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EFFECTS OF PHOSPHOENOLPYRUVATE ON DIVALENT CATION TRANSPORT BY RAT LIVER MITOCHONDRIA

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Chun-Fu Peng (1980) Effects of phosphoenolpyruvate on divalent cation transport by rat liver mitochondria. *Bull. Inst. Zool., Academia Sinica* **19**(2): 23-29. Isolated intact rat liver mitochondria are capable of accumulating a great amount of divalent cation, e. g. Ca^{2+}, Sr^{2+}, and Mn^{2+} in the presence of inorganic phosphate (Pi). Phosphoenolpyruvate (PEP) does not affect divalent cation uptake and H^+ production by rat liver mitochondria. However, PEP causes the efflux of Ca^{2+} but not Sr^{2+} and Mn^{2+} from mitochondria. The stimulation of Ca^{2+} efflux by PEP from Ca^{2+}—loaded rat liver mitochondria is associated with an enhanced respiratory rate and ATP hydrolysis. These results suggest that (i) mitochondrial Ca^{2+} uptake and release exist as two independent pathways, (ii) Sr^{2+} and Mn^{2+} do not share a similar pathway as Ca^{2+} efflux from mitochondria, (iii) the efflux of Ca^{2+} from mitochondria is regulated by intramitochondrial ATP level.

It is well known that uptake of Ca^{2+}, Sr^{2+} and Mn^{2+} by isolated intact mitochondria is supported by respiration or ATP hydrolysis[15], and is mediated by a ruthenium red and La^{3+}—sensitive carrier[17,21,24]. The specificity of divalent cation accumulation by mitochondria is in a order of $Ca^{2+} > Sr^{2+} > Mn^{2+}$. The respiration-linked uptake of Ca^{2+} and ejection of H^+ is influenced by the initial concentration of Ca^{2+} and Pi[15,27], uncoupling agents[4,15], oligomycin[23] as well as adenine nucleotide translocase inhibitors, i. e., atractyloside and bongkrekic acid[19,25]. Recent studies of Chudapongse and Haugaard[9], Peng *et al.*[18] demonstrated that PEP is yet another factor that can influence the mitochondrial Ca^{2+} movement. Since transport of Sr^{2+} and Mn^{2+} across the mitochondrial membrane is generally believed via a similar mechanism as that of Ca^{2+}. It would seem pertinent to investigate the effect of PEP on Sr^{2+} and Mn^{2+} accumulation by rat liver mitochondria.

MATERIALS AND METHODS

Liver mitochondria were prepared from adult male Sprague-Dawley rats in a medium containing 0.25 M Sucrose, 5 mM Hepes (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer pH 7.4, as previously described[18]. After the final wash, mitochondria were suspended in a medium containing 0.2 mM Hepes buffer pH 7.4 with 0.25 M Sucrose. All of the experiments were performed within two hours after the harvest of mitochondia. Mitochondrial protein was determined by the biuret method[14]. Oxygen consumption was determined polarographically with the Clark oxygen electrode[8]. The ejection and uptake of H^+ during the influx and efflux of Ca^{2+}, Sr^{2+}, or Mn^{2+} were followed with a sensitive pH electrode[11]. Appropriate corrections were made for the buffering capacity of the medium and suspened mitochondria by addition of standardized HCl or NaOH in each experiment. Divalent cation uptake and efflux were followed with $^{45}Ca^{2+}$, $^{85}Sr^{2+}$ and $^{54}Mn^{2+}$ as previously described[18].

Aliquots of the incubation mixture were withdrawn at selected time intervals after the addition of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, or $^{54}Mn^{2+}$ and rapidly filtered through a millipore filter[20]. The radioactivity of $^{45}Ca^{2+}$ of each sample was determined with a liquid scintillation spectrometer. The radioactivity of $^{85}Sr^{2+}$ and $^{54}Mn^{2+}$, being remittors, were counted in a Nuclear Chicago Model 8725 gamma counter. Mitochondrial ATP content was determined parallel to cation trasport studies. In all cases, the reaction mixture were removed at the desginated time and was centrifuged. A half milliliter of 20% trichloroacetic acid (TCA) was then added to the supernatant and mitochondrial pellets, respectively. The TCA extracts were then used to determine the amount of mitochondrial ATP content by the firefly luciferase reaction[26]. All reagents were analytical grade. PEP were obtained from Sigma Chemical Co. $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ were purchased from New England Nuclear Co. and $^{85}Sr^{2+}$ was obtained from International Chemical and Nuclear Corp.

