THE INHIBITOR-SENSITIVITY AND PATHWAYS OF P_i UPTAKE DURING CALCIUM AND STRONTIUM ACCUMULATION IN LIVER MITOCHONDRIA

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Received 3 September 1979

1. Introduction

P_i is transported by two different mechanisms in liver mitochondria [1–4]:

1. Via the P_i carrier together with protons (which process may in reality be a phosphate-hydroxyl exchange). This process is sensitive to NEM or to low concentrations of mercurials.
2. Via the dicarboxylate carrier in exchange for some dicarboxylate anions (malate, succinate or malonate), this process being inhibited by 2-butylmalonate and similar substituted dicarboxylates, as well as by high concentrations of mercurials.

It has been shown that inhibitors of the P_i carrier prevented largely the stimulation of respiration caused by addition of calcium (or strontium) plus P_i [5,6]. The small stimulation of respiration observed in the presence of P_i carrier inhibitors was proportional to, and due to the intramitochondrial P_i content [7]. It was inferred from these that the transport of P_i linked to calcium (or strontium) uptake proceeds via the P_i carrier in liver mitochondria.

It was reported recently that even if the P_i carrier was completely inhibited by NEM or mersalyl, respiring mitochondria took up some P_i if either calcium or strontium was added [8–10]. On this experimental basis a special transport system for calcium plus P_i was postulated (the lanthanide-sensitive calcium phosphate porter). An alternative possibility of P_i uptake, the participation of the dicarboxylate carrier in the uptake of P_i was however not investigated in [8–10].

Abbreviations: NEM, *N*-ethyl maleimide; BM, 2-butylmalonate; TMPD, *N,N,N',N'*-tetramethylparaphenylene diamine

Here we report that the mersalyl- and NEM-resistant uptake of P_i which follows the addition of either calcium or strontium to respiring liver mitochondria is completely absent if both the P_i carrier and the dicarboxylate carrier are simultaneously inhibited. The dicarboxylate carrier is thus responsible for the P_i uptake found in NEM- or mersalyl-treated mitochondria and there is no need to postulate an additional transport system.

2. Methods

Rat liver mitochondria were isolated as in [7]. For measurement of P_i and calcium uptake the mitochondria were incubated in open vessels at 25°C under constant magnetic stirring under 2 different conditions.

(1) Mitochondria were incubated in the presence of either succinate or ascorbate + TMPD and $Sr(NO_3)_2$ or $CaCl_2$ was added to the already respiring mitochondria (state 4–3 transition).
(2) Mitochondria were incubated in the presence of $CaCl_2$ and ascorbate but because of the lack of TMPD they did not respire: the uptake of calcium and P_i was initiated by the subsequent addition of TMPD (state 2–3 transition).

P_i and calcium uptake was measured by analysis of centrifuged mitochondria [7]. Aliquots were removed from the incubation mixtures and layered on top of 6 ml ice-cold 0.5 M sucrose, containing 5 mM Tris–HCl and 60 μM mersalyl. The tube was immediately centrifuged at 18 000 × *g* for 90 s in a Janetzki K24 refrigerated centrifuge. The supernatant was discarded, the pellet rinsed with 0.25 M sucrose and extracted at

0°C with 2.5 ml 1 N HCl. P_i determination in the extract was performed according to [11] and calcium was measured by atomic absorbtion spectrometry.

Intramitochondrial pH was determined by the [^{14}C]acetate distribution using the flow dialysis technique as in [12].

3. Results and discussion

Table 1 represents an experiment in which $Sr(NO_3)_2$ was added to mitochondria already respiring (state 4–3 transition). In these types of experiments the endogenous Ca^{2+} that had leaked out during the storage period of mitochondria was already taken up during the 2 min preincubation period, and the changes in the mitochondrial P_i content were due to Sr^{2+} addition. In the absence of any added inhibitor the addition of Sr^{2+} was followed by a proportional increase of the P_i content. Mersalyl (31.2 μM = 12 nmol/mg protein) inhibited strongly but not completely the P_i uptake. Thus, in agreement with the data of [8,9], significant P_i uptake was found even if sufficient mersalyl was present to inhibit the P_i carrier completely [4,13]. Also in the presence of mersalyl, larger addition of Sr^{2+} was followed by larger P_i uptake. 2-butylmalonate alone had no measurable effect on P_i uptake. The combination of 2-butylmalonate and mersalyl, however, abolished almost completely the P_i uptake.

In the experiment of table 2, the mitochondria were preincubated in the presence of Ca^{2+}, P_i and ascorbate, but there was no respiration because of lack of a suitable electron mediator (TMPD). The addition of TMPD started respiration and the uptake of both Ca^{2+} (endogenous plus added) and P_i (state 2–3 transition). Either mersalyl or NEM inhibited P_i uptake by about 50%. The combination of 2-butylmalonate with either mersalyl or NEM eliminated P_i uptake virtually completely. As in the experiments with Sr^{2+}, 2-butylmalonate alone had no effect on P_i uptake at all.

