Factors that Influence Phosphoenolpyruvate-Induced Calcium Efflux from Rat Liver Mitochondria

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Received November 12, 1973

Summary: Stimulation by phosphoenolpyruvate (PEP) of Ca^{2+} efflux from pre-loaded rat liver mitochondria is associated with an enhanced respiratory rate and is dependent on the P_i concentration of the extramitochondrial medium. PEP-induced Ca^{2+} efflux is inhibited by concentrations of NEM, mersalyl, and DTNB that also inhibit the respiration-linked transport of P_i as well as by bongkrekic acid at concentrations that also inhibits the adenine nucleotide translocase. Since PEP can gain access to the mitochondrial matrix by means of a carrier mediated exchange with intramitochondrial adenine nucleotides, we conclude that the ability of PEP to induce Ca^{2+} efflux could be associated with adenine nucleotide efflux as well as P_i influx and that the stability of the accumulated Ca^{2+} is likely to be controlled by the intramitochondrial ATP/P_i ratio.

Introduction: The respiration-linked uptake and maintenance of accumulated Ca^{2+} by isolated mitochondria are markedly influenced by the initial concentrations of Ca^{2+} and phosphate (P_i) (1-6), uncoupling agents (4,5,7) as well as added ATP and ADP (6,8) and agents that influence the intramitochondrial levels of adenine nucleotides such as oligomycin (6), and atractyloside (5,7). Recent studies of Chudapongse and Haugaard (9) demonstrate that phosphoenolpyruvate (PEP) is yet another factor that can influence the movement of Ca^{2+} into mitochondria. PEP not only inhibits the accumulation of Ca^{2+} in presence of P_i but also stimulates the release of Ca^{2+} from previously loaded mitochondria. The inhibitory effect of PEP on Ca^{2+} accumulation is prevented by addition of atractyloside or ATP. This together with the observations of Shug and Shrago (10) that PEP can be accumulated by isolated rat liver mitochondria by an atractyloside-sensitive exchange with intramitochondrial ATP suggest that the decrease of intramitochondrial adenine nucleotides could bring about the Ca^{2+} efflux. Inasmuch as Ca^{2+} uptake and efflux by mitochondria have been implicated in the control of cellular Ca^{2+} transport (11) as

well as carbohydrate metabolism (12-15) a more detailed investigation of this PEP effect was undertaken.

Materials and Methods: Liver mitochondria were prepared from adult male Sprague-Dawley rats as described previously (16). Measurement of mitochondrial respiration was performed polarographically at 28° in an oxygraph with a Clark electrode (16). Mitochondria (4 mg of protein) were added to 1.25 ml (final volume) of medium saturated with air at 28° and containing 80 mM KCl and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4). β-Hydroxybutyrate (10 mM) served as the respiratory substrate. Ca^{2+}-stimulated respiration was monitored by addition of 400 nmoles of $^{45}CaCl_2$. The uptake of $^{45}Ca^{2+}$ was determined by withdrawal of 0.3 ml aliquots at various time intervals and centrifuging the mitochondria in a Beckman 152 Microfuge and estimating the radioactivity of the clear supernatant in a Nuclear-Chicago, Mark I Liquid Scintillation counting system (6). The effect of PEP on Ca^{2+}-preloaded mitochondria was evaluated as follows: Mitochondria were exposed to substrate plus varying amounts of KH_2PO_4 for 1 minute at 28°. $^{45}CaCl_2$ was then added resulting in a typical state 3 stimulated respiration. When all the $^{45}Ca^{2+}$ has been accumulated, the respiratory rate returned to state 4 level. PEP was added one minute after state 4 had been reached. Inhibitors of phosphate and adenine nucleotide transport, when used, were added 30 seconds prior to PEP addition. The concentration of K-phosphate (P_i), phosphoenolpyruvate (PEP), ADP, ATP, bongkrekic acid, N-ethylmaleimide (NEM), mersalyl and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) are indicated in legends. Protein was estimated by the biuret method of Layne (17).

Chemicals: ATP, ADP, DL-β-hydroxybutyrate (sodium salt), N-ethylmaleimide (NEM), mersalyl, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. Phosphoenolpyruvate was obtained from Calbiochem and $^{45}Ca^{2+}$ was from New England Nuclear. We are indebted to Prof. W. Berends, Technological University of Delft, The Netherlands for the gift of bongkrekic acid.

