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THE EFFECT OF INORGANIC PHOSPHATE ON CALCIUM INFLUX INTO RAT HEART MITOCHONDRIA

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SUMMARY: The effect of inorganic phosphate on the accumulation of Ca^{2+} by heart mitochondria has been reinvestigated. Inorganic phosphate has no effect on the initial rate of Ca^{2+} uptake supported by respiration on either ascorbate plus tetramethylenephenylene diamine, pyruvate plus malate, or glutamate plus malate, although it does increase the final amount of Ca^{2+} accumulated; evidence suggests that the latter phenomenon requires phosphate influx via the phosphate carrier. It is concluded that the earlier reports that phosphate augments the initial rate of Ca^{2+} influx reflects an effect of phosphate on succinate oxidation, which was employed in the previous studies, rather than an Ca^{2+} transport itself.

INTRODUCTION: There is considerable evidence that the accumulation of Ca^{2+} by mitochondria is a direct consequence of the electrical potential difference across the inner membrane, and occurs via a carrier that catalyses the passive movement of Ca^{2+} down its electrochemical gradient (2 - 6) i.e. with a net positive charge transfer of 2 for each Ca^{2+} transported. On the contrary, other mechanisms have been proposed recently. In these, the Ca^{2+} carrier catalyses a partially charge-compensated movement of Ca^{2+}, either by a 1 : 1 exchange between Ca^{2+} and H^+ (8) or by 2 Ca^{2+} plus HPO_4^{2-} symport (7), so that each Ca^{2+} enters with an effective net charge transfer of 1. The latter mechanisms were used to explain the observations (8, 9) that the initial rates of Ca^{2+} uptake by heart and liver mitochondria, respiring on succinate, are stimulated several fold by inorganic phosphate. Inorganic phosphate was considered to be either obligatory for Ca^{2+} transport (7) or to act indirectly by lowering the intramitochondrial pH during Ca^{2+} transport, thereby promoting association of H^+ with the carrier (8).

In this paper we reexamine the effects of inorganic phosphate on Ca^{2+} transport in heart mitochondria, in order to establish whether or not

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phosphate is required or modifies the system responsible for Ca^{2+} translocation. In order to facilitate interpretation of experimental data, the mitochondria were largely depleted of endogenous inorganic phosphate thus permitting experiments with very low concentrations of inorganic phosphate in the external medium. It is concluded that inorganic phosphate has no direct effect on the Ca^{2+} transport system.

METHODS: Mitochondria from female, Wistar, rat hearts were prepared as described previously (9). The mitochondria were largely depleted of endogenous inorganic phosphate (25 - 30 nmoles·mg protein^{-1}) by passing the suspension (containing 50 - 100 mg of protein) through a Sephadex G-25 column (30 x 1.7 cm) at 20 - 25° C (flow rate, about 1 ml/min). Mitochondria treated in this way contained 1 - 4 nmoles inorganic phosphate·mg protein^{-1}.

Changes in the extramitochondrial concentration of free Ca^{2+} were measured with a Ca^{2+}-selective electrode (10) using calibration procedures that have been described in detail previously (9). The standard reaction medium contained 120 mM KCl, 5 mM N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonate buffer (K^+ salt; pH 7.0), 1 μg of rotenone·mg protein^{-1} and mitochondria (0.2 - 0.5 mg protein·ml^{-1}). Further additions of inorganic phosphate and $CaCl_2$ are described in the figure legends.

Inorganic phosphate was assayed according to the method of Stewart (11). The content of inorganic phosphate in the extramitochondrial medium before and after Ca^{2+} uptake was determined by centrifuging 0.3 ml of the mitochondrial incubate for 2 min at 15,000 rpm in an Eppendorf bench centrifuge and measuring the phosphate content of the supernatant.

Phosphate carrier activity was determined as the rate of mitochondrial swelling in 120 mM ammonium phosphate plus 1 μg of rotenone·mg protein^{-1} (12); the mitochondria were preincubated for 2 min at 25° C with various concentrations of mersalyl (see fig. 3) before use.

RESULTS AND DISCUSSION: Fig. 1 shows the uptake of Ca^{2+} by phosphate-depleted heart mitochondria following the addition of ascorbate-tetramethylenephenylenediamine (TMPD) as respiratory substrate. It was not possible to completely remove inorganic phosphate from the mitochondrial preparation and some residual phosphate does appear in the suspending medium during the preincubation period; in these experiments the extramitochondrial phosphate concentration was about 0.2 μM 15 secs before Ca^{2+} uptake was started. Trace (a) shows that, in the absence of mersalyl, the mitochondria accumulated about 30 nmoles Ca^{2+}·mg protein^{-1} within about 2 min.

