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INVOLVEMENT OF ENDOGENOUS PHOSPHOLIPASE A_2 IN Ca^{2+} AND Mg^{2+} MOVEMENTS INDUCED BY INORGANIC PHOSPHATE AND DIAMIDE IN RAT LIVER MITOCHONDRIA

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SUMMARY: Addition to rat liver mitochondria of 2 mM inorganic phosphate or 0.15 mM diamide, a thiol oxidizing agent, induced a respiration dependent efflux of Mg^{2+} which was prevented by both antimycin A and tetracaine. Tetracaine also inhibited the release of respiration induced by phosphate or diamide. Endogenous Ca^{2+} were retained by mitochondria until 50-60 per cent of endogenous Mg^{2+} has been lost. Tetracaine retarded Ca^{2+} release. The involvement of mitochondrial phospholipase A_2 is demonstrated both by its inhibition by tetracaine and its activation by diamide or phosphate. The failure of these compounds to activate phospholipase A_2 in the presence of added Ca^{2+} makes reasonable the assumption that the activation of phospholipase A_2 is secondary to respiration dependent Ca^{2+} movements, which would favour the interaction Ca^{2+}-phospholipase A_2.

INTRODUCTION

Recently we have shown that addition to rat liver mitochondria of 2 mM Pi or 0.15 mM diamide, a mild oxidizing agent, induces an efflux of endogenous Mg^{2+} dependent on coupled respiration (1-2), which was completely prevented by EGTA, ruthenium red, La^{3+} and NEM. These reagents also inhibited the release of respiration induced by both Pi and diamide (2). No net Ca^{2+} release occurred under these conditions. It was assumed that Mg^{2+} efflux is dependent on a cyclic in and out movement of Ca^{2+} across the inner mitochondrial membrane, in which the passive efflux induced either by Pi or diamide is compensated by a continuous energy linked reuptake (1,2). This interpretation would explain the dependence of Mg^{2+} efflux on coupled respiration, as well as the concomitant release of respiration in state 4.

Considering that mitochondrial phospholipase A_2 is activated by Ca^{2+}(3), a possible activation of endogenous phospholipases comes into play as an additional cause for Mg^{2+} efflux. Moreover, assuming that mitochondrial phospholipids represent a binding site for divalent cations (4), or the first step for their transmembrane transport (5), it appeared interesting to study whe-

Abbreviations: Diamide = diazenedicarboxylic acid bis(N,N-dimethylamide); FCCP = p-trifluoromethoxyphenylhydrazone; Hepes = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; Pi = inorganic phosphate; Tetracaine = 4-(butylamino)benzoic acid 2-(dimethylamino)ethyl ether hydrochloride ; NEM = N-ethylmaleimide.

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ther mitochondrial phospholipase A_2 was in some way involved in the cation movements induced by Pi or diamide. The results reported in the present paper demonstrate that diamide and Pi effectively induce an activation of mitochondrial phospholipase A_2. This is shown both by direct measurement of mitochondrial phospholipase A_2 activity and by inhibition of the diamide and Pi effects by tetracaine, a local anesthetic capable to inhibit phospholipase A_2 activity (6,7).

MATERIALS AND METHODS

[1,2-^{14}C]ethanolamine hydrochloride was obtained from New England Nuclear, diamide from Calbiochem (San Diego, California) and tetracaine from Hoechst (Italy).

Rat liver mitochondria were isolated in 0.25 M sucrose according to Schneider (8). Protein concentration was determined by the biuret method (9). Mg^{2+} and Ca^{2+} movements were estimated by atomic absorption spectroscopy of the supernatant (10) and cation contents at zero time were assayed in acid extracts of the pellet (11). Oxygen uptake was measured with a Clark oxygen electrode.