Table I

Effect of PEP on Ca^{2+} Retention by Rat Liver Mitochondria

PEP added to Medium (mM)	$^{45}Ca^{2+}$ Retained in Mitochondria (nmoles/mg protein)	
	2 min	4 min
0	96	97
0.6	96	85
1.2	95	26
2.4	85	23

Mitochondria, 4 mg of protein; $^{45}CaCl_2$, 400 nmoles; and initial KH_2PO_4, 0.8 mM. PEP at the indicated concentrations was added 1 min after the respiration returned to the initial resting state (State 4). $^{45}Ca^{2+}$ retained in mitochondria was measured at 2 and 4 minutes after PEP addition. Methods are detailed in text.

Results: The data presented in Table I demonstrate that the addition of 0.6 mM PEP to calcium and phosphate-loaded mitochondria causes release to the external medium of approximately 10% of the previously accumulated Ca^{2+} within 4 minutes and that higher concentrations of PEP caused nearly complete efflux. Inorganic phosphate is also released and the molar Ca/P ratio is 1.3 - 1.7. These results essentially confirm those of Chudapongse and Haugaard (9).

Previous studies of Drahota et al (7) demonstrated that higher concentrations of phosphate alone would induce efflux of accumulated Ca^{2+}. The data presented in Table II - Exp. 1 - indicate that the addition of up to 6.0 mM PEP to mitochondria that had accumulated Ca^{2+} in the absence of added phosphate did not induce Ca^{2+} efflux during the 4 minute reaction period. Exp. 2 shows the effect of PEP on the efflux of Ca^{2+} that had been previously accumulated in the presence of varying amounts of P_i. The data reveal that only when Ca^{2+} accumulation occurred in the presence of an initial concentration of 0.6 or 0.8 mM P_i did the subsequent addition of PEP induce Ca^{2+} efflux. For each of the systems employed in Exp. 2, the maximum amounts of Ca^{2+} and P_i accumulated prior to addition of PEP were 97 and 65 nmoles per mg protein respectively. Thus under these conditions Ca^{2+} efflux occurs only when the con-

Table II

The Phosphate-Dependent Effect of PEP in Inducing Ca^{2+} Efflux from Rat Liver Mitochondria

Exp.	Additions to medium (mM)		$^{45}Ca^{2+}$ retained in mitochondria (nmoles/mg protein)	
	Phosphate	PEP	2 min	4 min
1	0	1.2	96	97
	0	3.0	97	98
	0	6.0	97	97
2	0.2	1.2	97	97
	0.4	1.2	97	97
	0.6	1.2	96	29
	0.8	1.2	66	16

Incubation conditions were as described for Table I and Methods.

centration of P_i of the extramitochondrial phase at the time of PEP addition was 400 μM or higher.

The apparent dependence of Ca^{2+} efflux on the P_i concentration of the external medium suggests that P_i transport across the mitochondrial membrane could be involved in activation of Ca^{2+} efflux. The data presented in Table III demonstrate that mersalyl, NEM, and DTNB markedly suppress the efflux of Ca^{2+} in the presence of P_i and PEP. These three sulfhydryl reagents are known to inhibit the P_i^-/OH^- exchange carrier of the mitochondrial membrane (18-22). It would thus appear that P_i transport linked to mitochondrial respiration is required to demonstrate the PEP induced efflux of Ca^{2+}.

The data presented in Table III show that bongkrekic acid is also a potent inhibitor of the PEP induced Ca^{2+} efflux. This compound is a potent inhibitor of the adenine nucleotide translocase of the mitochondrial membrane (23-25). In view of these findings, it would seem reasonable to conclude that the uptake of P_i from the external medium as well as the efflux of intramitochondrial nucleotide are involved in the PEP induced Ca^{2+} efflux process.

Table III

Effect of Inhibitors of Mitochondrial Transport Systems for Phosphate and Adenine Nucleotides on PEP-induced Ca^{2+} Efflux from Rat Liver Mitochondria

Additions to Medium (mM)		$^{45}Ca^{2+}$ Retained in Mitochondria (nmoles/mg protein)	
		2 min	4 min
None		95	26
Mersalyl	(0.2 mM)	93	89
NEM	(0.3 mM)	96	96
DTNB	(0.61 mM)	99	99
Bongkrekic acid	(0.008 mM)	95	93

The incubation conditions were as described for Table I and Methods. PEP (1.2 mM) was added 30 seconds after addition of the inhibitors and 1 minute after the respiration returned to State 4.

