Participation of Endogenous Fatty Acids in Ca^{2+} Release Activation from Mitochondria

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Abstract. A correlation between the rate of H^+/Ca^{2+} exchange and the content of free fatty acids in mitochondria has been found. Fatty acids were isolated from mitochondria with different activities of H^+/Ca^{2+} exchange. It has been shown that these free fatty acids are able to induce Ca^{2+} release in exchange to protons after being added to freshly isolated mitochondria.

Key words: Calcium — Free fatty acids — Mitochondria

Introduction

Regulation of Ca^{2+} distribution between mitochondrial matrix and extramitochondrial medium is determined by two Ca^{2+} transporting systems i.e. electrogenic energy-dependent Ca^{2+} accumulation and electroneutral passive Ca^{2+} efflux from mitochondria through a system of H^+/Ca^{2+} exchange (Nicholls and Crompton 1980; Fiskum and Lehninger 1980). Studies on the energy-dependent Ca^{2+} accumulation system have indicated a participation of mitochondrial glycoproteins in this process (Carafoli and Sottocasa 1974). The system of H^+/Ca^{2+} exchange is not clearly understood. Little information is available on the molecular organization and regulatory mechanisms of the electroneutral H^+/Ca^{2+} exchange system. Lehninger et al. (1978) and Mglova (1981) have shown that in freshly isolated mitochondria the H^+/Ca^{2+} exchange system is inactive. Intensive Ca^{2+} release from mitochondria has been observed after calcium loading. Periodical activation and inhibition of the H^+/Ca^{2+} exchange system have been observed during mitochondrial oscillations of the fluxes (Gyulkhandanyan et al. 1978).

Evtodienko (1979) has suggested that the formation of the Ca^{2+} release system in the mitochondrial membrane is associated with the activation of mitochondrial phospholipase A_2, resulting in an elevation of the products of phospholipid hydrolysis, such as fatty acids and lysophospholipids. The participation of phospholipase A_2 in the activation of H^+/Ca^{2+} exchange has also been confirmed by the data of Angielski et al. (1980) and Harris and Cooper (1981) showing that exogenous fatty acids and lysophospholipids activate Ca^{2+} release from mitochondria. However, no direct evidence for the participation of endogen-

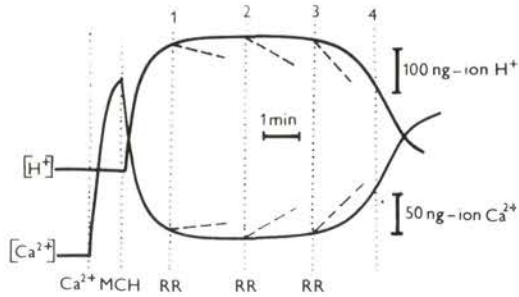

Fig. 1. Increasing H^+/Ca^{2+} exchange during calcium retention. MCH — mitochondria (2 mg of protein/ml); Ca^{2+} — $CaCl_2$, 5×10^{-4} mol/l; RR — Ruthenium Red, 10^{-6} mol/l. Incubation media: sucrose 0.25 mol/l, potassium succinate 0.005 mol/l, tris-HCl 0.002 mol/l, pH 7.5.

ous products of phospholipase action in the activation of H^+/Ca^{2+} exchange has been obtained. In the present work a comparative analysis of mitochondria with different activity of H^+/Ca^{2+} exchange system has been carried out.

Materials and Methods

Liver mitochondria were prepared from male Wistar rats weighing 190—200 g. Mitochondria were isolated by the method of Schneider (1948). The isolation medium consisted of 0.3 mol sucrose and 5 mmol Tris-HCl, pH 7.5. After centrifugation, the mitochondria were not washed. The pellet was suspended in a ratio of 10g of tissue/ml isolation medium. Protein was determined according to Lowry (Lowry et al. 1951). Protein concentration of the final mitochondrial suspension was 80—100 mg/ml. Ion fluxes of H^+ and Ca^{2+} were registered using ionselective electrodes. All experiments were conducted at 25°C in a thermostated vessel with a total volume of 2 ml. For conditions of incubation, see legends to figures.