Mitochondrial phospholipase A_2 activity was detected as follows: female rats were injected intraperitoneally with 10 μC of [1,2-^{14}C]ethanolamine dissolved in isotonic saline solution 1 hr before sacrifice. Liver mitochondria were then isolated and 2.5 mg labeled mitochondrial proteins were incubated in 2.5 ml medium containing 170 mM sucrose, 10 mM Hepes, 1.25 μM rotenone and 10 mM succinate. Other additions are described in the legend to the Table I. At the times indicated, the mitochondria were rapidly centrifuged (2 min at 0°C) and the pellet suspended in 4 ml of 0.3 M HCl and 0.05 M EGTA. Phospholipids were then extracted twice with 2 ml of n-butyl alcohol (12). The lipid extracts, evaporated under nitrogen stream and resuspended in $CHCl_3$-CH_3OH (2:1) were applied to 0.5 mm layers of silica gel (Merck) to separate phosphatidylcholine, phosphatidylethanolamine and their respective lysoderivatives. The chromatograms were developed in chloroform-methanol-acetic acid-water (65:15:10:4, by vol.) (13). After detection of the radioactive phospholipids with iodine vapours the areas of the chromatograms containing radioactive material were scraped off and transferred to liquid scintillation vials. The scintillation mixture was Packard Insta-gel-water (10:2.5 v/v). Radioactivity was determined in a Beckman LS-100 C liquid scintillation spectrometer.

RESULTS

Fig.1 (A and B) shows the time course of Mg^{2+} efflux from rat liver mitochondria induced by 2 mM Pi and 0.15 mM diamide respectively. It must be outlined that Pi induced Mg^{2+} efflux at pH ranging from 6.4 to 7, with optimum at pH 6.8. At pH 7.4 the Pi effect was neglegible. Other permeant anions, like acetate, failed to induce Mg^{2+} efflux at any pH.

As previously described Mg^{2+} efflux induced either by Pi or diamide, was completely prevented by antimycin A (see also Fig.1) as well as by FCCP, ruthenium red and NEM (2). Also tetracaine, as shown in Fig.1, completely prevented Mg^{2+} efflux promoted by diamide and partially that induced by Pi. The different pHs used in the experiments with Pi (6.8) and diamide (7.4) could account for the different extent of tetracaine inhibition.

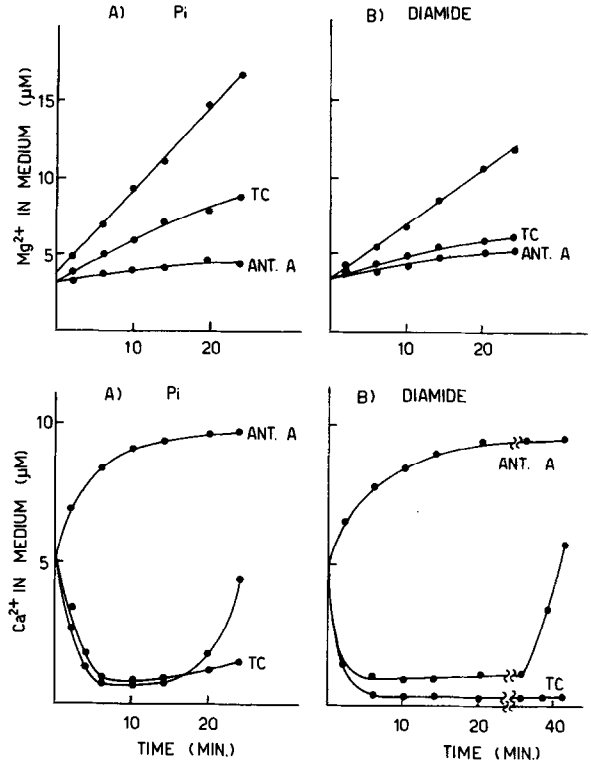

Figure 1 - Mg^{2+} and Ca^{2+} movements in liver mitochondria induced by phosphate (A) or diamide (B). Effect of tetracaine.

A - Rat liver mitochondria (1 mg/ml medium) were suspended in a medium containing: 170 mM sucrose, 10 mM Hepes pH 6.8, 10 mM sodium succinate, 1.25 μM rotenone, 2 mM sodium phosphate pH 6.8. Temperature 25°C. When present 0.5 mM tetracaine (TC), 1 μM antimycin A (Ant.A).

B - Experimental conditions and incubation medium as in A, except for 10 mM Hepes pH 7.4. Phosphate was omitted and replaced by 0.5 mM diamide.

At the concentrations of Pi and diamide used in the present study, Ca^{2+} present as contaminants in the incubation medium (~5 μM) were quickly taken up and retained within mitochondria for about 20-35 minutes respectively, until approximately 60 per cent of endogenous Mg^{2+} have been lost (2). Tetracaine considerably prolonged this period (Fig.1, A and B). In the presence of antimycin A no external Ca^{2+} were taken up, while endogenous Ca^{2+} were quickly released (Fig.1, A and B). Very little, or no swelling, measured as turbidity decrease at 525 nm, was observed under all conditions. However a consistent swelling did take place as soon as mitochondria became unable to retain Ca^{2+}.