Since, however, any phosphate taken up with Ca^{2+} could exit, i.e. recycle, via the phosphate carrier (which catalyses $H_2PO_4^-$ plus H^+ symport, or its equivalent (12, 13)), the effect of inhibiting the

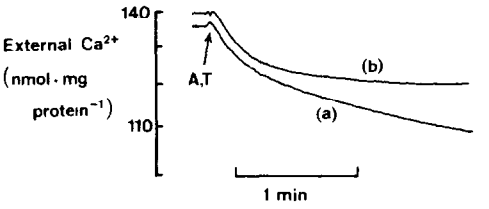
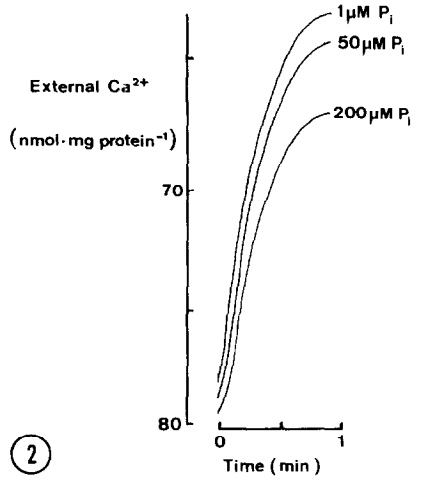


Fig. 1. The uptake of Ca^{2+} by phosphate-depleted mitochondria in the absence of added phosphate. The figure shows recorder tracings of the uptake of Ca^{2+} by heart mitochondria at 25° C suspended in standard reaction medium (see Methods Section) containing 30 μM $CaCl_2$. After a preincubation period of 5 min, uptake was initiated by ascorbate (2.5 mM) - tetramethylenephenylene diamine (25 μM) at the point marked A, T. Trace (a) was obtained in the absence of mersalyl and trace (b) with 20 nmoles of mersalyl·mg protein^{-1} added 2 min before Ca^{2+} uptake was started. The phosphate concentration in the extramitochondrial medium before Ca^{2+} uptake was 0.2 μM.

phosphate carrier was investigated. In this case, the mitochondria were preincubated with 20 nmole mersalyl·mg protein^{-1} which is sufficient to completely inhibit phosphate carrier activity (14, 15, fig. 3). Inclusion of mersalyl (trace b) does not change the initial rate of Ca^{2+} uptake, although it does decrease somewhat the total amount of Ca^{2+} accumulated. Nevertheless, even in the presence of mersalyl, the amount of Ca^{2+} accumulated (about 20 nmoles Ca^{2+}·mg protein^{-1}) greatly exceeded the phosphate content of the external medium (about 1.2 nmoles·mg protein^{-1}), so that almost all the Ca^{2+} influx is independent of phosphate flux. This conclusion is confirmed by the data of table 1 in which the net movements of Ca^{2+} and phosphate were compared. In the absence of mersalyl, the accumulation of Ca^{2+} does cause most of the residual extramitochondrial phosphate to be reaccumulated. In the presence of mersalyl, however, the uptake of Ca^{2+} occurred in the absence of any uptake of phosphate; in fact, very small losses of mitochondrial phosphate was repeatedly observed. These data indicate that Ca^{2+} influx into heart mitochondria does not require simultaneous movement of inorganic phosphate.

The previously observed ability of inorganic phosphate to stimulate the initial rate of Ca^{2+} uptake into heart mitochondria (9) utilizing succinate as respiratory substrate was reinvestigated with alternative



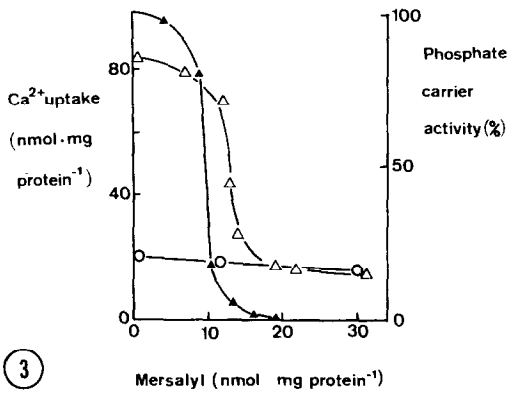


Fig. 2. The uptake of Ca^{2+} by phosphate-depleted mitochondria in the presence of mersalyl and different concentrations of phosphate. The experimental conditions were as described in fig. 1 with 20 nmoles mersalyl·mg protein^{-1}. Ascorbate (2.5 mM) - tetramethylenephenylene diamine (25 μM) was added at zero-time. The extramitochondrial phosphate concentration in the uppermost curve was 1 μM immediately before Ca^{2+} uptake was started. In the two other experiments, phosphate was added to give 50 μM and 200 μM phosphate as indicated.