The possible requirement of respiration for the PEP induced Ca^{2+} efflux which was suggested previously was investigated and the results are shown in Fig. 1. Ca^{2+} accumulation in the absence of added P_i was studied in Exp. A. PEP addition which under these conditions did not stimulate Ca^{2+} efflux (Table II) had only a minimal effect on respiration. P_i was included in the reaction system of Exp. B and in this case the addition of PEP (trace B-4) after a short lag period nearly doubled the respiratory rate over that observed when PEP was omitted (trace B-2). The addition of NEM plus PEP (trace B-1) or mersalyl plus PEP (trace B-3) produced inhibition of respiration as compared with that of PEP alone (trace B-4).

The data presented in Exp. C show that the addition of bongkrekic acid (trace C-1) at a concentration that will inhibit the PEP induced Ca^{2+} efflux will also inhibit the PEP-dependent respiration (trace C-2).

Discussion: The study described here confirms the results of Chudapongse and Haugaard (9) and provides some leads to the mechanism of the PEP induced Ca^{2+} efflux of preloaded rat liver mitochondria. PEP is a known constituent of isolated mitochondria (26) and presumably gains access by means of the atrac-

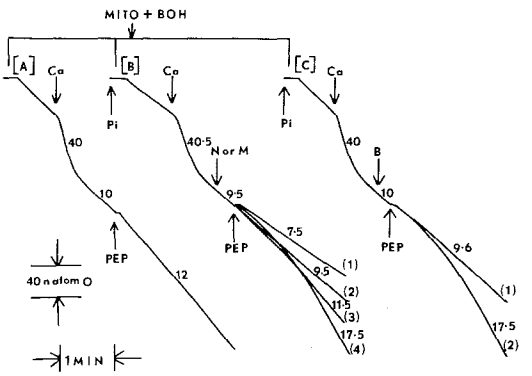

Fig. 1: PEP-induced Efflux of $^{45}Ca^{2+}$ from Rat Liver Mitochondria. The following additions were made at indicated times: Mitochondria 4 mg of protein; $^{45}CaCl_2$, 400 nmoles; K-phosphate, 0.8 mM; NEM (N), 0.3 mM; Mersalyl (M), 0.2 mM; and bongkrekic acid (B), 10 nmoles. The various numbers in traces indicate the rate of oxidation as ngatom O/min/mg protein. Experiment A-PEP, 6 mM and no phosphate present. Experiment B-trace B-4 represents effect due to PEP (1.2 mM) alone in presence of phosphate; traces B-1 and B-3 represent effect of NEM (N) and mersalyl (M) respectively on system represented by trace B-4; trace B-2 is obtained in the absence of PEP, NEM and mersalyl. Experiment C-trace C-2 represents effect due to PEP (1.2 mM) alone in presence of phosphate; trace C-1 represents effect of bongkrekic acid added to system represented by trace C-2. Methods are detailed in text.

tyloside- and bongkrekic acid-sensitive adenine nucleotide translocase as well as the tricarboxylate carrier systems (10,27-29). Chudapongse and Haugaard (9) showed that the PEP induced efflux of Ca^{2+} from preloaded mitochondria is inhibited by atractyloside and the present communication shows inhibition by bongkrekic acid. Since these compounds have no effect on the tricarboxylate carrier, it would seem reasonable to conclude that PEP uptake per se is not as significant as PEP uptake in exchange for intramitochondrial adenine nucleotide - possibly ATP. Thus a decrease of intramitochondrial ATP could be a factor involved in rendering the accumulated Ca^{2+} available for efflux.

An additional factor related to this process is the respiration-linked transport of P_i into the mitochondrial matrix. Agents such as mersalyl, NEM, and DTNB which inhibit the P_i^-/OH^- exchange carrier (18-22) also inhibit the PEP-induced Ca^{2+} efflux as well as the corresponding increase of respiration. P_i-induced efflux of Ca^{2+} from preloaded mitochondria was demonstrated by

Drahota et.al. (7) but high concentration of added P_i were required in order to obtain Ca^{2+} efflux of the extent described here. Recent studies in this laboratory demonstrate that this process too is inhibited by NEM and DTNB. We conclude that Ca^{2+} efflux in the presence as well as the absence of PEP requires P_i transport into the mitochondrial matrix but that addition of PEP allows efflux to occur at lower P_i concentrations.

From these results we suggest that the magnitude of the intramitochondrial ATP/P_i ratio influences the stability of the accumulated Ca^{2+}(or possibly calcium phosphate) of rat liver mitochondria. According to this view, any metabolic parameters that could decrease the intramitochondrial ATP level or increase the intramitochondrial P_i level would stimulate efflux of calcium phosphate by either a passive or a carrier-mediated process. These relationships are currently being investigated.

Acknowledgements: This investigation was supported by grant GB-15383 from the National Science Foundation.

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