Lipids were extracted by the method of Folch et al. (1957). The fraction of neutral lipids was obtained either by the method of Kates (1972) or by method of preparative thin layer chromatography (TLC) on Merck silica gel H plates after separation in the chloroform-methanol-water (65:25:4) solvent system. For preparative and analytical separation components of the neutral lipids, petroleum ether-diethyl ether-acetic acid system (80:20:1) was used. Spots corresponding to cholesterol, diglycerides, free fatty acids, triglycerides and cholesterol esters were identified. Estimation of chromatograms was performed on a Shimadzu CS-910 TLC Scanner equipped with a recorder and an analog integrator according to Heyneman et al. (1972) and Peter and Wolf (1973). The level of free fatty acids in lipid extracts was evaluated also additionally according to the method of Nixon and Chan (1979).

Results

It is known that, after calcium loading, mitochondria retain the accumulated calcium for some time followed by spontaneous Ca^{2+} efflux (Fig. 1). This Ca^{2+}

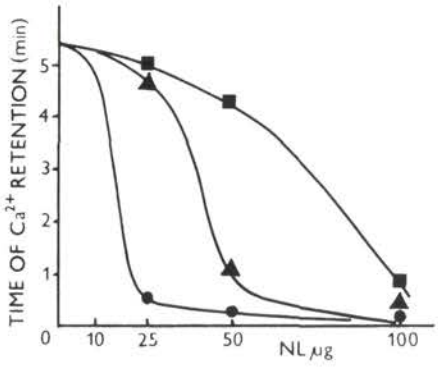

Fig. 2. Effect of neutral lipids on the time of calcium retention. For incubation medium see legend to Fig. 1. NL — neutral lipids from mitochondria: at point 1 (■), at point 3 (▲), at point 4 (●).

discharge is associated with H^+/Ca^{2+} exchange system activation. When the system of electrogenous Ca^{2+} accumulation is inhibited by Ruthenium Red an increase in the rate of H^+/Ca^{2+} exchange can be seen during the retaining of calcium (Fig. 1).

To elucidate the role of lipid compounds in the activation of Ca^{2+} release from mitochondria extracts of neutral lipids were obtained from preparations of mitochondria with different activity of H^+/Ca^{2+} exchange. Four points were chosen for the analysis: immediately after Ca^{2+} uptake (point 1), 4—5 minutes later (point 2), at the beginning (point 3) and in the middle of the spontaneous Ca^{2+} release (point 4) (Fig. 1).

In the first series of experiments we studied the effect of neutral lipid fractions isolated from mitochondrial preparations and having different rates of H^+/Ca^{2+} exchange (points 1—4) on the time of calcium retention. Upon the addition of 0.5 mmol $CaCl_2$ to mitochondrial suspension (2 mg of protein/ml) the time of calcium retention was 8—10 minutes. Preparations of neutral lipids were added to the incubated mitochondria, dissolved in ethanol before the addition of $CaCl_2$. In the presence of 20 μl ethanol (the maximal volume of addition) the time of calcium retention was 6—8 minutes.

An analysis of the effect of neutral lipids, isolated from mitochondria at different points, on the time of calcium retention has indicated that 25 μg of the neutral lipids fraction, isolated from point 4, drastically reduced the time of calcium retention by mitochondria. A similar effect was produced by the fraction of neutral lipids from mitochondria at point 3, however in an amount of 50 μg. The fraction of neutral lipids isolated from mitochondria at point 1 was effective only in an amount of 100 μg. Data of these experiments are presented in Fig. 2.

