibited by mersalyl [1] (table 1) but not by butylmalonate [1] (fig. 1). These findings indicate that the uncoupler-induced efflux of P_i is mediated by the P_i/OH^- translocator. FCCP, like other uncouplers, conducts protons across the mitochondrial membrane [12] and other membranes [2,12,13]. The fact that uncouplers stimulate the efflux of P_i, but not that of dicarboxylate [7,8,14], that is only that of a

Table 1
Stimulation of P_i efflux from mitochondria by FCCP and valinomycin: effect of pH and mersalyl. Experimental procedure was as described in the legend to figs. 1 and 2. Amount of mitochondria used, 7 mg protein. Mersalyl (25 nmoles/mg protein) was added in the preincubation mixture 30 sec after 2 mM P_i; in this case mitochondria were centrifuged through the second layer 2 min after the addition of mersalyl.

Additons	P_i efflux (nmoles)			
	at pH		+ mersalyl at pH	
	7	7.5	7	7.5
FCCP, 0.3 μM	12	10	2	2
Valinomycin, 0.2 μg/ml	6	3	8	0
FCCP + valinomycin	29	31	10	9

substrate whose translocation is protoncoupled, would suggest that the proton-conducting properties of the uncoupler plays a role in this stimulation [3]. The efflux of P_i in exchange diffusion with OH^- causes a transmembrane ΔpH. The uncoupler by conducting H^+ inward can dissipate, at least in part, the ΔpH and stimulate in this way the efflux of P_i. The results presented here are consistent with this mechanism. The ratio between the moles of FCCP added and the moles of P_i driven out of the mitochondria by the uncoupler is, at pH 6.5–7.5, higher than one. The inward conduction of H^+ by the uncoupler [12, 15] and the resulting efflux of P_i would be limited by the establishment of a transmembrane potential. This potential (positive inside) would be discharged by the addition of valinomycin, which could promote an electrogenic efflux of K^+ [12, 13]. According to this mechanism, the stimulation of the efflux of P_i by FCCP and valinomycin would potentiate each other. This was, indeed, found (fig. 2).

Nigericin mediates a K^+-H^+ exchange-diffusion [13]. In a K^+-free medium it will therefore cause an influx of H^+ in exchange for mitochondrial K^+. Since this exchange is not electrogenic, nigericin should not potentiate the extra efflux of P_i induced by FCCP. This was, indeed, the experimental result (see fig. 2).

Van Dam and Kraaijenhof [16] have proposed that uncoupler anions are transported into the mitochondria via the substrate anion translocators. On the basis of their proposal, it is difficult to see how uncoupler could induce the efflux of P_i except by an exchange-diffusion between P_i and the uncoupler anion. However, this does not easily explain the finding that whereas the stimulations by valinomycin and by FCCP of P_i efflux potentiate each other, those by nigericin and FCCP do not.

Acknowledgements

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