RESULTS

A typical example of oxygen and pH electrode tracing of mitochondrial respiration and H^+ movement is shown in the Fig. 1. The addition of divalent cation stimulates mitochondrial respriation and causes H^+ ejection from mitochondria. The stimulated respiration and H^+ production ceased when divalent cation uptake was complete. It is noted in the Fig. 1 that the rate of mitochondrial respiration and H^+ ejection is in the order of $Ca^{2+} > Sr^{2+} > Mn^{2+}$ (The number adjacent to each tracing represents the rate of oxygen con sumption or H^+ ejection after Ca^{2+}, Sr^{2+} or Mn^{2+} was added). The steady state amount of cation accumulation by mitochondria is shown in the numbers included in the parentheses, which is obtained by the radioisotope determination as described in the

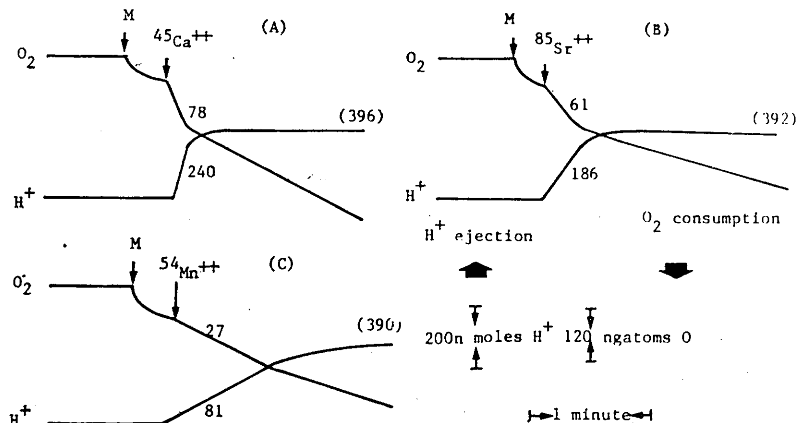

Fig. 1. Oxygen and pH electrode tracing showing the effect of Ca^{++}, Sr^{++} and Mn^{++} on mitochondrial oxygen consumption and H^+ movement. Incubation mixture contained 0.2 mM Hepes Buffer, pH 7.4 Sucrose 100 mM, KCl 100 mM, Glutamate (2.5 mM), Malate (2.5 mM), Pyruvate (2.5 mM), Inorganic phosphate (1 mM), M (mitochondria 5.0 mg protein), Ca^{++}, Sr^{++}, and Mn^{++} (400 n moles each) was added to the incubation mixture as indicated in a final volume of 2.2 ml at 30°C. The number anjacent to each oxygen tracing repesents the rate of oxygen consumption by mitochondria (ng atoms 0/mg/min). The number adjacent to each pH tracing represents the rate of H^+ ejection /mg/min. The number included in the parentheses represents the amount of divalent cation accumulated by mitochondria.

Methods Section. Almost all added divalent cation were accumulated by mitochondria at indicated time after Ca^{2+}, Sr^{2+} or Mn^{2+} was added. (396, 392 and 390 n moles out of 400 n moles added Ca^{2+}, Sr^{++} and Mn^{2+} were accumulated, respectively). Fig. 2 shows that the addition of 1.0 mM PEP does not affect Ca^{2+} induced mitochondrial respiration and H^+ ejection. However, PEP enhenced a second phase of respiratory stimulation and a H^+ up-