The results obtained allow us to conclude that neutral lipids of mitochondria

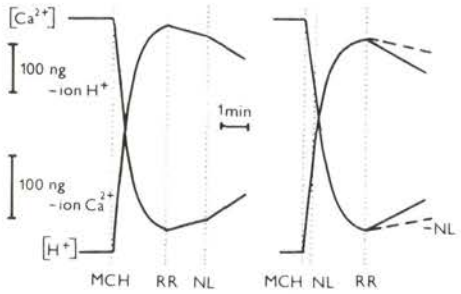

Fig. 3. Effect of neutral lipids on the rate of H^+/Ca^{2+} exchange. MCH — mitochondria (2 mg of protein/ml); RR — Ruthenium Red, 10^{-6} mol/l; NL — neutral lipids. Incubation medium: sucrose 0.1 mol/l; KCl 0.1 mol/l; potassium succinate 0.005 mol/l; KH_2PO_4 7×10^{-4} mol/l; tris-HCl 0.002 mol/l; pH 7.5.

contain product(s) which change the permeability of the inner mitochondrial membrane, the amount of the product(s) being increased during the calcium retention.

In another series of experiments, effects of neutral lipid fractions obtained from mitochondria at point 1 and 4 on H^+/Ca^{2+} exchange has been studied. The neutral lipid fractions were obtained by preparative TLC. Total mitochondrial lipids (2.4 mg) were applied on the chromatographic plate. After separation, neutral lipids were eluted from the chromatogram with a chloroform-methanol (2 : 1) mixture, the solvents were evaporated, and the neutral lipids were dissolved in 100 μl of ethanol. To estimate the rate of H^+/Ca^{2+} exchange, mitochondria were loaded by calcium and the energy-dependent calcium accumulation was inhibited by Ruthenium Red. Lipids were added in volumes of 20 μl (14—15 μg) either immediately after the addition of mitochondria, or 2—2.5 minutes after the addition of Ruthenium Red (Fig. 3). In both cases, the activation of H^+/Ca^{2+} exchange rate by neutral lipids isolated from mitochondria at point 4 was higher than that at point 1. The results are presented in Table 1.

To elucidate which component of the neutral lipids makes the greatest contribution to the activation of H^+/Ca^{2+} exchange, the following series of experiments was carried out. Neutral lipids isolated from 2.4 mg of the total mitochondrial lipids were subjected to preparative TLC separation in petroleum ether-diethyl either acetic-acid system. The components obtained were eluted with chloroform-methanol (2 : 1) from silicagel, dried, dissolved in 100 μl of ethanol and 20 μl of the solution was added to mitochondria.

It was found that free fatty acids have a pronounced effect on the H^+/Ca^{2+} exchange system, while other neutral lipid components produced little or no effect on it. As seen from Table 1, the activating effect of fatty acids isolated from

Table 1. Effects of neutral lipids and free fatty acids on the rate of H^+/Ca^{2+} exchange in mitochondria

Additions	Rate of H^+/Ca^{2+} exchange	
	ng ions H^+	ng ions Ca^{2+}
	min . mg of protein	min . mg of protein
—	10.4 ± 2.4	11.4 ± 2.0
NL fraction point 1*	22.9 ± 2.8	20.4 ± 2.1
NL fraction point 4*	31.8 ± 3.1	32.0 ± 2.8
NL fraction point 1**	10.6 ± 1.4	9.3 ± 1.2
NL fraction point 4**	19.4 ± 1.2	20.8 ± 1.0
FFA fraction point 1*	14.6 ± 1.8	13.1 ± 2.0
FFA fraction point 4*	19.6 ± 2.0	17.4 ± 1.8

For the incubation medium, see the legend to Fig. 3

NL — neutral lipids, FFA — free fatty acids.