As shown in Fig.2 (A and B) Pi and diamide, the former at pH 6.8, significantly released the respiration in state 4; tetracaine abolished the effect of diamide and significantly decreased that of Pi.

TABLE I

Stimulation by phosphate (A) and diamide (B) of liver mitochondrial phospholipase A_2 activity. Effect of tetracaine.

A		B	
Additions	Per cent of phosphatidyl-ethanolamine hydrolysis	Additions	Per cent of phosphatidyl-ethanolamine hydrolysis
none	3.2 ± 0.10	none	4.1 ± 0.15
Pi	5.1 ± 0.25	diamide	6.3 ± 0.05
Pi + TC	3.2 ± 0.15	diamide + TC	4.9 ± 0.20
$CaCl_2$	5.8 ± 0.00	$CaCl_2$	6.4 ± 0.10
$CaCl_2$ + Pi	5.3 ± 0.10	$CaCl_2$ + diamide	6.5 ± 0.05
FCCP	3.6 ± 0.20	FCCP	4.5 ± 0.15

Rat liver mitochondria were incubated at 20° for 30 minutes: A medium as in Fig. 1 A, B medium as in Fig. 2 B.
Additions: 2 mM Pi, 0,5 mM diamide, 0.5 mM tetracaine (TC), 2 mM $CaCl_2$ and 0.8 μM FCCP. The data are the mean values of two measurements and are representative of the results obtained with 20 different mitochondrial preparations.

Table I (A and B) shows that both Pi and diamide significantly stimulated $[^{14}C]$-lysophosphatidylethanolamine formation in liver mitochondria previously injected with uniformly labelled $[^{14}C]$-ethanolamine. The formation of lysophosphatidylethanolamine was assumed as a proper index for mitochondrial phospholipase A_2 activity on the basis of the observation that this enzyme preferentially hydrolyses phosphatidylethanolamine (14). As percentage of total mitochondrial phosphatidylethanolamine the increase of lysophosphatidylethanolamine induced by the two agents could appear relatively modest, but it should be considered that even small changes of endogenous phospholipids, especially if limited to a key locus in the mitochondrial structure, might induce critical modifications in mitochondrial functions (15). On the other hand the stimulation of mitochondrial phospholipase A_2 activity by Pi or diamide has been constantly observed in all the numerous experiments performed. It should also be outlined that the increase of phosphatidylethanolamine hydrolysis became evident after about 20 minutes of incubation, that is at the time when Pi or diamide treated mitochondria started to release accumulated Ca^{2+} (Fig.1, A and B). Tetracaine prevented phosphatidylethanolamine hydrolysis (Table I). In the presence of added Ca^{2+} the activity of mitochondrial phospholipase A_2 resulted, as expected, slightly increased but insensitive either to Pi or diamide. FCCP did not induce any significant stimulation of phosphatidylethanolamine hydrolysis.

DISCUSSION

The observation that Mg^{2+} efflux from rat liver mitochondria induced by Pi or diamide is inhibited by antimycin A (Fig.1) as well as by uncouplers, ruthenium red and NEM (2) would indicate that the process is dependent on Ca^{2+} and Pi movements supported by coupled respiration. On the other hand the inhibition by tetracaine (Fig.1) suggests that also mitochondrial phospholipase A_2 is somehow involved in the respiration dependent Mg^{2+} efflux. Tetracaine is known to inhibit phospholipase A_2 (6,7), probably by displacing divalent cations from binding sites of biological membranes and precisely from negatively charged phospholipids (16). It is conceivable that also in our case tetracaine could interfere with the ternary complex: phospholipase A_2-Ca^{2+}--phospholipids. As shown in Table I both Pi and diamide induced a significant stimulation of mitochondrial phospholipase A_2, which is abolished by tetracaine.

It would appear that this stimulation is indirect, probably mediated by respiration supported Ca^{2+} movements which could unmask phospholipase activity by making available Ca^{2+} necessary for the activity of the enzyme. The failure of Pi and diamide to stimulate phospholipase A_2 activity in the presence of added Ca^{2+} supports this assumption.