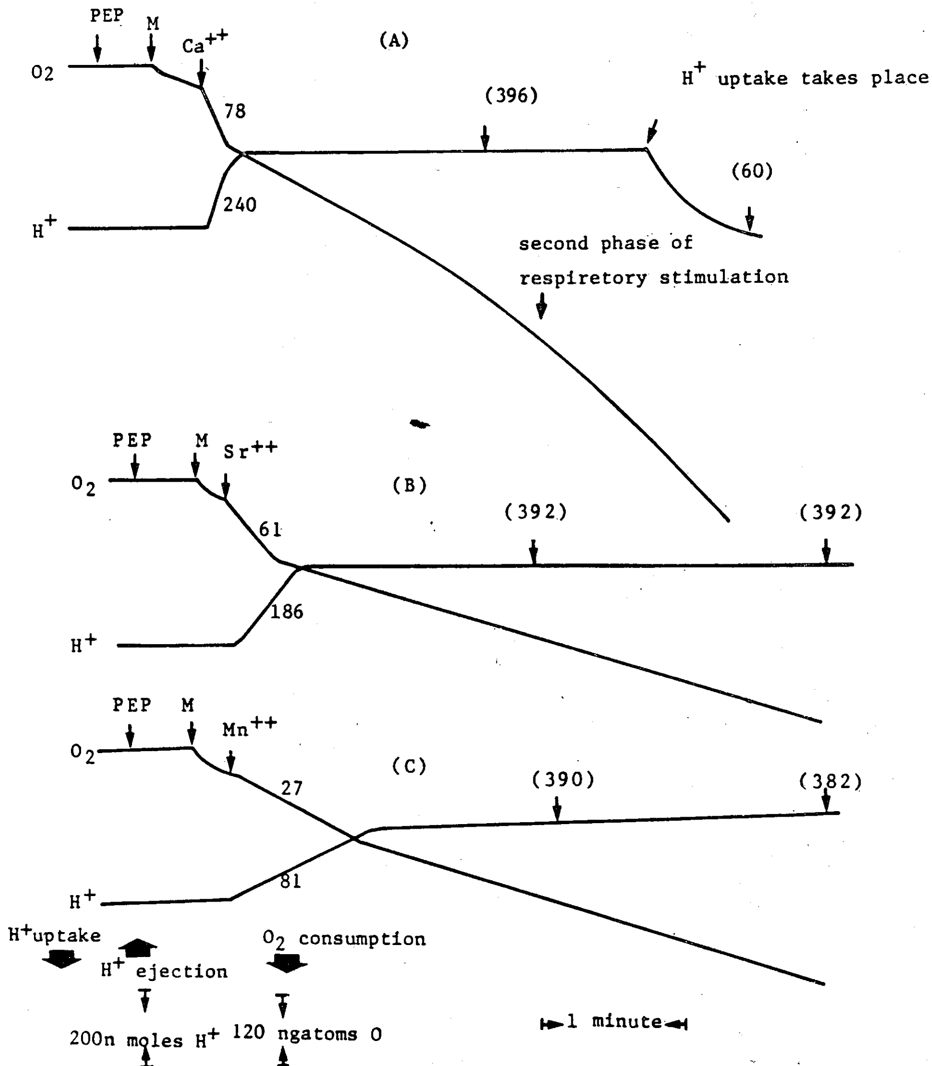

Fig. 2. Oxygen and pH electrode tracing showing the effect of PEP on mitochondrial oxygen consumption and H^+ movement. The experimental conditions were the same as shown in the Fig. 1. PEP (1.0 mM) was added before the addition of Mitochondria (M). The numbers adjecent to each tracing have the same indication as shown in the Fig. 1. The numbers included in the parenthesis represent the steady state amount of cation accumulation.

take by mitochondria. In addition, accumulated Ca^{2+} was discharged from mitochondria after PEP was added. Accumulated mitochondrial Ca^{2+} was decreased from 396 nmoles 60 nmoles (Fig. 2A) Conversely, Fig. 2B and 2C shows that PEP did not induce a second phase of respiratory stimulation and H^+ uptake by mitochondria when Sr^{2+} or Mn^{2+} was added. Furthermore, accumulated Sr^{2+} or Mn^{2+} was not discharged from mitochondria by the addition of PEP.

It has been noted that adenine nucleotides may be required in maintaining accumulated cation in mitochondria[15]. The effect of PEP on mitochondrial ATP content was thus studied. Fig. 3 demonstrates that a decrease of ATP concentration occurred when PEP was added in the Ca^{2+}-loaded mitochondria. Conversely, PEP did not cause a reduction of ATP concentration when mitochondria was loaded with Sr^{2+} or Mn^{2+}. There was no detectable ATP found in the external medium. Most of ATP were found in the mitochondrial pellets suggesting that ATP hydrolysis, not ATP-PEP exchange, occurred during the efflux of Ca^{2+} when PEP was aded. This speculation was further studied by the addition of oligomycin, an inhibitor of the mitochondrial ATPase. Fig. 4 shows that the addition of oligomycin after PEP was added totally blocked ATP hydrolysis and Ca^{2+} efflux from mitochondria. Moreover, the second phase of the respiratory stimulation and H^+ uptake during the efflux of Ca^{2+} as demonstrated in the Fig. 2A were also prevented by oligomycin (Oxygen and pH tracing figure was not shown).