Fig. 3. The effect of mersalyl on the extent of Ca^{2+} uptake and the phosphate-carrier activity of phosphate-depleted heart mitochondria. The final amount of Ca^{2+} accumulated from the standard medium containing 93 nmoles Ca^{2+}·mg protein^{-1} was measured from traces of the type shown in fig. 1. Phosphate carrier activity was measured as described in the Methods Section. Symbols: ○, Ca^{2+} accumulated in the absence of added phosphate (extramitochondrial phosphate, 0.3 μM); △, Ca^{2+} accumulated in the presence of 200 μM phosphate; ▲, phosphate carrier activity expressed as a percentage of that in the absence of mersalyl.

sources of respiratory energy. Fig. 2 reports data obtained with ascorbate-TMPD as respiratory substrate in the presence of different concentrations of inorganic phosphate. A lag phase of about 10 secs follows the addition of substrate and, thereafter, Ca^{2+} influx attains a velocity of about 1 nmole Ca^{2+}·mg protein^{-1}·sec^{-1} irrespective of the phosphate concentration. These values are close to those measured previously with succinate as respiratory substrate (about 1.5 nmole Ca^{2+}·mg protein^{-1}·sec^{-1} (9)). With mersalyl present, inorganic phosphate causes a small decrease in the total amount of Ca^{2+} accumulated. It should be noted

Table 1

A comparison of the Ca^{2+} and phosphate accumulated by phosphate-depleted heart mitochondria

Conditions	Ca^{2+} uptake (nmole Ca^{2+}·mg protein^{-1})	Extramitochondrial phosphate (nmole·mg protein^{-1})	
		Before Ca^{2+} uptake	After Ca^{2+} uptake
1. Without mersalyl	35	1.6, 1.8	0.3, 0.2
2. With mersalyl	25	2.5, 2.2	3.6, 4.0

The experimental conditions were as described for fig. 1; different mitochondrial preparations were used in the different experiments. The extramitochondrial phosphate was determined 15 secs before Ca^{2+} uptake was started and again, after a further 2½ min, when Ca^{2+} uptake was essentially complete; duplicate values are reported.

that this is not due to complexation of Ca^{2+} by phosphate in the external medium since the complexation that does occur is less than the error of experimental reproducibility (about 5 %).

Other experiments (not shown) revealed that phosphate does not affect the rate of Ca^{2+} uptake by heart mitochondria respiring either pyruvate (5 mM) plus malate 5 mM) or glutamate (5 mM) plus malate (5 mM) with extramitochondrial Ca^{2+} concentrations between 8 μM and 40 μM i.e. with a range of Ca^{2+} concentration in which any change in K_m or V_{max} of the carrier in the presence and absence of phosphate would be apparent (K_m for Ca^{2+} 12 - 15 μM, (9)). On the contrary, the previously reported stimulation of the rate of succinate-supported Ca^{2+} uptake by inorganic phosphate (9) has been reconfirmed. Indeed, the net Ca^{2+} influx into the phosphate-depleted mitochondria in the absence of phosphate was never greater than 5 nmoles Ca^{2+}·mg protein^{-1} i.e. much less than the uptake obtained with ascorbate-TMPD, glutamate-malate or pyruvate-malate (15 - 35 nmoles Ca^{2+}·mg protein^{-1}). It is concluded that the stimulation by phosphate of succinate-supported Ca^{2+} uptake reflects an action on succinate oxidation rather than the Ca^{2+} transport system, since phosphate has no effect when other respiratory substrates are used.

It is extensively documented, however, that inorganic phosphate does markedly augment the total amount of Ca^{2+} accumulated by respiring mi-

tochondria (see (16, 17) for reviews). This phenomenon was clarified with respect to the proposed lack of any direct effect of phosphate on Ca^{2+} transport.

Fig. 3 shows the final amount of Ca^{2+} accumulated by phosphate-depleted heart mitochondria in the presence and absence of added phosphate. With phosphate present, about 85 nmoles of Ca^{2+}·mg protein^{-1} were accumulated. This amount is diminished by inclusion of mersalyl, and with about 18 nmoles mersalyl·mg protein^{1} the amount of Ca^{2+} taken up is similar to that which occurred in the absence of added phosphate (about 17 nmoles Ca^{2+}·mg protein^{-1}).

Fig. 3 also shows the inhibition of the phosphate carrier activity by mersalyl. It is evident that the amount of mersalyl required to completely inhibit the phosphate carrier is not detectably different from that needed to completely prevent the augmentation of Ca^{2+} uptake by phosphate. These data support the previous proposal (18) that phosphate increases Ca^{2+} accumulation via transport by the phosphate carrier i.e. by entering essentially as H_3PO_4 and thereby lowering the intramitochondrial pH which tends, otherwise, to become very alkaline during respiration-supported Ca^{2+} uptake (19, 20, 21), i.e. phosphate uptake under these conditions is driven by the pH gradient across the inner mitochondrial membrane. It should be noted that a strict coincidence between the complete titration curves of Ca^{2+} uptake and phosphate carrier activity in fig. 3 would not be predicted since phosphate transport would need to be substantially inhibited before any effect on the final amount of Ca^{2+} accumulated is seen.

In summary, the present data indicate that Ca^{2+} and phosphate fluxes in heart mitochondria are interlinked only indirectly and that phosphate has no direct effect on the activity of the Ca^{2+} transport system. Furthermore, although such indirect coupling, via the transmembrane pH gradient is manifest, with time, in the velocity of net Ca^{2+} uptake, interpretation of this phenomenon requires many aspects to be considered, e.g., effects of intramitochondrial alkalinity on substrate oxidation (in particular, of succinate) and the electrical potential difference across the inner membrane, and intramitochondrial calcium phosphate precipitation, before any conclusions may be drawn about the properties of the Ca^{2+} transport system per se.

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