The calcium accumulation itself was not much influenced by mersalyl or mersalyl plus 2-butylmalonate or NEM. This fact is in accord with earlier observations in which the complete absence of P_i (and other proton-carrying anions) Ca^{2+} uptake was not seriously affected [14,15]. It was however consistently found that the combination of NEM with 2-butylmalonate decreased the extent of P_i uptake.

The P_i uptake in the presence of mersalyl was specific for the divalent cations Ca^{2+} and Sr^{2+}. When P_i uptake was linked to K^+ transport facilitated by valinomycin, mersalyl alone inhibited always com-

Table 1

P_i uptake (nmol/mg protein) of respiring mitochondria after addition of $Sr(NO_3)_2$

Inhibitor added	$Sr(NO_3)_2$ added (nmol/mg protein)	
	25	50
–	30.8	63.0
Mersalyl	10.0	26.7
2-Butylmalonate	34.9	65.2
2-Butylmalonate + mersalyl	1.1	2.1

Mitochondria (6 mg protein) were incubated at 25°C with continuous magnetic stirring in the presence of 247 mM sucrose, 5 mM KCl, 3 mM $MgCl_2$, 0.5 mM Tris–HCl, 0.3 mM Tris–phosphate, 1 μM rotenone, 6 μg oligomycin, 3.2 mM Na-ascorbate 70 μM TMPD and the inhibitors indicated, at pH 7.0 in 2.3 ml final vol. The pH of the suspension was recorded and an aliquot was taken for P_i determination. Then 25 or 50 nmol/mg protein of $Sr(NO_3)_2$ was added and when the proton ejection ceased an aliquot was again taken for a second P_i determination. The concentration of the inhibitor was: 2-butyl malonate 8.7 mM; mersalyl 31.2 μM. The initial P_i content of the mitochondria before Sr^{2+} addition was in nmol/mg protein: 40.9 with no added inhibitor; 36.1 with mersalyl; 35.4 with 2-butylmalonate; 24.8 with mersalyl + 2-butylmalonate

Table 2
P_i and Ca^{2+} content and uptake of centrifuged mitochondria following the addition of TMPD

Conditions: Inhibitor present at the time of TMPD addition	P_i (nmol/mg protein)		Ca^{2+} (ng atom/mg protein)	
	Content	Uptake	Content	Uptake
No inhibitor, no TMPD	28.3	–	20.1	–
No inhibitor	78.0	49.7	76.0	55.9
BM	77.4	49.1	82.5	62.4
Mersalyl	51.1	22.8	76.5	56.4
Mersalyl, BM	32.2	3.9	69.7	49.6
NEM	48.8	20.5	68.6	48.5
NEM, BM	28.6	0.3	51.2	31.1

Mitochondria (6 mg protein) were incubated at 25°C with continuous magnetic stirring, in the presence of 247 mM sucrose, 5 mM KCl, 3 mM $MgCl_2$, 0.5 mM Tris–HCl, 0.3 mM Tris–phosphate, 1 μM rotenone, 6 μg oligomycin and 3.2 mM Na-ascorbate at pH 7.0 in 2.3 ml final vol. Additions were made at 30 S intervals. 66 nmol $CaCl_2$/mg protein was added after the inhibitor indicated and Ca^{2+} uptake was started by adding 70 μM TMPD to start respiration (no TMPD was added to the control in the first line of the experiment). When the ion uptake was finished (as controlled by pH recording) the mixtures were supplemented with those inhibitors not yet added and aliquots of the suspension were centrifuged for P_i and Ca^{2+} determinations. The concentration of inhibitor was: 31.2 μM mersalyl; 8.7 mM 2-butyl malonate; 320 μM NEM, this last acting for 60 s.

pletely the P_i movement. The mechanism for this different behavior of the transport processes is not yet clear: two factors may be responsible:

1. The true intramitochondrial free P_i concentration and activity is probably low after Ca^{2+} or Sr^{2+} accumulation as most of the intramitochondrial P_i forms a precipitate with the divalent cations. This creates favorable conditions for the exchange of intramitochondrial dicarboxylates with the extramitochondrial P_i on the dicarboxylate carrier and the P_i content of the matrix increases. In contrast with this, when K^+ is accumulated in the presence of valinomycin, the free intramitochondrial P_i content and activity is high and the high P_i concentration inhibits the dicarboxylate exchange competitively [16].
2. In the absence of P_i added (state 6), the intramitochondrial pH value was always somewhat higher after Ca^{2+} accumulation than after valinomycin-facilitated K^+ accumulation. In one typical experiment the values were pH 8.8 and 8.3, respectively. The higher intramitochondrial alkalinity after Ca^{2+} accumulation might be connected with the mersalyl-insensitive P_i uptake.

4. Conclusion

The mersalyl- and NEM-resistant P_i uptake coupled to Ca^{2+} and Sr^{2+} transport is completely abolished by 2-butylmalonate and very probably is catalyzed by the dicarboxylate carrier of the liver mitochondria. It is therefore not necessary to postulate a separate calcium-phosphate porter.

Acknowledgements

The work is part of the research project 'Carrier Mechanisms of Biological Membranes' and was supported by grant no. 1-01-0306-01-1/F of the Hungarian Ministry of Health. The authors thank Mrs Julianna Szilassy for expert and devoted technical assistance.

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