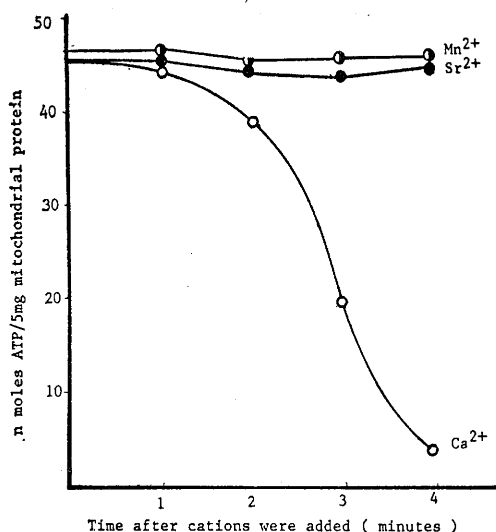

Fig. 3. Effect of PEP on endogenous mitochondrial ATP concentration in the present of Ca^{2+}, Sr^{2+}, or Mn^{2+}. Experimental conditions were the same as described in the Fig. 2. Incubation mixture was withdrawn and centrifuged. Samples were taken 1, 2, 3, and 4 minutes after the addition of divalent cation. Trichloroacetic acid (TCA) was added to mitochondrial pellets and the supernatant fraction, respertively. TCA extracts were then used to determine ATP concentration in the mitochondrial pellets is shown in this figure. No detectable ATP was found in the supernatant fraction.

DISCUSSION

This study demonstrates that phosphoenolpyruvate (PEP) does not affect uptake of Ca^{2+}, Sr^{2+} or Mn^{2+} by isolated rat liver mitochondria but does induce Ca^{2+} but not Sr^{2+} or Mn^{2+} efflux from mitochondria. These results suggest that while uptake of Ca^{2+}, Sr^{2+} and Mn^{2+} is mediated through a ruthenium red sensitive carrier[17,21,24], the efflux of Sr^{2+} and Mn^{2+} do not share a similar pathway as Ca^{2+} efflux from mitochondria. Inasmuch as Ca^{2+} uptake and efflux by mitochondria have been implicated in the control of cellular Ca^{2+} transport[2] as well as carbohydrate metabolism[10,12,22,28], this study presents two important information. First of all, Ca^{2+} and Mg^{2+} but not Sr^{2+} and Mn^{2+} are the major physiological divalent cation. It is very likely that biological nature is arranged in such a way that mitochondria do not possess an efflux pathway for Sr^{2+} and Mn^{2+} since both divalent cations do not play any physiological role as Ca^{2+} does. Secondly, PEP is an important intermediate of glycolytic and gluconeogenic pathways and is the only known physiological