Parce et al. (17) have recently suggested that in coupled mitochondria phospholipase A_2 is silent because inaccessible to Ca^{2+} due to the organization of mitochondrial membranes and the location of the enzyme within the mitochondria. It is also possible that phospholipase A_2 appears silent because its activity is normally compensated by a concomitant reacylation of lysocompounds. It is only when the two processes become unbalanced, either because phospholipase A_2 activity is increased, or because, as in deenergized mitochondria, reacylation process is lowered or blocked, that phospholipase A_2 activity becomes evident. In our conditions the stimulation of phospholipase A_2 activity is detectable only after 20 minutes of incubation when mitochondria become no more capable to completely retain accumulated Ca^{2+}. It is possible that at this stage also reacylation process is no longer able to compensate accelerated phospholipids hydrolysis. However the possibility that Pi or diamide could evoke phospholipase activity simply by rendering mitochondria less coupled and therefore less efficient in supporting the reacylation rate, can be ruled out by the observation that uncouplers fail to induce any stimulation of phospholipase activity as well as Mg^{2+} efflux.

It is known that a consistent portion of mitochondrial Mg^{2+} is present in the intermembrane space bound to either of the two membranes (18), that is in the same compartment where phospholipase A_2 is located (19,20). It is then possible that when phospholipase A_2 is activated, digestion of phospho-

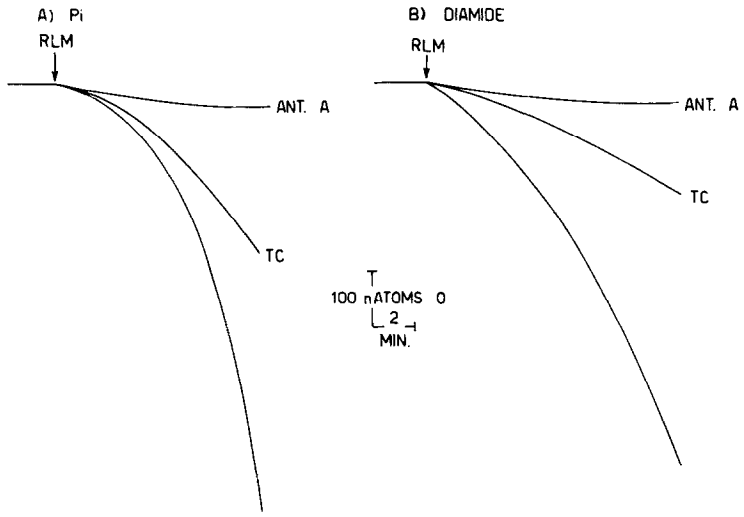

Figure 2 - Release of respiration induced by phosphate (A) or diamide (B). Effect of tetracaine.

A - Incubation medium as in Fig. 1 A.

B - Incubation medium as in Fig. 1 B. When present 0.5 mM tetracaine, 1 μM antimycin A.

lipids might cause a release of bound Mg^{2+}. This release, together with Mg^{2+} displaced from their binding sites by Ca^{2+} movements (21), might contribute to the overall Mg^{2+} efflux.

As to the mechanism by which diamide induces the described effects, it is tempting to speculate that by oxidizing some pairs of membrane thiol groups this agent facilitates a passive leakage of mitochondrial Ca^{2+} compensated by an energy consuming reuptake in virtue of a sufficient transmembrane electrochemical potential. This interpretation agrees with an increased respiration in state 4 (Fig.2) and the capability of diamide treated mitochondria to retain accumulated Ca^{2+}. Reed and Lardy (22) have already proposed analogous interpretation to explain Mg^{2+} efflux mediated by ionophore A23187.

Pi induced the same effect as diamide provided the pH of the external medium is below 7. Considering that Pi can support Ca^{2+} transport probably by transferring protons into the matrix space (23), it is conceivable that a low pH would favour Ca^{2+} flux across inner membrane. This interpretation explains the release of respiration induced by Pi at low pH as well as the inhibitory action of NEM and ruthenium red (2).

The possibility that low pH could affect per se the permeability of mitochondrial membrane seems not tenable, since other permeant ions (acetate, β-hydroxybutyrate, etc.) were completely uneffective.

The circumstance that also tetracaine strongly prevents the efflux of Mg^{2+} from mitochondria (Fig.1), as well as the release of respiration induced by diamide or Pi (Fig.2) can be interpreted as the consequence of an interference of the anaesthetic with the binding of Ca^{2+} to inner membrane phospholipids.

Finally, considering that the Pi concentrations used in these experiments are in the physiological range and that the pH imposed can be easily reached in vivo in conditions of anoxia or ischemia (24), present results might be of some value in interpreting the biochemical events occurring in these pathological states.

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