* — the fraction was added during Ca^{2+} accumulation (*100* ng ions/mg of protein)

** — the fraction was added 2—2.5 min after the addition of Ruthenium Red (Ca^{2+} −80 ng ions/mg of protein)

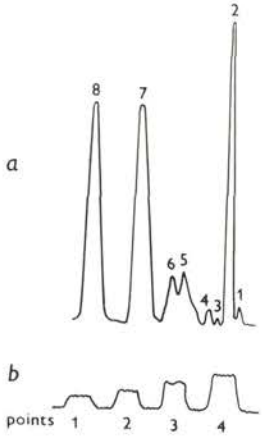

Fig. 4. *a*) Thin layer chromatographic separation of neutral lipid components of mitochondria. 1) 1,2 — diglycerides; 2) cholesterol; 3) 1,3-diglycerides; 4) free fatty acids; 5—6) not identified; 7) triglycerides; 8) cholesterol esters. *b*) Result of recording only free fatty acids in neutral lipid samples obtained from mitochondria at points 1—4. Scanning was carried out perpendicular to the direction of the chromatographic development.

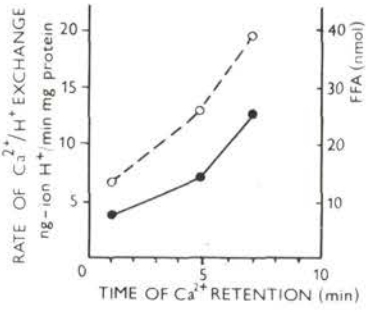

Fig. 5. Correlation between the rate of H^+/Ca^{2+} exchange and the amount of free fatty acids. (○) — rates of H^+/Ca^{2+} exchange; (●) — amounts of free fatty acids (FFA).

preparations of mitochondria at point 4 is higher than that at point 1. Also the contents of free fatty acids in the lipid extracts of mitochondria at points 1—4 was estimated. Results of a thin layer chromatographic analysis of neutral lipids obtained from mitochondria at points 1—4 are shown in Fig. 4. It can be clearly seen that at point 4 the content of free fatty acids was more than twice than at point 1. The concentration of other components was practically unchanged. The data obtained allow us to consider free fatty acids as a factor determining the rate of Ca^{2+} release from mitochondria.

Fig. 5 shows changes in the H^+/Ca^{2+} exchange rate and the contents of free fatty acids in mitochondria as determined according to the method of Nixon and Chan (1979), during calcium retention. There is a correlation between the activation of H^+/Ca^{2+} exchange rate and the increase in the contents of free fatty acids.

Discussion

Our data indicate that there is a good correlation between the content of fatty acids and the intensity of H^+/Ca^{2+} exchange. It was found that upon the addition to calcium loaded mitochondria, endogenous free fatty acids stimulate H^+/Ca^{2+} exchange. In this case, the amount of fatty acids added corresponds to that in the mitochondrial preparations with active system of H^+/Ca^{2+} exchange.

It is known that Ca^{2+} may also be released from mitochondria under the action of commercial preparations of fatty acids (Angielski et al. 1980) or lysophosphatidylcholine (Harris and Cooper 1981). However, lysophosphatidylcholine can be subjected to the action of lysophospholipase, and the fatty acids formed during this process can be an effective component.

It should be noted that Pfeiffer et al. (1979) have not observed any correlation between Ca^{2+} release and the swelling of mitochondria during the accumulation of

fatty acids. However, in these experiments, the H^+/Ca^{2+} exchange was not estimated, and thus the efflux of cations and swelling could have been due to a nonspecific increase in mitochondrial permeability during the spontaneous efflux of cations. It can be supposed that free fatty acids are directly involved in the activation of H^+/Ca^{2+} exchange since they are able to bind Ca^{2+} and H^+ and penetrate into the hydrophobic phase of the mitochondrial membrane (Angielski et al. 1980). However, the possibility that free fatty acids can facilitate the functioning of an unidentified H^+/Ca^{2+} exchange system by a modification of physicochemical properties of the hydrophobic phase of the membrane, cannot be ruled out. In this case, free fatty acids can serve as a regulator of Ca^{2+} release which emerge in mitochondria during calcium accumulation in matrix and phospholipase A_2 activation (Severina and Evtodienko 1981). Definite evidence for the direct participation of free fatty acids in the activation of the H^+/Ca^{2+} exchange system can be obtained in model experiments with fatty acids introduced into lipid bilayers.

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