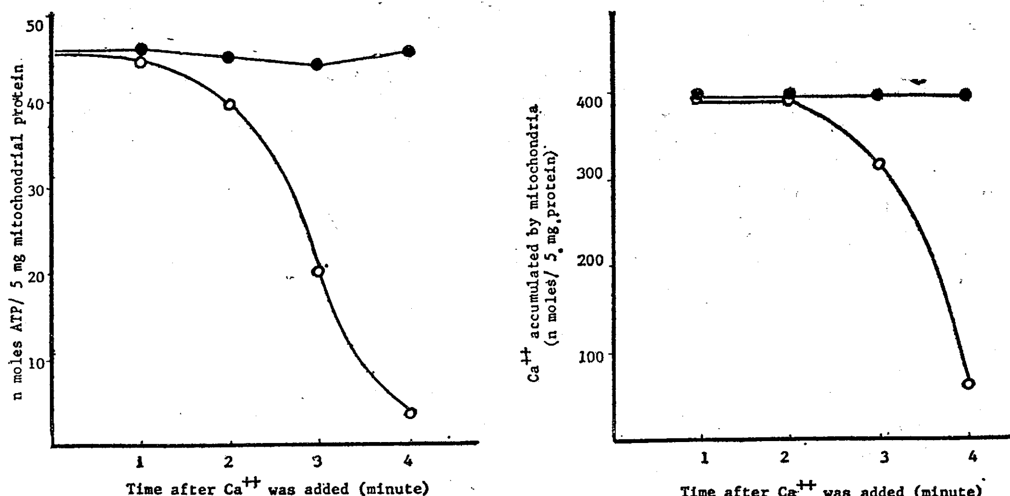

Fig. 4. Effect of oligomycin on PEP induced ATP hydrolysis and Ca^{++} efflux by mitochondria: The experimental conditions were the same as described in the Fig. 2. Oligomycin (4/μg) was added after the addition of PEP and mitochondria (M, 5/mg protein). Ca^{2+} was then added to initiate the reaction. Both mitochondrial ATP concentration and Ca^{++} accumulation were determined 1, 2, 3, and 4 minutes after the addition of Ca^{2+}, •—• with oligomycin, ∘—∘ without oligomycin.

metabolite to induce Ca^{2+} efflux from mitochondria. Several physiological functions have been proposed for the mitochondrial Ca^{2+} transport process. Mitochondia may play a role in skeletal calcification[1,16], in cardiac muscle contration and relaxzation where sarcoplasmic reticulum is poorly developed[7,13], and in regulating cellular Ca^{2+} concentration[3,5]. However, these physiological roles of mitochondria are not proven at the present time, although the ability of mitochondria to relax myofibrils has been demonstrated[6]. The demonstration of Ca^{2+} efflux induced by PEP tempts to suggest that PEP may play an important role in these physiological functions in regulating cytosolic Ca^{2+} concentration through efflux process.

The hydrolysis of ATP by mitochondria during Ca^{2+} efflux in the presence of PEP supports the hypothesis of the intramitochondrial ATP/Pi ratio controlling the stability of accumulated Ca^{2+}[18]. The second phase of the mitochondrial respriation in the presence of Ca^{++} after the addition of PEP is due to a continuous phosphorylation of ADP immediately after ATP is hydrolyzed. Accordingly, any metabolic parameters that could decrease the intramitochondrial ATP content would stimulate efflux of Ca^{2+} by either a passive or a carrier-mediated process. Recently, Shug and Shrago[25] have shown that PEP can be accumulated by isolated rat liver mitochondria by an atractylosidesensitive carrier exchange with intramitochondrial ATP. Their study appears to suggest that the reduction of intramitochondrial ATP by PEP through an exchange process could bring about the Ca^{2+} efflux. However, this study does not find any detectable ATP in the external medium, in stead, almost all of intramitochondrial ATP were hydrolyzed as ADP and Pi during the efflux of Ca^{2+}. Furthermore, olgomycin does not block ATP-PEP exchange but does inhibit ATP hydrolysis by mitochondria. The inhibition of PEP induced-second phase of the respriatory stimulation, H^+ uptake and Ca^{2+} efflux by oligomycin strongly suggests that Ca^{2+} efflux is mediated and regulated through a Ca^{2+}-specific mitochondrial ATPase.

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磷醇丙酮酸 (Phosphoenolpyruvate) 對粒線體兩價正離子傳遞的影響

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粒線體在有負離子，如磷酸 (inorganic phosphate) 的情況下有能力吸收 (uptake) 大量的正離子，如鈣、鍶和錳。磷醇丙酮酸 (phosphoenolpyruvate) 不會影響粒線體吸收正離子的能力；它可致使被粒線體吸收了的鈣離子釋放到粒線體外，但却無能力將鍶和錳離子釋放出粒線體外。當鈣離子由粒線體排出時，粒線體消耗氧的速率也隨著增加，同時 ATP 也被水解。這種現象會被粒線體膜上 ATPase 的抑制物，Oligomycin 所抑制。這些結果顯示：(1)鍶和錳離子由粒線體排出的機制與鈣離子不同，(2)鈣離子的排出與粒線體膜上的 ATPase 有關，粒線體內 ATP 濃度的高低會影響鈣離子的吸收或排出。許多學者認為粒線體能吸收與釋放鈣離子有其生理意義，例如骨骼肌之鈣化過程、心臟肌之伸張與收縮，和細胞質內維持低濃度的鈣離子均需粒線體之參與。而磷醇丙酮酸是目前所知的，唯一對粒線體鈣離子的傳遞有控制作用的生理代謝物質 (physiological metabolites)。